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Phoenixin Attenuates Monosodium Glutamate-Induced Testicular Toxicity in Rats: A Potential Protective Approach

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Keywords

- Phoenixin
- Monosodium Glutamate
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Abstract

Background: Monosodium glutamate (MSG), a widely utilized food additive, has been implicated in the provocation of testicular toxicity through oxidative stress and hormonal dysregulation. **Aim:** This study explores the potential role of phoenixin in ameliorating MSG-induced testicular toxicity. **Methods:**Forty male Wistar rats were randomly assigned to four distinct groups (n=10 per group): Control, Phoenixin, MSG, and Phoenixin+MSG, and were subjected to the experimental protocol over30 days.Semen samples were analyzed for sperm count, motility, and viability. In serum, Gonadotropin-releasinghormone (GnRH), Luteinizinghormone (LH), follicle-stimulating hormone (FSH), testosterone, and kisspeptin were quantitatively assessed.The activity of the testicular steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD, together with markers of testicular oxidative stress and tumor necrosis factor-alpha (TNF-α), were assessed. Additionally, the testicular levels of silent information regulatorof transcription 1(SIRT1), phosphorylated AMP-activated protein kinase (p-AMPK), andcaspase-3 immunoreactivity were estimated. **Results:**MSG exposure significantly impaired sperm parameters, disrupted hormonal balance, and triggered oxidative stress and inflammatory responses in the MSG group compared to the Phoenixin and control groups. In contrast, co-administration of phoenixin in the Phoenixin+MSG group significantly restored sperm function, improved serum sex hormone profile, enhanced testicular antioxidant defenses, upregulated p-AMPK and SIRT1 expression, while attenuating TNF-α expression and caspase-3 immunoreactivity compared to the MSG group. Our findings underscore the pivotal role of phoenixin in ameliorating testicular dysfunction and promoting steroidogenic activity in our experimental model. **Conclusion:**Phoenixin effectively protected against MSG-provoked testicular toxicity through the restoration of the hypothalamic-pituitary-gonadal(HPG) axis, enhancement of steroidogenesis, attenuation of oxidative stress, inflammation, and apoptotic cascade, which was enforced by its upregulation ofthe SIRT1/AMPK signaling. These results imply a beneficial role of phoenixin in mitigating the reproductive dysfunction associated with MSG testicular toxicity.

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Introduction

The reproductive systems of both males and females are highly vulnerable to a variety of harmful environmental factors. Poor nutrition, medical disorders, industrial pollutants, and food additives are among numerous factors that have been linked to the onset and progression of reproductive dysfunction [1].

The synthetic sodium salt monosodium glutamate (MSG) is made of glutamate,anon-essential amino acid. MSG is usually added to food to enhance its flavor and is typically utilized unlabeled in various culinary items. As a result, it is impossible to determine how much MSG a typical individual consumes daily **[2]** Several biological systems are susceptible to elevated MSG levels; meanwhile, one extremely susceptible MSG target is the reproductive system. It is more vulnerable to peroxidative and excitotoxic damage because it contains large amounts of polyunsaturated fatty acids, glutamate receptor abundance, and low antioxidant reserves **[3].** When given orally to rats, MSG results in oxidative glutamate damage, hemorrhage in the testes, and low sperm count **[4].**

Phoenixin, a recently discovered neuropeptide localized primarily in the hypothalamus, has an emerging role in regulating reproductive function, neuroprotection, energy homeostasis, and the gut-brain axis**[5].** Phoenixin-14, a 14-amino acid version, is the most researched form of the new peptide hormone. The hypothalamus, along with other regions of the brain and peripheral tissues involving the reproductive system, possesses significant levels of its expression**[5].**

The axis between the hypothalamus, pituitary, and gonadal, which governs reproductive function, is regulated by phoenixin. The hypothalamus and pituitary gland express their receptor, GPR173**[6].**In addition, phoenixin was reported to act locally on the ovarian mitochondrial machinery and overexpress the gonadotropin-releasing hormone (GnRH) receptors, through which it could elevate the ability of ovarian cells to withstand oxidative, inflammatory, and apoptotic damage, with a sequel of alleviating obesity-induced reproductive impairment **[7].**

Although the literature on phoenixin's impact on testicular dysfunction is scarce, Yilmaz et al.,**[8]**reported that phoenixin therapy could mitigate torsion-detorsion of the testes. The authors attributed the protective effect of phoenixinto itsanti-inflammatory effect and antioxidative ability, highlighting the potential for further investigation into its therapeutic role in improving other forms of testicular injury.

The NAD-dependent deacetylase protein, SIRT1, controls several sperm biological functions, such as repair of DNA, survival of the cell, autophagy, apoptosis, and energy homeostasis through adenosine monophosphate-activated protein kinase (AMPK) activation **[9].** As far as we are aware, there are no findings on how MSG and phoenixin affect the testicular SIRT1 and its signaling cascades.

These aforementioned data could provide a scientific rationale for considering phoenixin as a potential protective agent against MSG-induced testicular deterioration. The scarcity of scientific research examining phoenixin's preventive benefits

against testicular dysfunction linked to MSG further underscores the significance of our study in addressing this gap.

