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The antioxidant and therapeutic effects of *Malva sylvestris* extract on testicular tissue and sperm quality in varicocele-induced adult Wistar rats

Fatemeh Koohkan¹, Mahnaz Azarnia¹ and Latifeh Karimzadeh Bardeei^{1*}

Abstract

Background Varicocele is a major male infertility issue, and *Malva sylvestris* shows promise as a treatment due to its antioxidant properties. The present study evaluated the protective effects of *Malva sylvestris* on testicular health, sperm quality, and oxidative stress-related gene expression. Adult male Wistar rats were randomly assigned into five groups ($n=8$): the control group, the varicocele model group, the varicocele group with partial occlusion of the left renal vein treated with 750 or 1500 mg/kg *Malva sylvestris* for 21 days, and a surgical sham group. The epididymal content and Histological analyses of animals testicular tissue were examined to evaluate fertility parameters, and qRT-PCR was employed to determine the expression of *SIRT1*, *FOXO1*, *NRF2*, *NF-κB*, and *TGF-β* genes.

Results Varicocele leads to the induction of apoptosis, the occurrence of DNA damage, a reduction in *SIRT1* and *FOXO1*, and an increase in *NRF2*, *TGF-β*, and *NF-κB* gene expression. According to Histological morphometric analysis, treatment with *Malva sylvestris* showed the increased thickness of Mean Seminiferous Tubule Diameter and Epithelial Thickness in the spermatogenic epithelium, as well as the presence of a greater number of germ cells and mature sperm. In this study, *Malva sylvestris* treatment showed antioxidative effects since it upregulated the expression of *SIRT1* and *FOXO1* genes and downregulated the expression of *NF-κB*, *NRF2*, and *TGF-β* genes.

Conclusion Based on the findings, *Malva sylvestris* as cytoprotective agent modulate key antioxidant pathways, including upregulation of *SIRT1* and *FOXO1* (linked to cellular repair and OS resistance) and downregulation of pro-inflammatory mediators; then, *Malva sylvestris* as a promising natural antioxidant for managing varicocele-related infertility, offering a potential adjunct or alternative to conventional therapies.

Keywords Infertility, *Malva sylvestris*, Oxidative stress, Sperm, Testis, Varicocele

Résumé

Contexte La varicocèle est un problème majeur d'infertilité masculine, et *Malva sylvestris* est prometteuse en tant que traitement, en raison de ses propriétés antioxydantes. La présente étude a évalué les effets protecteurs de *Malva sylvestris* sur la santé testiculaire, la qualité du sperme et l'expression des gènes liés au stress oxydatif. Des rats Wistar mâles adultes ont été répartis au hasard en cinq groupes ($n = 8$) : groupe témoin, groupe modèle varicocèle, groupe

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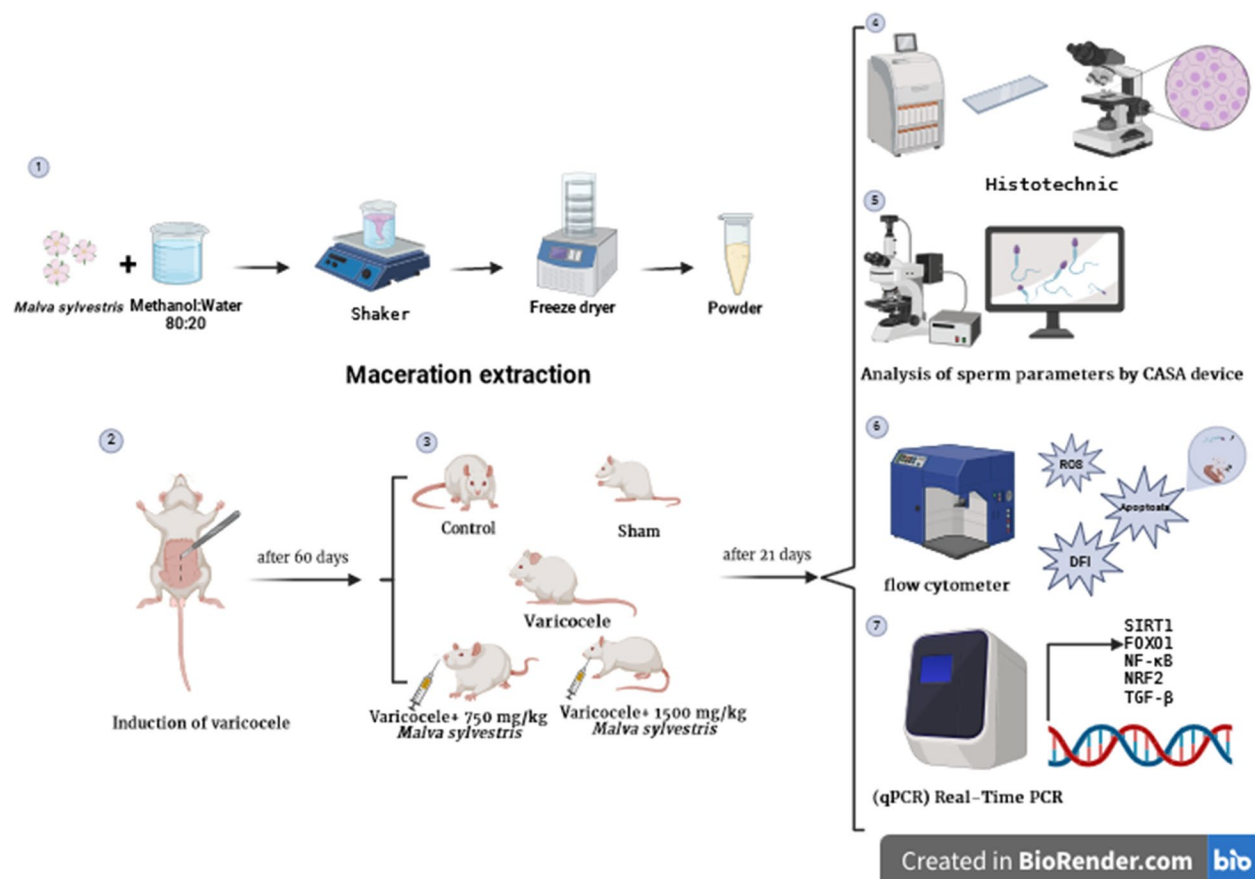
varicocèle avec occlusion partielle de la veine rénale gauche traité avec 750 ou 1500 mg/kg de *Malva sylvestris* pendant 21 jours, et groupe simulé chirurgicalement. Le contenu épидидymaire et les analyses histologiques de tissu testiculaire des animaux ont été examinés pour évaluer les paramètres de fertilité, et la qRT-PCR a été utilisée pour déterminer l'expression des gènes *SIRT1*, *FOXO1*, *NRF2*, *NF-κB* et *TGF-β*.

Résultats La varicocèle entraîne l'induction d'une apoptose, l'apparition de dommages à l'ADN, une réduction de *SIRT1* et *FOXO1*, et une augmentation de l'expression des gènes *NRF2*, *TGF-β* et *NF-κB*. Selon l'analyse morphométrique histologique, le traitement par *Malva sylvestris* a montré une augmentation de la moyenne de l'épaisseur du tube séminifère et de l'épaisseur épithéliale de l'épithélium séminifère, ainsi que la présence d'un plus grand nombre de cellules germinales et de spermatozoïdes matures. Le traitement par *Malva sylvestris* a montré des effets antioxydants puisqu'il régulait à la hausse l'expression des gènes *SIRT1* et *FOXO1* et à la baisse l'expression des gènes *NF-κB*, *NRF2* et *TGF-β*.

Conclusions Sur la base des résultats, *Malva sylvestris*, en tant qu'agent cytoprotecteur, module les principales voies antioxydantes, y compris la régulation à la hausse de *SIRT1* et *FOXO1*, et la régulation à la baisse des médiateurs pro-inflammatoires. *Malva sylvestris*, en tant qu'antioxydant naturel prometteur pour la gestion de l'infertilité liée à la varicocèle, offre un complément potentiel ou une alternative aux thérapies conventionnelles.

Mots-clés Infertilité, *Malva sylvestris*, Stress oxydatif, Spermatozoïdes, Testicules, Varicocèle

Graphical Abstract



Introduction

The primary function of the male reproductive system is the generation of spermatozoa and hormones. Male infertility can be caused by various factors, particularly deviations in sperm parameters, which represent as spermatogenic dysfunctions, such as the complete absence of sperm (azoospermia), diminished sperm count (oligozoospermia), anomalous sperm morphology (teratozoospermia), or impaired sperm motility (asthenozoospermia). It is worth noting that the prevalence of male factor infertility varies geographically, ranging from 20 to 70% [1].