2. Materials and methods

2.1. Drugs and chemicals

Phoenixin-14 amide was procured from Sigma-Aldrich Corporation, headquartered in St. Louis, Missouri, USA. The MSG, a crystalline powder, was formulated by dissolving a measured amount in distilled water to achieve the designated concentration. All additional chemicals were sourced from Sigma Chemical Company (MO, USA).

2.2 Animals

In February 2025, a study was conducted at Tanta University using 40 albino rats (Wistar strain, males) with a weight range of 180 to 220 grams. The animals were distributed randomly into adequately ventilated cages, with five rats per enclosure, and housed under standardized conditions (22 ± 2°C temperature and a 12-hour alternating light and dark schedule). For the entire duration of the study, the animals were provided unrestricted ad libitum access to a standard laboratory rodent diet and drinking water. The study protocols received ethical approval fromthe Medical Research Ethics Committee of Tanta University's Faculty of Medicine (Approval Code: [36264PR1050/1/25]).

2.3. Experimental design

Following a seven-day acclimation phase, the rats were randomly allocated into four experimental groups, each comprising ten animals, and subjected to daily treatments for 30 days in all groups:

- ***Group I (Control):**Received distilled water and isotonic saline daily via oral gavage.
- ***GroupII (Phoenixin):** Received phoenixin at a dosage of 100 ng/g body weight after dissolution in sterile isotonic salinevia gastric gavage daily, as established by **[10].**
- ***Group III (MSG):** Treated with monosodium glutamate (MSG) at a dosage of3 g/kg/day after dissolution in distilled water via oral gavage, a regimen previously shown to induce testicular toxicity and reproductive impairment in rats as reported by **[11, 12].**
- ***Group IV (Phoenixin + MSG):** Received combined phoenixin and MSG treatments, following the same protocols as groups II and III, administered concurrently.

2.4. Experimental procedures

Upon completion of the 30-daysexperiment, rats were givenan intraperitoneal (IP) injection of sodium pentobarbital administered at a dosage of 60 mg/kg **[13],**then were sacrificed via cervical dislocation. Blood was collected via cardiac puncture, then serum was collected and cryopreserved at −80°C for later biochemical testing.

Following this, a laparotomy was performed to excise the testes and epididymis. The organs were rinsed in 1.15% potassium chloride (KCl)solution at 0°C, dried completely, and weighed. The epididymal tissue was processed for *semen evaluation.*Seminal fluid specimens were obtained from the cauda region of the epididymis to evaluate sperm motility, concentration, and viability. The cauda region was surgically excised, weighed, and finely minced in 2 mL of physiological saline. The tissue was then

maintained at ambient temperature to allow spermatozoa to disperse into the saline solution **[14].**

Then, each testis was cut into three parts. The first part was homogenized in 1.15% KCl and centrifuged at (3000 rpm, 10 minutes, 4°C) to prepare supernatants for *biochemicalanalysis* The second portion was maintained in a frozen state at −80°C for future *RNA extraction and molecular assay.* The third portion was preserved in Bouin's solution for 24 hours. Subsequently, the tissue was dehydrated using progressively concentrated ethanol solutions, cleared, and then embedded in paraffin. Thin sections (5 μm in thickness) were then sliced and stained with hematoxylin and eosin (H&E) for *histopathological* and *immunohistochemical assay.* Testicular protein levels were quantified using the Bradford method **[15].**

2.4.1Sperm function evaluation:

-Formotility assessment, a droplet of the sperm sample was placed onto a microscope slide preheated to a temperature of 37 degrees Celsius. The proportion of motile sperm was analyzed via examining ten randomly selected fields at 400x magnification, with the average motility calculated using the methodology outlined in **Cheng et al.[16]**

-Sperm Viability Assessment: Seminal smears were prepared with eosin-nigrosin staining, a method described by **Filler [17].** Microscopic analysis was performed to differentiate live (unstained) from dead (stained) spermatozoa, enabling the calculation of the viability percentage based on the alive-to-dead ratio.

-Sperm count analysis: The quantification of sperm count per milliliter was conducted following the protocol outlined by **Robb et al. [18].** Semen samples were diluted at a 1:5 ratio (volume/volume) using normal saline supplemented with 40% formalin to immobilize spermatozoa. The total sperm count was subsequently determined using a Neubauer hemocytometer.

2.4.2Biochemical assays:

2.4.2.1Assessment of hormonal profile:

Serum levels of **Gonadotropin-releasing hormone (**GnRH) and **luteinizing hormone (**LH) were quantified using Enzyme-Linked Immunosorbent (ELISA) kits from LifeSpanBioSciences, Inc. (Washington, USA; Catalog No: LS-F13002 and LS-F27508, respectively). **Testosterone** was analyzed utilizing a kit from Aviva Systems Biology (San Diego, USA; Catalog No: OKEH02537),**follicle-stimulating hormone** (FSH)was determined by using a kit from Cloud-Clone Corp. (Houston, USA; Catalog No: CEA830Ra), and Kisspeptin was analyzed using a kit supplied by CUSABIO Life Science (Maryland, USA; Code: CSB-E13434r).

2.4.2.2Assessment of testicular oxidative stress biomarkers:

Malondialdehyde (MDA), a recognized biomarker of oxidative lipid damage, was measured in testicular tissue homogenates using a thiobarbituric acid-based spectrophotometric technique, as outlined in the protocol by**Mihara and Uchiyama [19].** Concurrently, the enzymatic **activity of glutathione peroxidase (GPx)**, an antioxidant enzyme, was evaluated by

Biodiagnostic Co.'s colorimetric assay kit** (Giza, Egypt, Catalog No:GP 2524) to assess antioxidant capacity. Furthermore, oxidative DNA damage was evaluated by quantifying the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG)in the homogenate via an ELISA kit (Catalog No: E-EL-0028, Elabscience, USA), providing insight into DNA oxidation.

2.4.2.3 Assessment of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD enzymes activity:

The enzymatic activity of 3β-HSD and 17β-HSD, critical enzymes in androgen synthesis and steroidogenic machinery, was measured in testicular homogenates using methodologies adapted from **Talalay et al [20]** and **Jarabak et al [21]**, respectively.

2.4.2.4 Assessment of testicular p-AMPK levels:

Testicular phosphorylated AMP-activated protein kinase (p-AMPK) concentrations were quantified via an ELISA kit (Catalog No. MBS1602983, MyBioSource, San Diego, USA).

2.4.2.5Real-time quantitative PCR (qPCR) of testicularSIRT-1and tumor necrosis factor-α (TNF-α)

Extraction of total RNA from frozen testicular tissue using Thermo Scientific GeneJET RNA Purification Kit (comparable to Catalog No: K0731, Waltham, MA, USA) was carried out, followed by estimation of RNA concentration and purity using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, NC, USA).

The cDNA synthesis from 5 µg of total RNA was performed using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA; Catalog No: EP0451). Subsequently, 2 µL of the resulting cDNA was amplified via SYBR Green-based quantitative PCR (qPCR) utilizing Applied Biosystems' StepOne Plus Real-Time PCR platform. The primer sequences utilized in the reactions are presented in **Table 1.**

Table 1. Primer sequences for qRT-PCR.

Gene	GenBank Accession No.	Forward	Reverse
SIRT-1	NM_001372090.1	5′-GTG GCA GTA ACA GTG ACA GTG-3'	5′-GTC AGC TCC AGA TCC TCC AG-3′
TNF-α	NM_036807.1	5′-CCCTGGTACTAACTC CCAGAAA-3'	5′-TGTATGAGAGGGACGGAA CC-3'
GADPH	NM_017008.4	5′- CATGCCGCCTGGAGAAACCTGCCA-3′	5′-GGGCTCCCCAGGCCCCTC CTGT− 3′.

The thermal cycling settings were: An initial step of activation, subsequent denaturation, annealing, and extension. The study analyzed quantitative PCR data by first determining the cycle threshold (Ct) measurements for the genes of interest. These values were normalized against the Ct of the housekeeping gene GAPDH to account for variations in sample loading. Relative gene expression was then quantified as fold change using the $2^{-\Delta\Delta Ct}$ formula, following the Livakmethod**[22].**

2.4.3Histological study:

Both seminiferous tubules and interstitial spaces were included in the samples. Following a 24-hour fixation period in Bouin's solution, a tissue fragment was dried by a series of graded ethanol, cleared, and embedded in paraffin wax**[23]**. Serial paraffinsections were cut at 5 μm thickness, stained byHematoxylin and Eosin (H&E), and subjected tohistopathologicalexamination**[24]**.

2.4.3.1Immunohistochemical analysis for caspase 3

Following dewaxing and rehydration, paraffin-embedded tissue sections were washed with phosphate-buffered saline (PBS) for immunohistochemistry. The slides were incubated in a water bath maintained at 95–98°C for 20 minutes after being submerged in 10 mM sodium citrate buffer (pH 6.0) to retrieve the antigen. The slides underwent treatment using 3% hydrogen peroxide for 10 minutes at ambient temperature to stop the endogenous peroxidase activity. Following this, the slides underwent three PBS washes, each lasting five minutes **[25]**.

The sections were treated with a rabbit-derived polyclonal antibody targeting caspase-3, an apoptosis biomarker **(26)** for a whole night at 4°C in a humidified incubation chamber (Abcam, Cambridge, MA, USA) with antibodies diluted at a ratio of 1:1000. The study then used the Avidin-Biotin-Peroxidase method for the immunohistochemistry identification of caspase-3 **[27]**. Tissue sections were treated without the main antibodies for negative controls **[25]**. Caspase-3-positive cells showed distinct brown cytoplasmic staining, indicating immunoreactivity **[28]**.

2.5. Statistical analysis:

The data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to compare differences across multiple groups, with Tukey's post hoc test utilized for pairwise comparisons. Statistical significance was established at $P < 0.05$. Data analysis was performed using IBM SPSS Statistics software (Version 23.0, NY, USA)."

3. Results

3.1. Impact of phoenixin and MSG on semen analysis (sperm count, motility &viability)

Relative to the control and phoenixin groups, the MSG group's sperm count, motility, and viability were significantly lower. However, as Figure 1 illustrates, co-administration of phoenixin and MSG significantly mitigated these parameters in comparison to the MSG-treated group.