Varicocele, characterized by the dilation of veins that carry blood from the testicles, spermatic veins and the pampiniform plexus, is a common cause of infertility in men seeking treatment at infertility centers. Varicocele is prevalent in 11.7% of couples and affects 15% of the general male population. It is the cause of infertility in 25.4% of infertile men, occurring in 35% of men with primary infertility and 75% of men with secondary infertility [2–4].

Approximately 30 to 80% of sterile males exhibit levels of reactive oxygen species (ROS) in their semen that exceed the levels of antioxidants present, which in turn impacts sperm motility and functionality and contributes to the development of varicocele disease. Hypoxia and thermal stress are the primary elements responsible for the increased generation of ROS within germ cells, consequently interfering with the maturation of spermatozoa and the normal operation of epididymal cells. This imbalance leads to DNA damage, which triggers the initiation of the apoptotic pathway, resulting in a decreased sperm count and ultimately leading to male infertility [5–7].

The previous studies underscored that oxidative stress is a central mediator of sperm dysfunction via DNA damage, lipid peroxidation, mitochondrial impairment, and apoptosis; ROS (e.g., superoxide anion, hydrogen peroxide) directly attack sperm DNA, leading to strand breaks and base modifications. High DNA fragmentation reduces fertilization potential and increases the risk of embryonic abnormalities. On the other hand, ROS target polyunsaturated fatty acids (PUFAs) in sperm membranes, triggering lipid peroxidation. Loss of membrane fluidity and acrosome integrity, impairing sperm motility and egg-binding capacity. Also Sperm rely on mitochondrial ATP for motility. Excessive ROS disrupts mitochondrial electron transport, reducing energy production and leading to Asthenozoospermia (reduced sperm motility) and premature sperm exhaustion. ROS activate pro-apoptotic pathways (e.g., Bax/Bcl-2 imbalance,

caspase-3) in germ cells, leading to premature cell death and Oligozoospermia and disrupted spermatogenesis. ROS impair Leydig cell steroidogenesis, lowering testosterone production. Testosterone deficiency further exacerbates spermatogenic failure [8–10].

Microsurgery is currently considered the most effective approach for performing varicocelectomy, as it is associated with fewer complications and better pregnancy results [11]. Nevertheless, as surgical interventions are financially challenging for some individuals [12], various types of medications, such as selective estrogen receptor modulators and antioxidants, are used as an alternative to treat varicocele. The use of antioxidants derived from medicinal plants is one approach for reducing oxidative stress levels and enhancing fertility [13, 14].

Malva sylvestris (M.S) is one of these medicinal plants which has long been used in traditional herbal medicine and cosmetic procedures [15]. The physiological function of the foliage of M.S is contingent upon various chemical components, such as polyphenols, vitamins C and E, beta-carotene, gossypetin 3, and glucoside, which play a central role in its biological efficacy [16]. The molecular mechanism by which antioxidants derived from M.S alleviate varicocele involves modulation of SIRT1, FOXO1, and pro-inflammatory mediators; addressing oxidative stress and inflammation—key contributors to varicocele pathogenesis. Antioxidants (e.g., flavonoids, phenolic acids) in M.S enhance SIRT1, a NAD⁺-dependent deacetylase. This may occur via increased NAD⁺ availability or direct compound-SIRT1 interaction. SIRT1 deacetylates and activates FOXO1, amplifying its antioxidant functions in testicular tissues. SIRT1 deacetylates NF-κB subunits (e.g., p65), inhibiting their nuclear translocation and transcriptional activity, thereby suppressing pro-inflammatory genes. By inhibiting NF-κB via SIRT1, M.S antioxidants reduce expression of cytokines (TNF-α, IL-6), COX-2, and iNOS, curtailing inflammation. SIRT1-FOXO1 axis enhances cellular resilience to oxidative stress, while NF-κB inhibition reduces inflammation. This dual action protects spermatogenesis and testicular function in varicocele [17–20].

The present study aimed to investigate the protective effects of M.S on alleviating testicular injury in an experimental varicocele model and clarify the precise underlying mechanism of action; The objective of this study is to advance prior work by demonstrating not only histological improvements (e.g., reduced seminiferous tubule degeneration) but also functional recovery in sperm quality (motility, count, morphology), bridging the gap between antioxidant intervention and reproductive outcomes.

Materials and methods

Preparation and evaluation of the antioxidant content of *M.S* extract

M.S flowers were collected from Karaj, Alborz Province, Iran. First, the leaves were separated, desiccated at room temperature, and finely ground. The resulting powder was then placed in 2 L 96% ethanol (Razi, Iran) and stored at 25 °C for 48 h. Subsequently, the mixture was agitated by a shaker (Hoilph, Germany), filtered, and centrifuged at 1000 ×g for 8 min (Hanil, South Korea). After the dissolved solvent was evaporated at room temperature, the extracted powder was refrigerated at 4 °C until further analysis. The DPPH test method was used to assess the antioxidant potential of *M.S*. To formulate a DPPH solution, 4 g of pure powder was dissolved in 100 mL of methanol. Then, 6 mL of DPPH was added, and the mixture was allowed to rest for 30 min at 37 °C. Finally, the absorbance of each sample was measured at 517 nm, and the percentage of inhibition of free radicals was calculated using the following formula: $1\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$. The reference sample consisted of 3 mL of *M.S* powder and 3 mL of methanol.

Experimental design and animals

Forty male Wistar rats weighing 200 to 250 g were sourced from the Animal House of Kharazmi University and kept in a controlled environment (22 ± 2 °C, 45–50% humidity, and a 12-h light/dark cycle) with access to water and standard chow ad libitum. All animal experiments comply with the ARRIVE guidelines and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. The study was approved by the Ethics Committee of Kharazmi University under code IR.KHU.REC.1402.091. After a one-week acclimatization under standard conditions, the rats were divided into five groups ($n = 8$). Group 1 (control) had no varicocele, group 2 had induced varicocele, and groups 3 and 4 (varicocele + *M.S*) received *M.S* at 750 and 1500 mg/kg (intraperitoneal), respectively, for 21 days following varicocele induction for eight weeks [21–23]. A surgical sham group (group 5) also underwent all procedures except spermatic vein ligation. However, data from this group were excluded from comparative analysis due to a lack of significant difference from the control group.

Procedures for varicocele induction

Male rats were induced with experimental varicocele using Turner's method [24] under sterile conditions at room temperature (25 °C), as described previously. The rats were anesthetized intraperitoneally with 50 mg/kg ketamine and 10 mg/kg xylazine [25]. A 2–4 cm vertical incision was made along the midline of the abdomen to access the left

internal spermatic vein and the left renal vein of the inferior vena cava. The surgical procedure continued with creating a tunnel near the left renal vein and separating the vein from the surrounding fatty tissues. This was followed by partial occlusion of the renal vein through suturing with a 4–0 nonabsorbable silk suture with the assistance of a rod. Upon completion of the suturing, the procedure was ended by withdrawing the auxiliary rod and closing the incision layers by stitching. After an experimental period of eight weeks, all the animals were euthanized through CO₂ gas inhalation, and the epididymal tissue and testicles were extracted.

Sperm parameter analysis

The epididymal tubes were dissected in 1 mL of culture medium. Afterward, 10 µL of the solution was placed on a Neubauer slide, fixed with 2% formalin, and examined by the enumeration of five fields at 400 × magnification using a microscope (Zeiss, Germany).

For viability assessment, an aliquot of 5 µL of sperm suspension was placed on the slide, and 2.5 µL of 1% eosin was added to it. Following a 30-s incubation at 37 °C, 2.5 µL of 10% nigrosin was added to the suspension. Subsequently, 1000 viable (unstained) and nonviable spermatozoa (red) were counted under a microscope, and 5 µL of the suspension was placed on a Leja slide with a depth of 20 µL. The slide was preheated to 37 °C prior to assessing the motility rate and other sperm characteristics using Computer-Assisted Sperm Analysis (CASA, Eclipse E-200 microscope, Nikon Co., Tokyo, Japan). Sperm motility parameters consisted of Curvilinear Velocity (VCL), Average Path Velocity (VAP), Straight-Line Velocity (VSL), Linearity Index (LIN), Straightness Index (STR), Wobbling Index (WOB), Beat Cross Frequency (BCF), and Amplitude of Lateral Head displacement (ALH) [26]. Furthermore, progression assessment was conducted according to WHO4 and WHO5 criteria, considering factors such as velocity, head area, and round cells.