3.2. Impact of phoenixin and MSG on the HPG axis

As displayed in Figure 2, the MSG-treated rats' serum levels of GnRH, FSH, LH, testosterone, and Kisspeptin levels exhibited a significant decline relative to those of phoenixin and the control groups. In the meantime, these hormones were significantly raised by the simultaneous administration of MSG and phoenixinas opposed to the MSG group. According to these findings, Phoenixin intervention could reverse the disruption in the HPG axis, which was reflected by improvement of the MSG-induced reproductive dysfunction.

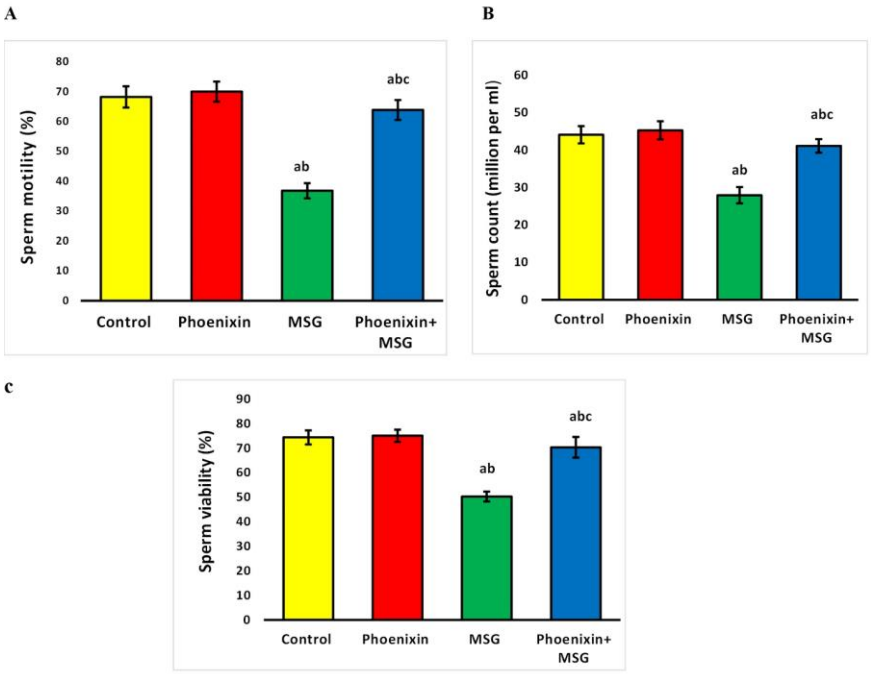

Figure 1. Impact of phoenixin and MSG on semen analysis. **(A)** sperm motility, **(B)** sperm count, **(C)** sperm viability. Note: Data are presented as mean ± SD (n = 10). [a] p<0.05 Vs. Control, [b]p<0.05 Vs Phoenixin, [c] p<0.05 Vs. MSG group. MSG, monosodium glutamate.

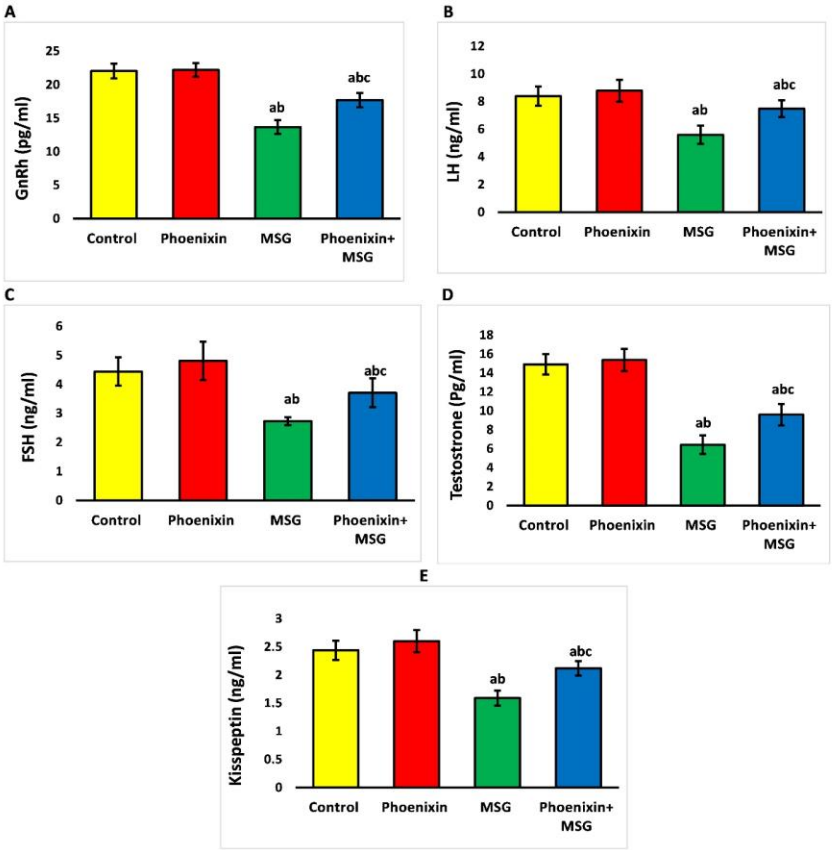

Figure 2. Influence of phoenixin & MSG on HPG axis and kisspeptin levels. **(A)** GnRH, **(B),** LH **(C),** FSH **(D)** Testosterone, and **(E)** kisspeptin. Note: Data are expressed as mean± SD (n=10). [a] p <0.05 Vs. Control, [b]p<0.05 Vs Phoenixin, [c]<0.05 Vs MSG group.MSG, monosodium glutamate; FSH, Follicle-Stimulating Hormone; LH, Luteinizing Hormone; GnRH, Gonadotropin-releasing hormone.

3.3. Impact of phoenixin and MSG on steroidogenic testicular enzymes (3B-HSD and 17B-HSD)

As displayed in Table 2 and comparable to the control and phoenixingroups, the MSGgroup's enzyme activities of 3B-HSD and 17B-HSD were significantly lower. However, rats given MSG and phoenixin together showed significantly greater 3B-HSD and 17B-HSD activity than those in the MSGgroup.

3.4. Phoenixin ameliorated MSG-induced testicular oxidative stress and inflammation

MSG supplementation for 30 days led to significantly greater levels of MDA, 8.OHdG, and TNFα, along with lower levels of GPx, in contrast to the phoenixin and control groups. But, as seen in Table 3, when phoenixin and MSG were given in combination, the previously mentioned characteristics were inverted.

3.5. Phoenixin reversed the MSG impact on the testicular SIRT1/AMPK axis

Comparable to the control and phoenixin groups, the MSG group demonstrated a significant downregulation of testicular SIRT1 and p-AMPK, as illustrated in Figure 3. In the meantime, this pathway was significantlyupregulated when phoenixin and MSG were administered at the same time. This may lend credence to the idea that SIRT/AMPK plays a part in phoenixin'sprotection against MSG-induced testicular damage.

3.6 Impact of phoenixin on the testicularhistological structure:

Histological analysis of testicular tissue from both the control and phoenixingroups demonstrated structurally normal seminiferous tubules inthe testis (Figure 4 A-F). Seminiferous tubules were rounded to oval and lined with stratified germinal epithelium. Their lumina are filled with mature spermatozoa. Interstitial tissue contains clusters of Leydig cells and seminiferous tubules with intact basement membranes. Sertoli cells and spermatogonia rest on the basement membrane. Seminiferoustubulesare lined by spermatogonia, spermatocytes, and mature spermatids. Interstitial tissue contains clusters of Leydig cells, and seminiferous tubules are surrounded by myoid cells. There are large irregular pale nuclei of Sertoli cells, darkly stained spermatogonia, spermatocytes with large nuclei, round and elongated spermatids, and mature spermatozoa in the lumen.

However, the histological examination of the MSG group (Figure 4 G-I) demonstrated structural alterations in the seminiferous tubules, including irregular tubules, some appear with irregular germinal epithelium, and others show the separation of epithelium from the basement membrane. Eosinophilic material was noted within vacuolated Leydig cells. The lumen had few sperm with hypoplasia of the cells.The lumen had few sperm, and the interstitial tissue showed congested and dilated blood vessels. Spermatogenic cells showed cytoplasmic vacuolation and dark, small pyknotic nuclei. Wide intercellular spaces can be seen.

Meanwhile, the phoenixin + MSG group (Figure 4 J-L) exhibited restoration of the seminiferous tubule architecture, although intertubular spaces remained widened, and the spermatogenic cells appeared nearly normal. Stratified epithelium extends from the base to the lumen, with the lumen filled with slight edema between tubules.

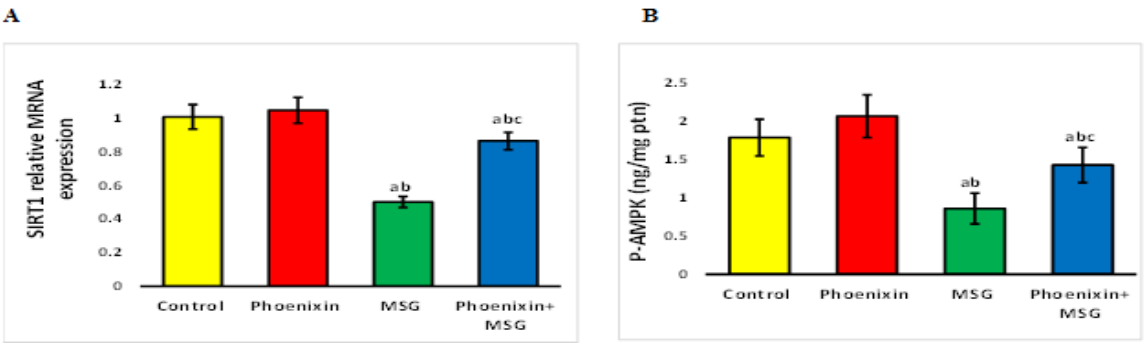

Figure 3. Impact of phoenixin and MSG on the testicular SIRT/AMPK axis. (A) SIRT1 relative mRNA expression and (B) p-AMPK. Note: Data are stated as mean ± SD (n = 10). [a] p<0.05 Vs. Control I, [b] p<0.05 Vs. Phoenixin, [c] p<0.05 Vs. MSG group. MSG, monosodium glutamate,SIRT1: Silent information regulator 1, P-AMPK: phosphor-adenosine monophosphate-activated protein kinase.