Histopathological evaluations

Varicocele was induced through ligation of the left renal vein in rats, and the outcomes were assessed after eight weeks. After all the animals were anesthetized using previously outlined procedures, their testes were excised for further examination. The left testis was carefully dissected, rinsed with cold saline, and then immersed in a 10% formalin solution for 24 h. The tissue samples were subsequently dehydrated through a series of increasing alcohol concentrations, followed by clarification with toluene. Thin transverse Sects. (4 µm thick) were mounted on glass slides and stained with hematoxylin and eosin (H&E). The histopathological alterations within the testicular tissue were then assessed under a light microscope. For quantitative

assessment of testicular morphometry, 100 seminiferous tubules exhibiting circular or maximally round cross-sectional profiles were selected. Tubule diameter was measured as the minor axis using ImageJ software. MSTD was calculated as:

$$\text{MSTD} = \sum (\text{diameters of all measured tubules}) / \text{Number of tubules measured}$$

Epithelial thickness was defined as the perpendicular distance between the basement membrane and luminal edge (excluding the lumen). Measurements were taken at five equidistant points per tubule using ImageJ's straight-line tool, averaged per tubule, and MSET derived as [27]:

$$\text{MSET} = \sum (\text{average epithelial thickness per tubule}) / \text{Number of tubules measured}$$

Sperm chromatin structure assay

First, semen samples were thinned at a ratio of 1:10 with phosphate-buffered saline (PBS; pH 7.4) to examine the quality of sperm chromatin. Next, they were centrifuged at 2300 rpm for 5 min at 4 °C to eliminate seminal plasma. Afterward, they were treated with 70% ethanol for 10 min and went through centrifugation and suspension in PBS again. A total of 10,000 sperm cells were preserved at 28 °C to further analyze their chromatin structure using a mixture of 2 mg/mL acridine orange, 0.15 M NaCl, 126 mM Na₂HPO₄, 1 mM ethylenediaminetetraacetic acid, and 37 mM citric acid buffer at a pH of 7.4. Finally, the sperm cells were stained, and Sperm Chromatin Structure Assay (SCSA) was conducted to examine the DNA fragmentation index (DFI%) and the percentage of sperm with high-density staining (HDS%) using a flow cytometer [28–30]. In SCSA, a DFI of $\geq 25\%$ was considered abnormal [31].

Investigation of the ROS content in epididymal tissue

To measure ROS levels, flow cytometric assessment was utilized with 2',7'-dichlorofluorescein diacetate (DCFH-DA) to detect hydrogen peroxide (H₂O₂) and dihydroethidium (DHE) to identify superoxide anion (O₂⁻). The suspensions derived from epididymis in the previous step were incubated with DCFH-DA (10 mmol) and DHE (1.25 μ mol) for 15 min at 37 °C. The samples were then centrifuged at 350 rpm in the dark until the incorporation of probes into the cellular membranes. After a triple wash with PBS, DCFH was transformed into dichlorofluorescein (DCF), and DHE was transformed into 2-hydroxyethidium (HE). The samples were then analyzed using a flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA), which measured green fluorescence of DCF within the range of 500–530 nm in the FL-1 channel and red fluorescence of HE within the

range of 590–700 nm in the FL-2 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm [32].

Examining the mitochondrial membrane potential of testicular tissue with JC-1 dye

To measure the mitochondrial membrane potential (MMP), which indicates the activity of this intracellular organelle, flow cytometry was used. The MMP was assessed using a lipophilic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) that selectively entered mitochondria. A sample contain-

ing 1×10^6 /mL sperm was incubated with JC-1 in the dark for 10 min at 37 °C under 5% CO₂ pressure within an incubator, followed by a PBS wash. Changes in membrane potential led to the transition of JC-1 dye from green to orange, allowing differentiation between normal and damaged cells based on the presence of orange granules [33, 34].

Analysis of sperm apoptosis by flow cytometry

Membrane translocation of phosphatidylserine (PS) and membrane integrity were assessed using the Annexin V technique. The external identification of PS through Annexin V conjugates was carried out using flow cytometry. Seminal samples containing 5×10^6 spermatozoa per mL were washed and centrifuged (800 $\times$ g for 5 min) in HEPES buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). The samples were subsequently resuspended in HEPES buffer, followed by the separation of 5 L of allophycocyanin-annexin V and 0.1 L of 0.005 mM stock solution, after which green nucleic acid-stained DNA dye (Sytox, Molecular Probes, Eugene, OR, USA) was introduced. Each 100 L of sperm cell suspension in this mixture was incubated for 15 min at room temperature, followed by repeated washing and centrifugation steps (800 $\times$ g for 5 min). Both positive (apoptosis induction with H₂O₂) and negative (HEPES buffer lacking Ca²⁺) controls were included. Finally, 500 L of HEPES buffer was added, and the samples were promptly subjected to flow cytometry analysis. The configuration included 488 and 635 nm excitation with a 30/530 nm bandpass filter for the detection of the fluorescein isothiocyanate (FITC) conjugate (Sytox) and a 16.661 nm bandpass filter for allophycocyanin (Annexin V) [35].

Analysis of Sperm Apoptosis by Flow Cytometry. It should be supported with representative photomicrograph.

RT-qPCR

Total RNA was extracted from testicular tissue using TRIzol reagent (Invitrogen, USA). The extracted RNA was subsequently reverse transcribed to generate cDNA using a commercially procurable kit (Thermo Fisher Scientific, Waltham, MA, USA) in conjunction with RT-PCR (Real-time Rotor gene, Germany). The Qrt-PCR was performed in a total volume of 20 μ L, comprising 2 μ L of cDNA (five times diluted), 0.5 μ L of 5 mmol/L solutions of both forward and reverse primers (Table 1), and 10 μ L of 2X SYBR DNA PCR. Each sample was subjected to duplicate analyses. The sequences of the forward and reverse primers were as follows. Melt curve analysis was conducted for each iteration to examine the existence of nonspecific PCR products and primer dimers. The expression ratio was determined using the relative formula derived from the comparative CT method ($\Delta\Delta CT$).

Statistical analysis

The analysis was conducted using GraphPad Prism software (version 9). Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. The data are presented as mean $\pm$ SD. Statistical significance was set at $P < 0.05$.

Results

Measurement of the antioxidant properties of *M.S* leaves

The antioxidant properties of *M.S* leaves were evaluated using the DPPH test method. Based on the findings, the 70% ethanol extract of *M.S* showed an overall efficacy of 42.372% in suppressing DPPH radicals. The antioxidant activity of *M.S* was computed using the formula $[(Ac - As) \div Ac] \times 100$, where Ac represents the absorbance

of the control and As represents the absorbance of the sample, resulting in an antioxidant activity of 42.372%. Vitamin C was also used as an indicator of antioxidant activity, and its IC_{50} was measured at 2.3 mg, while the IC_{50} of the *M.S* extract was measured at 1.18 mg. The closer the IC_{50} is to that of vitamin C, the greater the antioxidant potential.

Varicocele induction and testicular histopathology evaluation

The H&E staining revealed that varicocele disrupts the architecture of testicular tissue (Fig. 1A; a, b, c). Compared to the control group, rats in the varicocele model exhibited substantial impairment of testicular spermatogenesis and sperm maturation disorders, resulting in thinner and more irregular seminiferous tubules (Fig. 1B). The mean seminiferous tubule diameter (MSTD) was $397.743 \pm 7.6 \mu m$ in the control group, whereas in the varicocele group, it was $196.808 \pm 13.2 \mu m$ ($P < 0.01$). There was also a significant difference in the mean seminiferous epithelial thickness (MSET) between the control group ($5.131 \pm 2.6 \mu m$) and the varicocele group ($2.947 \pm 1.3 \mu m$), indicating a reduction in the varicocele model group. Compared to the varicocele model group, treatment with *M.S* showed clear reparative effects, as evidenced by the increased thickness of MSTD and MSET in the spermatogenic epithelium, as well as the presence of a greater number of germ cells and mature sperm (Fig. 1C). Although there was a minor enhancement in grams per kilogram, it did not reach statistical significance. Moreover, the testicular structure did not fully revert to its original state. The results of cell enumeration within 100 spermatogenic tubes are detailed in (Table 2).

Evaluation of epididymal sperm parameters

The results of the CASA investigation, which juxtaposes sperm characteristics across four control sets, varicocele, and treated sets, are illustrated in Figs. 2 and Table 3. The viability, motility, and morphology of the sperm from the left testis were notably lower in the varicocele group than in the control group ($P < 0.001$). However, no significant difference was noted compared to the control group.