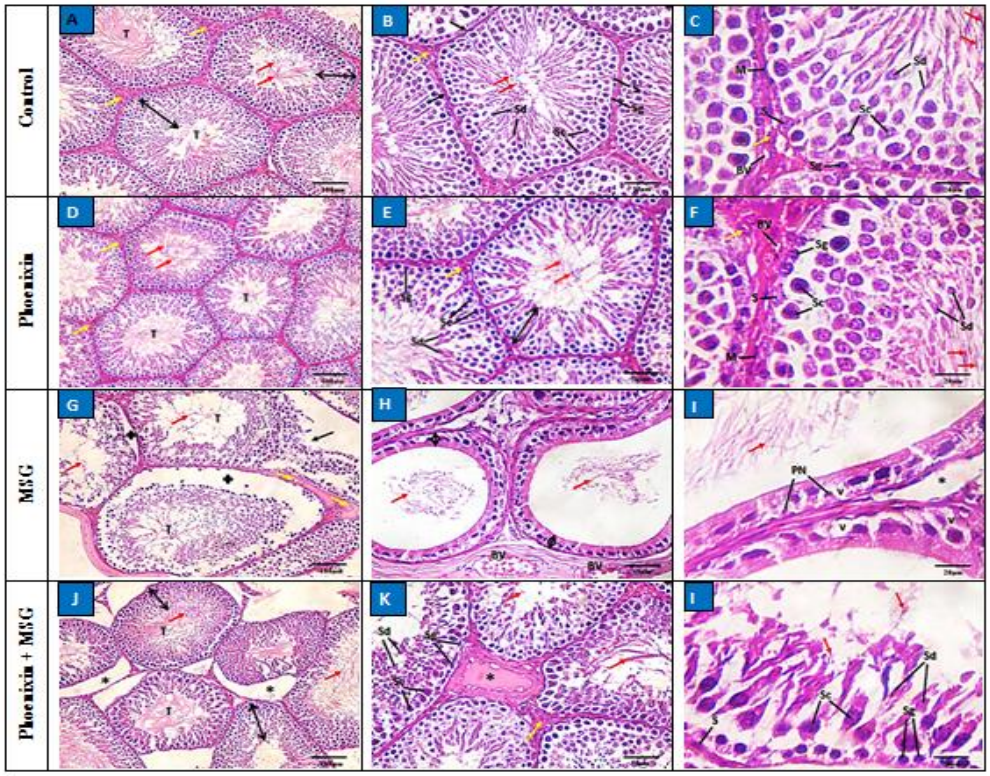

Figure 4: Histological examination of the testis from the groups studied. **(A-C)The control group** demonstrated: **(A)** Rounded to oval seminiferous tubules (T) lined with stratified germinal epithelium (double head black arrow). Their lumina are filled with mature spermatozoa (red arrows). Interstitial tissue containing clusters of Leydig cells (yellow arrows), **(B)** Seminiferous tubules with intact basement membrane (black arrows). Sertoli cells (S) and spermatogonia (Sg) rest on basement membrane. Seminiferous tubule lined by spermatogonia, spermatocytes (Sc), spermatids (Sd), and mature spermatozoa (red arrows). Interstitial tissue contains clusters of Leydig cells (yellow arrow), and **(C)** Seminiferous tubules are surrounded by myoid cells (M). There are large irregular pale nuclei of Sertoli cells (S), darkly stained spermatogonia (Sg), spermatocytes (Sc) with large nuclei, round and elongated spermatids (Sd), and mature spermatozoa (red arrows) in the lumen. Interstitial Leydig cells (yellow arrow) can be seen. **(D-F)Phoenixin group** showed: **(D)** Seminiferous tubules (T) with lumen filled with spermatozoa (red arrows). Interstitial tissue contains clusters of Leydig cells (yellow arrows), **(E)** Spermatogenic cells extend from the base to the lumen (double-headed black arrow). There are spermatogonia (Sg), spermatocytes (Sc), spermatids (Sd), and spermatozoa in the lumen (red arrows). Interstitial tissue contains clusters of Leydig cells (yellow arrow), **(F)** Seminiferous tubule surrounded by myoid cells (M). There are irregular elongated pale nuclei of Sertoli cells (S) and darkly stained spermatogonia (Sg) resting on the basement membrane. Spermatocytes (Sc), round and elongated spermatids (Sd), and a lumen filled with spermatozoa (red arrows). The interstitial Leydig cells (yellow arrow) and blood vessels (BV) can be seen. **(G-I)** The **MSG group showed**: **(G)** Irregular tubules (T), one appears with irregular germinal epithelium (black arrow) and others show the separation of epithelium from the basement membrane (+). Eosinophilic material with vacuolated Leydig cells (yellow arrows). The lumen has few sperms (red arrows), **(H)** Hypoplasia of cells (double head black arrow). The lumen has few sperms (red arrows). Interstitial tissue has congested and dilated blood vessels (BV), **(I)** Spermatogenic cells show cytoplasmic vacuolation (V) and dark small Pyknotic nuclei (PN). Wide intercellular spaces (*) can be seen. The lumen has few or no sperms (red arrow). **(J-L)Phoenixin + MSG group**showed: **(J)** Apparently normal tubules (T) with wide spaces (*). Stratified epithelium extends from base to lumen (double head black arrow). The lumen is filled with spermatozoa (red arrows), **(K)** Slight edema (*) between tubules. Spermatogonia (Sg), spermatocytes (Sc), spermatids (Sd), and a lumen filled with spermatozoa (red arrows). Interstitial tissue contains clusters of Leydig cells (yellow arrow), **(L)** Sertoli cells (S), basal darkly stained spermatogonia (Sg), large spermatocytes (Sc), rounded or oval spermatids (Sd) and mature spermatozoa (red arrows). Hematoxylin and eosin staining (A, D, G & J) (×200, scale bar = 100 µm), (B, E, H & K) (×400, scale bar = 50 µm) and (C, F, I & L) (×1000, scale bar = 20 µm).

3.7 Impact of phoenixin on the testicular caspase-3 immunohistochemistry

Immunohistochemical analysis of specimens fromthe control and phoenixin groups revealednegative caspase-3 immunostaining in spermatogenic cells. Conversely,the MSG group exhibited strong positive caspase-3 immunostaining within all spermatogenic cell layers in most of the seminiferous tubules, meanwhile, the Phoenixin + MSG group showed variable reaction to immunostaining. Some spermatogenic cells have negative reactions, while others appear with positive reactions, as displayed in **Figure 5.**

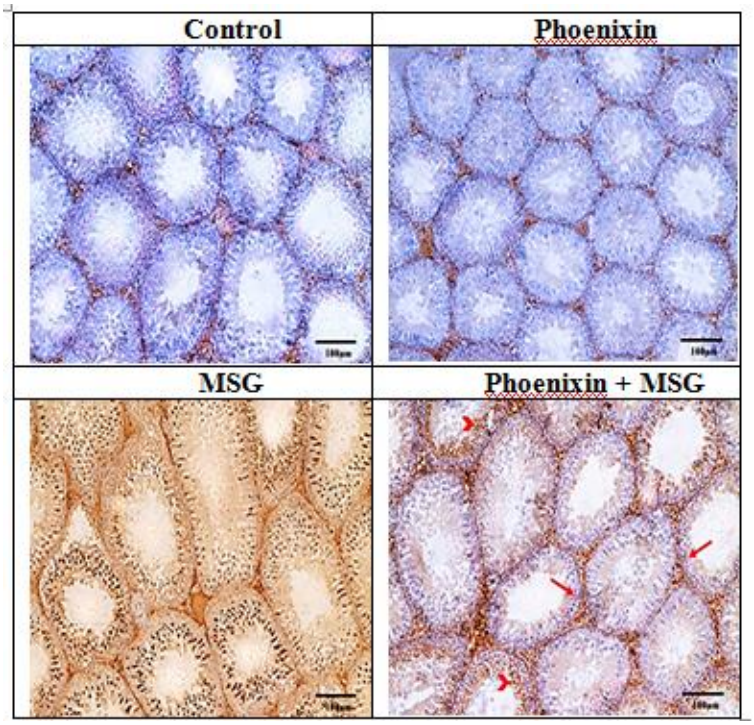

Figure 5: Photomicrograph of caspase-3 immunostaining in the rats' testes demonstrated the protective role of phoenixin against MSG-induced testicular injury. **Control and Phoenixin groups** exhibited negative caspase-3 immunostaining in spermatogenic cells, the **MSG group** showed strong positive caspase-3 immunostaining within all spermatogenic cell layers in most of the seminiferous tubules, meanwhile, the **Phoenixin + MSG** group showed variable reaction to immunostaining. Some spermatogenic cells show negative reactions (arrows), while others appear with positive reactions (arrow heads). Original magnification x100, scale bar 200 μm.

4. Discussion:

The foremost outcome of our research is that the HPG axis imbalance, coupled with inflammatory, oxidative stress, and apoptotic cascade, could be the main fundamental causes of MSG-induced testicular toxicity. Meanwhile, the simultaneous administration of phoenixin and MSG could alleviate the MSG-provoked testicular toxicity, as evidenced by the mitigation of the disturbed hormonal profile, testicular architecture, and sperm analysis parameters.

Our results showed that MSG signified a real challenge for spermatogenesis, as revealed by the MSG group's deteriorated sperm parameters relative to the control group.

Impaired spermatogenesis may result from inherent issues within germ cells, supporting Sertoli cells, or Leydig cells, or it may be attributed to the disrupted endocrine signals that regulate the entire spermatogenetic process **[29].**

At the central level, our findings highlighted that MSG interferes with the normal functioning of the HPG axis, mirrored by reduced serum GnRH, FSH, LH, and testosterone, which are mandatory for normal spermatogenesis and testicular function. This is most likely a consequence of excitotoxic neuronal destruction in the hypothalamus that could be a sequel of excess stimulation of N-methyl-D-aspartic acid (NMDA) receptors **[30].** This diminishes hypothalamic GnRH secretion, leading to a lack of LH and FSH, which negatively impacts testosterone production and spermatogenesis. MSG could act peripherally due to the existence of glutamate transporters and receptors within the testicular tissue. The expression of glutamateNMDA receptor, GluR1, GluR2/3, GluR2/3, and GluR5 mRNA has been documented in testicular tissue **[31]**