DFI sperm chromatin structure

Figure 3A depicts the comparison of the sperm DFI using the SCSA. Significant differences were observed in sperm chromatin following partial occlusion of the left renal vein. There was a significant increase in perm DFI in the varicocele group ($P < 0.001$). In contrast to the model group, the *M.S* 750 mg/kg treatment group exhibited a significant reduction in the epididymal sperm DFI ($P < 0.01$).

Table 1 Primer sequences

Gene	Sequence 5'–3'
Rat-Nrf2-F	CAGCACATCCAGACAGACAC
Rat-Nrf2-R	AATATCCAGGGCAAGCGACT
Rat-B2M-F	CGTCGTGCTTGCCATTACG
Rat-B2M-R	GCAGTTGAGGAAGTTGGGC
Rat-NF- κ B-F	ACAACATATGAGGTCTCTGGG
Rat-NF- κ B-R	CTATGTGCTGTCTTGTTGGAG
Rat-Sirt1-F	TTCAAGGCTGTTGGTTCCAG
Rat-Sirt1-R	AGGCGTCATCTTCAGAGTCT
Rat-FOXO1-F	ACTTCAAGGATAAGGGCGACA
Rat-FOXO1-R	ATTTCCCACTCTTGCTCCC
Rat-TGF- β -F	GACATGAACCGACCCTTCCT
Rat-TGF- β -R	GCCGTACACAGCAGTTCTTC

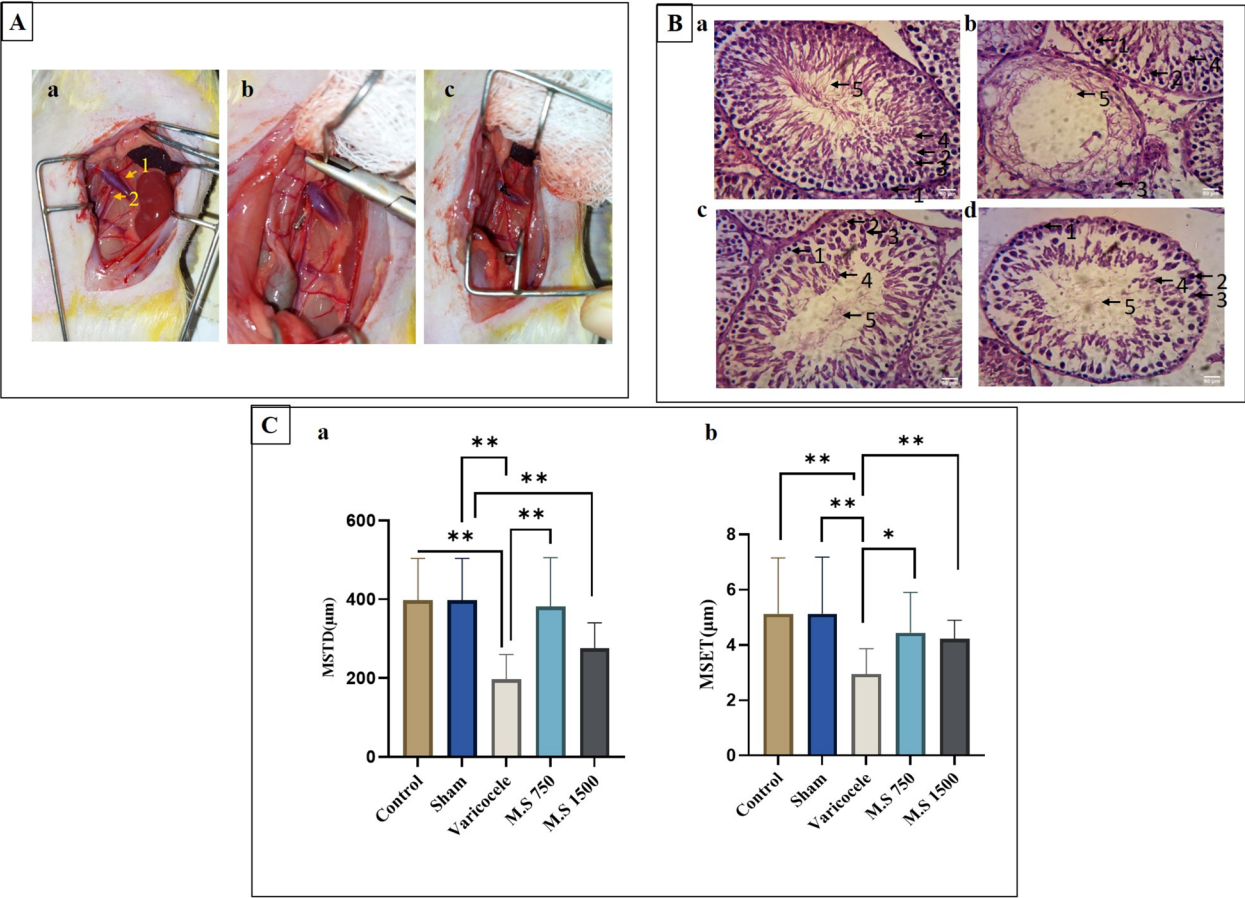


Fig. 1 **A** Surgery and induction of the varicocele model in adult male Wistar rats. The left renal vein, from which the internal spermatic vein branches off, was tied. **a**: Location of the renal vein and internal spermatic vein relative to the kidney, **b**: needle passage to isolate the renal vein, **c**: renal vein node. 1: Renal vein, 2: spermatic vein. **B** Histological analysis of the testicular tissue of the studied groups using hematoxylin and eosin staining. **a**: Control group, **b**: varicocele group, **c**: M.S 750 mg/kg treatment group, **d**: M.S 1500 mg/kg treatment group, 1: Spermatogonial cell, 2: primary spermatocyte cell, 3: secondary spermatocyte cell, 4: spermatid, 5: sperm. **C** Histological analysis of **a**: mean seminiferous tubule diameter, **b**: mean seminiferous epithelial thickness. **D** The number of spermatogonia, primary spermatocytes, and spermatids. The extent of destruction in the induction group and repair in the treatment group can be seen. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. All values are expressed as mean $\pm$ SD. A *p* value of ≤ 0.05 was considered significant. *P* < 0.05(*), *P* < 0.01(**), *P* < 0.001(***). M.S: *Malva sylvestris*

Table 2 Cell analysis spermatogenic tubes

Cell Type	Control	Sham	Varicocele	M.S 750	M.S 1500
Spermatogonia	58 $\pm$ 12	56 $\pm$ 02	22 $\pm$ 16***	59 $\pm$ 7***	56 $\pm$ 9***
Primary Spermatocyte	46 $\pm$ 10	44 $\pm$ 14	13 $\pm$ 8***	44 $\pm$ 12***	34 $\pm$ 7***
Spermatid	97 $\pm$ 13	94 $\pm$ 18	37 $\pm$ 15***	76 $\pm$ 11**	73 $\pm$ 6***

M.S *Malva sylvestris*. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. The data are presented as mean $\pm$ SD. Statistical significance was determined by *P* < 0.01(**), and *P* < 0.001(***)

ROS

Varicocele results in the disruption of local oxidative equilibrium, leading to the initiation of OS and the overproduction

of ROS. Oxidative stress in turn reduces and perturbs the total antioxidant capacity (TAC) of the overall seminal plasma and sperm parameters [36]. Compared to the control group, ROS levels significantly increased in rats induced with bilateral varicocele (*P* < 0.01). Compared to the varicocele model group, the average DCFH levels of epididymal sperm were significantly lower in the M.S 750 mg/kg intervention group (*P* < 0.001), as depicted in Fig. 3B.

JC-1

The evaluation of the MMP serves as a more accurate indicator of the performance of Mitochondria [33]. Compared to the control group, the varicocele group demonstrated the highest level of apoptosis in terms of MMP (*P* < 0.001). Moreover, the extent of MMP damage

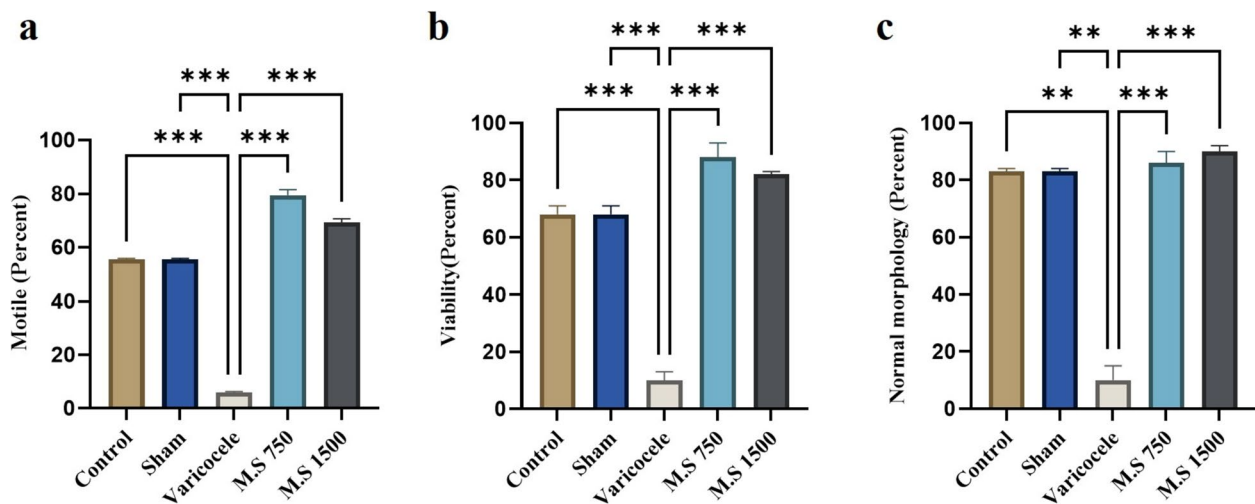


Fig. 2 Comparison of epididymal sperm parameters. a: Motility, b: viability, c: normal sperm morphology in the left testicle. The treatment groups showed a significant increase compared to the induction group. M.S 750: M.S 750 mg/kg, M.S 1500: M.S 1500 mg/kg, ns = 8. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. A p value of ≤ 0.05 was considered significant. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). M.S: *Malva sylvestris*

Table 3 Parameters used in sperm motility analysis

Parameters	Control	Sham	Varicocele	M.S 750	M.S 1500
PR (%)	31.08 $\pm$ 0.8	34.78 $\pm$ 3.80	11 $\pm$ 0.99***	51.11 $\pm$ 0.5****	45.17 $\pm$ 0.77***
NP (%)	24.19 $\pm$ 0.55	23.6 $\pm$ 2.05	5.53 $\pm$ 0.599***	28.36 $\pm$ 0.166***	24.71 $\pm$ 0.21***
IM (%)	44.73 $\pm$ 3.75	42.01 $\pm$ 0.75	94.21 $\pm$ 2.23***	20.52 $\pm$ 2.50***	30.12 $\pm$ 1.15***
M (%)	55.27 $\pm$ 0.59	52.32 $\pm$ 1.09	5.79 $\pm$ 0.30***	79.48 $\pm$ 2.01***	69.88 $\pm$ 2.30***
VCL (μ m/sec)	37.6 $\pm$ 2.02	35.6 $\pm$ 0.22	1.36 $\pm$ 1.03***	44.17 $\pm$ 1.5***	43.17 $\pm$ 2.5***
VSL (μ m/sec)	12.30 $\pm$ 1.05	12.27 $\pm$ 0.59	0.60 $\pm$ 0.53***	17.31 $\pm$ 1***	16.47 $\pm$ 0.5***
VAP (μ m/sec)	14.85 $\pm$ 2	15.78 $\pm$ 0.789	0.60 $\pm$ 0.60**	23.13 $\pm$ 0.13***	20.63 $\pm$ 1***
LIN (%)	15.68 $\pm$ 1.01	16.85 $\pm$ 2	3.7 $\pm$ 0.7***	18.37 $\pm$ 0.379***	17.54 $\pm$ 0.545***
STR (%)	36.73 $\pm$ 0.739	33.68 $\pm$ 1.21	13.33 $\pm$ 0.335***	38.01 $\pm$ 1***	32.46 $\pm$ 0.46***
WOB (%)	33.92 $\pm$ 0.92	31.92 $\pm$ 0.92	0.70 $\pm$ 0.5***	44.37 $\pm$ 0.37***	41.27 $\pm$ 0.27***
ALH (%)	2.59 $\pm$ 0.5	2.09 $\pm$ 0.6	0.21 $\pm$ 0.2*	2.97 $\pm$ 0.7***	2.87 $\pm$ 0.7***
BCF (%)	1.45 $\pm$ 0.5	1.32 $\pm$ 0.4	0.30 $\pm$ 0.3*	1.90 $\pm$ 0.5**	1.95 $\pm$ 0.5**

M.S *Malva sylvestris*, PR progressive, NP nonprogressive, IM immotile, M motile, VCL velocity of the curved line, VSL velocity of straight line, VAP velocity of average pathway, LIN linearity, STR straightness, WOB wobble index, ALH amplitude lateral head displacement, BCF beat frequency. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. The data are presented as mean $\pm$ SD. Statistical significance was determined by $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

was slightly greater in the 1500 mg/kg treatment group than in the 750 mg/kg treatment group. Interestingly, the orange fluorescence associated with varicocele was significantly more pronounced in the 750 mg/kg treatment group ($P < 0.001$), suggesting a reduction in apoptosis within this group (Fig. 3C).

Apoptosis

Apoptosis usually occurs during normal spermatogenesis, resulting in the loss of 75% of the potential sperm

count. As a result, only 25% of the primary spermatocytes expected from A spermatogonia are produced. Disruption of apoptosis at this stage results in a male infertility phenotype. A comparison was made between varicocele rats and those receiving treatment with M.S. The proportion of sperm cells exhibiting phosphatidylserine displacement, which was located in the lower right quadrant and denoting primary apoptosis, was significantly greater in the varicocele model than in the treatment groups (Fig. 4).

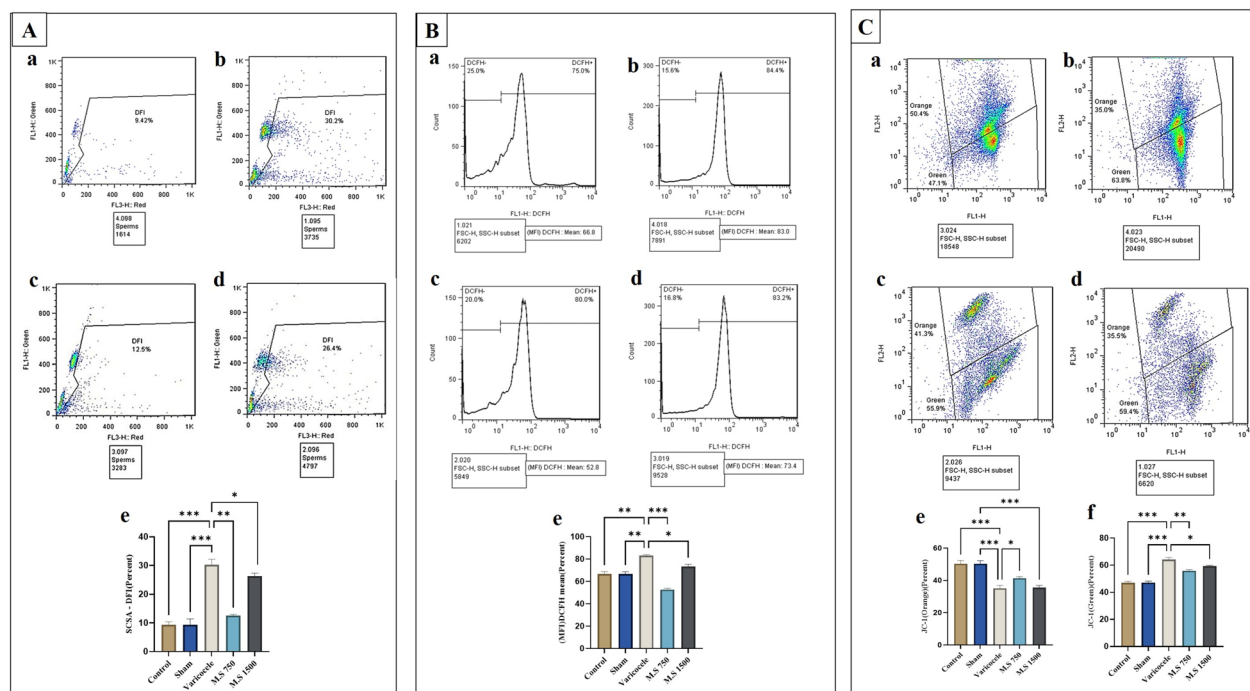


Fig. 3 Effects of *M.S* treatment (750 and 1500 mg/kg) on Sperm Chromatin Structure in varicocele-induced rats. **A** Comparison of sperm DFI strand damage through sperm chromatin structure analysis. **B** Comparison of DFI in the studied groups. **C** Comparison of the level of apoptosis by examining the MMP of the tissue testis with JC-1 dye in a: the control group, b: the varicocele group, c: the 750 mg/kg treatment group, and d: the 1500 mg/kg group. The extent of damage in the induction group and repair in the treatment group can be seen in this image, according to the chromatin structure (DFI) and mitochondrial membrane staining. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. A p value of ≤ 0.05 was considered significant. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). *M.S*: *Malva sylvestris*; DFI: DNA Fragmentation Index; SCSA: Sperm Chromatin Structure Assay; DFI: DNA Fragmentation Index

RT-qPCR

To investigate how epigenetic involvement could have potentially modulated the impact of *M.S* at a dose of 750 mg/kg in the testicular tissues of the varicocele model, the expression levels of *SIRT1*, *FOXO1*, *NRF2*, *NF-κB*, and *TGF-β* genes were assessed. The enzymatic activities of *SIRT1*, *FOXO1*, and *TGF-β* were significantly reduced in the testes following the development of experimental varicocele. However, after the 21-day administration of *M.S*, this trend was reversed (Fig. 5). *NF-κB* gene expression was upregulated in the varicocele group compared to the control group which experienced no significant changes following subsequent treatment with *M.S*. Furthermore, *NRF2* gene expression was significantly lower in the varicocele group than in the control group ($P < 0.05$) while in the treatment group, it was significantly lower than in the varicocele group ($P < 0.001$).

Discussion

This study underscores the complex interplay between oxidative stress (OS), genetic regulation, and apoptosis in varicocele-induced infertility, while highlighting the therapeutic potential of *M.S* extract in mitigating these

effects. The findings demonstrate that *M.S* administration, particularly at 750 mg/kg, significantly improves sperm quality, reduces DNA fragmentation, and restores mitochondrial membrane potential by modulating key antioxidant and anti-inflammatory pathways. Its efficacy is linked to the upregulation of *SIRT1* and *FOXO1*, which enhance cellular repair and antioxidant defenses, and the suppression of *NF-κB*, *TGF-β*, and *NRF2*, thereby attenuating OS and inflammation.

In comparison, the synthetic antioxidants butylated hydroxyl toluene (BHT) and ascorbic acid presented significantly higher FRAP values of 0.715 and 1.854, respectively, at the same concentration. Although the half-maximal effective concentration (EC₅₀) value of the *M.S* flower extract at 21.52 μg/mL was higher than the positive controls BHT and α-tocopherol at 3.23 and 2.25 μg/mL, respectively, it showed significant antioxidant properties by inhibiting active oxygen [37]. *M.S* exhibited an antioxidant activity of 42.372% and a DPPH half-maximal inhibitory concentration (IC₅₀) of 1.180. Ben Saad et al. (2017) investigated the impact of *M.S* extract on mitigating the detrimental effects of lithium carbonate on the testicles and heart.

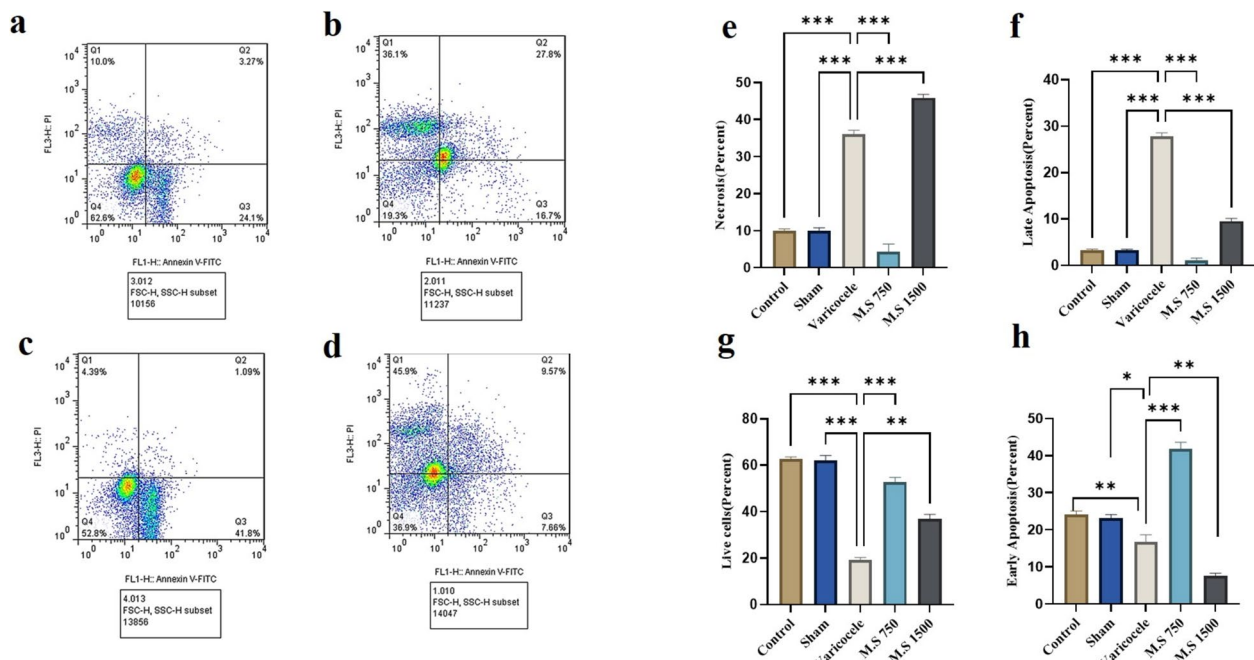


Fig. 4 Comparison of sperm cell apoptosis in the investigated groups. **a** Control group, **b** varicocele group, **c** 750 mg/kg treatment group, **d** 1500 mg/kg treatment group, **e** comparison of sperm cell necrosis in the investigated groups, **f** comparison of secondary apoptosis in sperm cells in the studied groups, **g** comparison of living sperm cells in the studied groups, **h** comparison of primary apoptosis in sperm cells in the studied groups. Differences in live and apoptotic cells are observed in the induction and treatment groups using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. A p value of ≤ 0.05 was considered significant. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). M.S 750: M.S 750 mg/kg, M.S 1500: M.S 1500 mg/kg. Q1: Necrosis, Q2: Late apoptosis, Q3: Early apoptosis, Q4: Live cells. M.S: *Malva sylvestris*

Their findings indicated that *M.S* effectively mitigates the harmful effects of lithium carbonate, which can be attributed to its interaction with intricate polysaccharides possessing antioxidative characteristics [22].

Recent studies have proposed that conditions such as ion imbalance, hypoxia, and alterations in blood circulation have the potential to elevate ROS in varicocele patients. ROS attack polyunsaturated fatty acids in sperm membranes, reducing motility and membrane integrity. ROS induce DNA strand breaks, leading to genetic abnormalities and reduced fertilization potential. Excessive ROS impair ATP production, essential for sperm motility. Antioxidants neutralize ROS, restore redox balance, and protect sperm integrity [38]. In the clinical setting, antioxidant compounds, such as kallikrein, L-carnitine, anthocyanin, silymarin, chrysin, selenium, resveratrol, and other similar agents are utilized for the management of varicocele. [39]. A clinical trial on 300 sub-fertile males showed that antioxidant blends significantly improved sperm count, motility, and reduced DFI in severe oligospermia cases [40, 41]. Examinations of various components of *M.S* have revealed that the leaves exhibit greater efficacy than the petiole. This disparity is attributed to flavonoid compounds with robust antioxidant properties. *M.S* flower extract has also displayed

major antioxidant capabilities, attributed to various compounds found within this botanical sample, such as phenols, flavonoids, and tocopherols [20, 37]. The assessment of the antioxidant potential of the methanolic extract of *M.S* flowers revealed a reduction of 0.355 at 0.5 mg/mL.

The present study examined the impact of *M.S* on sperm quality within a varicocele model. H&E staining, sperm count, and motility analyses indicated that *M.S* enhances spermatogenesis and mitigates testicular tissue injury. Sperm DFI serves as a quantitative measure for assessing sperm DNA damage, which is a significant contributor to male infertility. The increase in ROS levels can disrupt the inflammatory milieu, potentially raising DFI and impairing mitochondrial function and motility, which in turn leads to reproductive dysfunction [42, 43]. In the current study, sperm DFI was higher in the varicocele model group than in the control group, which weakened sperm motility. Following a 21-day treatment with *M.S*, a significant reduction was observed in sperm DFI.

Mitochondria play a key role in the production of ROS and superoxide radicals under low oxygen, thereby affecting sperm quality. The hypoxic-ischemic shock in varicocele can change sperm mitochondria, which leads to abnormal sperm and infertility. In addition, hyperpolarization of the sperm MMP increases superoxide

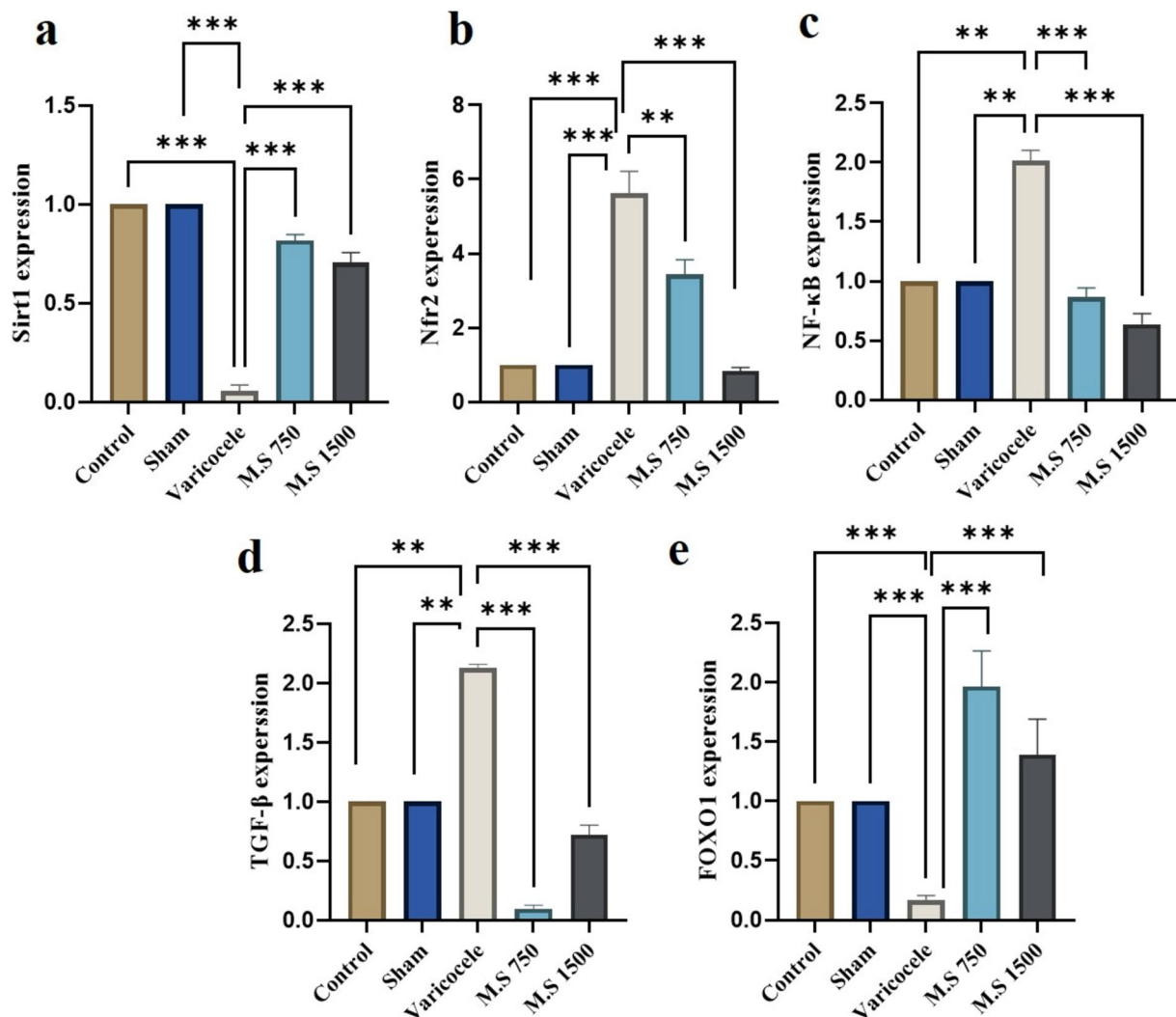


Fig. 5 Effects of M.S treatment (750 and 1500 mg/kg) on SIRT1, FOXO1, NF-κB, and TGF-β gene expression in the varicocele model. **a** SIRT1 gene expression, **b** NRF2 gene expression, **c** NF-κB gene expression, **d** TGF-β gene expression, **e** FOXO1 gene expression. *Malva Sylvestris* shows increased expression of SIRT1 and FOXO1 genes and decreased expression of NF-κB, TGF-β, and NRF2 genes. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) coupled with Tukey's post hoc test. A *p* value of ≤ 0.05 was considered significant. *P* < 0.05(*), *P* < 0.01(**), *P* < 0.001(***)). M.S: *Malva sylvestris*

production [33, 44]. In the present study, the relationship between mobility and MMP was evident. While there was a decline in mobility in the varicocele group compared to the control group, a marked improvement was observed in the mobility of *M.S* treatment groups compared to the varicocele group. Research has suggested that antioxidants facilitate the recovery of MMP and sperm motility. The results of the DPPH analysis showed that the level of antioxidants in *M.S* was satisfactory. Therefore, the antioxidant attributes of *M.S* can contribute to the restoration of MMP [35]. This study also found that ROS levels increase in varicocele, as increased thermal stress is associated with increased

generation of ROS by cellular organelles, such as mitochondria, the plasma membrane, the cytoplasm, and peroxisomes [45].

The bioactive compounds in *Malva sylvestris*—including flavonoids, polyphenols, and terpenoids—appear to directly inhibit NADPH oxidase (NOX enzymes), a major contributor to reactive oxygen species (ROS) generation in varicocele. Flavonoids such as quercetin and kaempferol may bind to critical NOX subunits, disrupting enzyme assembly and activity, thereby suppressing NOX-driven oxidative stress. By blocking NADPH oxidase, *Malva* diminishes the production of superoxide (O_2^-) and other ROS in testicular and sperm cells [15, 20].

Additionally, its phytochemicals act as direct ROS scavengers and enhance endogenous antioxidant defenses, such as superoxide dismutase (SOD) and glutathione, a mechanism supported by studies demonstrating *Malva*'s ROS-lowering effects in diabetic and inflammatory models [18, 46]. The reduction in ROS alleviates oxidative damage to mitochondrial membranes, preventing cytochrome c release and subsequent activation of caspase-9, which in turn suppresses downstream caspase-3 activity. This is critical, as elevated caspase-3 levels are strongly associated with sperm apoptosis in varicocele patients. Ultimately, the inhibition of caspase-3 helps preserve sperm DNA integrity and cellular viability, offering a protective mechanism against varicocele-induced infertility [47–49]. In this study, compared to the control group, the varicocele group presented diminished counts of viable cells and cells undergoing primary apoptosis. On the other hand, there was a significant increase in necrosis and secondary apoptosis levels in the varicocele group, indicating an advanced progression of apoptosis within the sperm cells, ultimately leading to necrosis. In the *M.S* 750 mg/kg treatment group, more viable cells and sperm cells undergoing primary apoptosis were detected than in the varicocele group. There was also a significant decline in the number of sperm cells undergoing secondary apoptosis and necrosis, which indicates the beneficial effects of *M.S* hydroalcoholic extract on spermatogenesis, and suggests that this extract, which functions as an antioxidant, possibly impedes the transition of sperm cells into subsequent apoptosis phases. However, the *M.S* 1500 mg/kg treatment group, compared to the varicocele group, presented notable increases in necrosis levels. There was also a significant decline in primary and secondary apoptosis in this particular group, signifying the progression of sperm cells toward necrosis. Therefore, this concentration of the extract did not yield optimal outcomes in managing the apoptosis process in varicocele pathology and potentially exerted cytotoxic effects.

The regulatory gene responsible for silencing information, known as *SIRT1*, plays a crucial role in enhancing the production of antioxidants, facilitating the repair of cells that have been compromised due to OS, and preventing cellular malfunction. Depletion of *SIRT1* results in impaired mitochondrial function accompanied by elevated levels of ROS, increased lipid peroxidation, and DNA harm in both male and female reproductive cells (sperm and eggs), ultimately causing infertility [50]. Matthew Coussens et al. (2008) revealed that spermatogenesis is significantly reduced as a result of *SIRT1* deficiency [51]. In another study, Taymour Mostafa et al. (2020) assessed *SIRT1* expression levels in males diagnosed with varicocele following surgical intervention, revealing a significant reduction in *SIRT1* in sperm, subsequently

leading to improvements in sperm parameters. Furthermore, this phenomenon was found to be associated with changes in seminal fluid composition [52]. Mostafa et al. reported that OS and *SIRT1* deficiency are the causes of male infertility in varicocele patients. Varicocele also induces OS, and *SIRT1* deficiency intensifies the effect of reducing the antioxidant defense of sperm [53, 54].

The *FOXO1* gene belongs to the FOXO protein family, which regulates the advancement of spermatogenesis, starting from the sustained self-renewal of spermatogonia to the onset of spermatogenesis and meiosis [55]. The reduction in *SIRT1/FOXO1* testicular expression levels in the varicocele group suggests a correlation between varicocele-related fertility disorders and the *SIRT1/FOXO1/OS* axis. The upregulation of *SIRT1* activity modulated by *M.S* in the experimental group implies a potential molecular interplay between *M.S* and *SIRT1* in varicocele-triggered OS, accompanied by increased *FOXO1* expression and antioxidant capabilities. The ability of *M.S* to increase *FOXO1* levels may be attributed to *SIRT1*-mediated *FOXO1* deacetylation, which facilitates the nuclear translocation, retention, and transcriptional function of its antioxidant enzymes. *SIRT1*-mediated deacetylation of proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) enhances mitochondrial biogenesis and metabolic oxidative process while suppressing proinflammatory pathways. Furthermore, nuclear *SIRT1* expression has a role in the modulation of nuclear factor erythroid 2-related factor 2 (*NRF2*) expression and the stimulation of robust transcription of antioxidant enzymes [19]. Yong Wang et al. (2018) revealed that grape seed proanthocyanidin extract (GSPE) can alleviate testicular oxidative damage resulting from varicocele by activating nuclear (erythroid-derived 2) antioxidant pathway (*NRF2*) [56].

NRF2 serves as a transcription factor responsible for upregulating the expression of antioxidant proteins, thereby exerting protective effects against OS-induced damage [57]. *SIRT1*, in conjunction with *NF-κB*, represents an additional prospective pathway that serves a significant function in diverse biological phenomena, such as inflammation, apoptosis, and OS [58]. Volkan Tuğcu et al. (2010) reported the *NF-κB* transcription factor in varicocele experimental models [59]. A link has been found between the reduced expression of *SIRT1* in the varicocele group and elevated levels of acetylated *NF-κB*, which can be attributed to compromised mitochondrial biogenesis, heightened OS, and increased inflammatory responses. Furthermore, the dysfunction of mitochondria, which leads to the disruption of MMP, has been found to strongly associate with irreversible alterations in permeability [60]. These alterations ultimately trigger a series of breakdown reactions

that result in increased apoptotic cell death capacity, as revealed in the present study.

Previous studies have also shown that transforming growth factor-beta (TGF- β) levels are elevated in individuals diagnosed with varicocele, indicating a possible link to diminished sperm characteristics and impaired spermatogenesis. A study conducted in 2008 examined epidermal growth factor (EGF) and TGF- β levels in infertile varicocele patients compared to fertile individuals to analyze the association between TGF- β and sperm plasma. The results indicated while the levels of EGF and TGF-Semen plasma β increased in varicocele patients, seminal parameters decreased in this group [61]. Dobashi et al. also found increased expression of TGF- β 1 in varicocele testes, indicating TGF- β 1 plays a role in seminiferous tubule fibrosis and potentially leads to spermatogenesis impairment [62]. A 2010 study further investigated TGF- β 1 expression in the pathogenesis of human testicular tissue. Monohistochemical studies have revealed the presence of TGF- β 1 and its specific receptors in Leydig cells in samples obtained from both normal tissue and from patients diagnosed with Sertoli cell-only syndrome (SCO) or hypospermatogenesis. Although the presence of Leydig cell hyperplasia (LCH) is not confirmed, it is suggested that its expression is likely to be elevated [63]. In another study, Kowalewski et al. reported higher TGF- β levels in the varicocele group compared to the control group [64].

The present study revealed a significant reduction in the expression of *SIRT1* and *FOXO1* genes in the varicocele group, demonstrating an association with the level of ROS. Furthermore, there was a significant increase in *NF- κ B*, *TGF- β* , and *NRF2* genes in the varicocele group. Previous research has indicated that antioxidative agents confer protection to testicular tissues from OS and apoptosis in varicocele-induced rat models [65, 66]. In essence, this study demonstrated notable alterations in *SIRT1*, *FOXO1*, *NRF2*, *NF- κ B*, and *TGF- β* gene expression, and in OS levels in rats induced by varicocele, which aligns with the findings of previous studies. The present findings indicated the cytoprotective capacity of *M.S* at 750 and 1500 mg/kg concentrations as a potent antioxidative agent in ameliorating varicocele-related complications through modulating OS and apoptotic pathways. *M.S* exhibited anti-inflammatory properties by upregulating *SIRT1* and *FOXO1* genes while downregulating *NF- κ B*, *TGF- β* , and *NRF2* genes, which consequently diminished ROS levels.

The findings from this study and related research position *M.S* as a promising adjunct therapy for managing OS-driven male infertility, particularly in conditions like varicocele. *M.S* offers a natural polyphenol-rich

alternative with synergistic compounds that enhance ROS scavenging and mitochondrial protection. Post-Varicolectomy surgical administration of *M.S* could mitigate residual OS, accelerate recovery of spermatogenesis, and reduce sperm DFI, potentially improving pregnancy rates. While further clinical validation is essential, its ability to modulate SIRT1/FOXO1, inhibit NADPH oxidase, and suppress apoptosis positions it as a compelling candidate for integrative fertility care.

The current study has some limitations. This investigation offers more substantial evidence regarding the antioxidant properties of *M.S* by analyzing alterations in the expression of proteins that play a role in cellular signaling pathways. Nevertheless, the research team encountered obstacles in acquiring the necessary antibodies because of financial and political limitations. Furthermore, the absence of advancements in functional organoid technology and testicular tissue constructs compelled the team to depend on animal models for this study.

Conclusions

The findings of this study, along with previous research, imply that *M.S* extract can alleviate tissue damage caused by OS due to varicocele in the testicular region. These findings provide fundamental insights into the positive effects of *M.S* extract on sperm characteristics in male rats with experimental varicocele. The findings also show that *M.S* substantially contributes to the management of OS, cell viability, and the enhancement of protective mechanisms on testicular tissue in male rats suffering from varicocele. Further studies are needed to ascertain whether *M.S* can improve fertility outcomes in varicocele patients.

Abbreviations

ROS	Reactive Oxygen Species
OS	Oxidative Stress
CASA	Computer-Assisted Sperm Analysis
VCL	Curvilinear Velocity
VAP	Average Path Velocity
VSL	Straight-Line Velocity
LIN	Linearity Index
STR	Oscillation Index
WOB	Wobbling Index
BCF	Beat Cross Frequency
ALH	Amplitude of Lateral Head
H&E	Hematoxylin And Eosin
PBS	Phosphate-Buffered Saline
SCSA	Sperm Chromatin Structure Assay
DFI	DNA Fragmentation Index
HDS	High-Density Staining
DCFH-DA	2',7'-Dichlorofluorescein Diacetate
H2O2	Hydrogen Peroxide
DHE	Dihydroethidium
DCF	Dichlorofluorescein
HE	2-Hydroxyethidium
MMP	Mitochondrial Membrane Potential
PS	Phosphatidylserine
FITC	Fluorescein Isothiocyanate
one-way ANOVA	One-way Analysis of Variance

MSTD	Mean Seminiferous Tubule Diameter
MSET	Mean Seminiferous Epithelial Thickness
TAC	Total Antioxidant Capacity
BHT	Butylated Hydroxyl Toluene
IC ₅₀	Half-maximal Inhibitory Concentration
EC ₅₀	Half-maximal Effective Concentration
PPARGC1A	Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
EGF	Epidermal Growth Factor
TGF-β	Transforming Growth Factor-Beta
LCH	Leydig Cell Hyperplasia
SCO	Sertoli Cell-Only syndrome

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Authors' contributions

Conceptualization and Project administration, writing review & editing: LKB, FK; Supervision, Validation: MA; Methodology, Investigation, Resources, writing – original draft: FK.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

There were no "human subjects" in this study. All animal experiments comply with the ARRIVE guidelines and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. The study was approved by the Ethics Committee of Kharazmi University under code IR.KHU.REC.1402.091.

Competing interests

The authors declare no competing interests.

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