On the other hand, cotreatment with MSG and phoenixinsuccessfully restored the functional integrity of the HPG axis, with a notable increase in GnRH secretion, which enhanced the gonadotropin production, as earlier supported**[32]**. Phoenixin enhanced GnRH's capacity to upregulate GnRH receptor expression in cultured dispersed male anterior pituitary cells, thus enhancing gonadotropin secretion **[33].**

In addition, the stimulatory effect of phoenixinon the GnRH release could be mediated by its enhancement in kisspeptin release, as evinced herein and previously supported **[34].** Kisspeptin is a hypothalamic neuropeptide that responds to paracrine cues from dynorphin and neurokinin B, acting upstream of GnRH to stimulate its release. It also increases LH production and LH pulsatile frequency **[35].**Phoenixin acts through the GPR173 receptor to activate both GnRH and kisspeptin neurons, leading to increased expression of Kiss1, which can restore the release of GnRH and enhance FSH and LH secretion **[36].**

At the testicular level, our study found that the MSG group displayed a substantially declined testicular activity of 3β-HSD and 17β-HSD enzymes, which are crucial in testosterone synthesis. The reduced activity of these enzymes contributed to the decline in serum testosterone levels, confirming our hormonal findings. In contrast, phoenixin substantially stimulated their enzyme activities, which could play a role in improving testosterone secretion.

This could be mediated via its SIRT1 upregulation by phoenixin, as demonstrated by our findings presented later herein. Sirt1 modulates autophagy to control testosterone production in Leydig cells **[37].** Sirt1 also promotes steroidogenesis via its anti-inflammatory effects and its coactivation of steroidogenic factor 1 **[38].** Nevertheless,the specific molecular mechanism through which phoenixin influences the testosterone synthesis pathway remains unclear and warrants further investigation.

The testicular oxidative stress provoked by glutamate was validated herein by the notable increase in MDA and 8-OHdG levels concomitant with a decline in testicular GPx activity, as matched previously **[39]**. The MSG-induced ROS production is incremental in the testicular cellular damage and mitochondrial dysfunction**[40].** The toxic effects of MSG can alter mitochondrial inner membranes, causing depletion of GSH levels, altering the oxidative defense systems, and

increasing lipid peroxidation. Testicular and sperm membranes are extremely vulnerable to reactive oxygen species, contributing to male infertility **[41].**

The MSG's pro-apoptotic activity was evinced herein by an increment of caspase 3 immuno-expression at staggering levels within the testicular tissues of MSG-exposed rats. This is linked to excessive glutamate intake, which over-activates glutamate receptors, leading to intracellular calcium waves and triggering apoptosis **[42].** MSG-induced oxidative stress also provokes apoptotic cell death with a sequel of testicular tissue damage**[43]**. The heightened apoptotic cell death in Leydig cells results in a reduction in testosterone production, potentially exacerbating germ cell apoptosis and increasing the risk of infertility**[44].**

In addition, our findings revealed significantly elevated levels of TNF-alpha in MSG-administered rats, which might be crucial in testicular dysfunction brought on by MSG. In harmony,**Leiseganget al. [45]**suggested a positive correlation between elevated levels of inflammatory cytokines and adverse effects on male reproductive function, such as disruption of the HPG axis and impairment of the steroidogenesis cascade, leading to hypogonadotropic hypogonadism."

On the other hand, and in alignment with previous studies **[7, 8, 46],** our findings demonstrate that phoenixinintervention signified a substantial antioxidant, antiapoptotic, and anti-inflammatory criterion, which could explain its testicular cytoprotection in the MSG-intoxicated rats. In harmony, **Yilmaz et al.'s** recent study **[8]** revealed that the potent antioxidant and anti-inflammatory potentials of phoenioxin alleviated the testicular damage provoked by torsion-detorsion in rats, raising the assumption of the potential therapeutic role of phoenixin in other models of testicular tissue damage.

Importantly, and considering our findings, we could hypothesize that the testicular protective impact of phoenixincould be mediated by its ability to counteract the MSG-induced downregulation of the SIRT1/AMPK signaling. This signifies the possible crucial role of this pathway in mediating the protective role of phoenixin in our model.

SIRT1 is fundamental for normal spermatogenesis and the protection of germ cells against the hazardous impacts of oxidative stress and apoptosis **[47,48].** Its activation leads to the deacetylation of key transcription factors, thus enhancing cellular resilience against oxidative and inflammatory insults **[5].**It activates and phosphorylates AMPK, which controls many cellular metabolic processes **[49].** Both molecules activate each other to enhance mitochondrial biogenesis and display antioxidant criteria [**49].** p-AMPK enhances ATP production, impacting sperm motility and acrosomal reaction while decreasing reactive oxygen species **[50].**

In harmony, **Zeng et al. [51]** documented that the modifying role of phoenixinon neuroinflammation and apoptotic cell death is SIRT1 dependent. Phoenixin demonstrated multiple hepatoprotective benefits, including anti-inflammatory and antioxidant potential, by activating the

AMPK/SIRT1 and NRF2/HO-1 pathways **[10],**aligning with our findings.

5. Conclusion

This research illustrated for the first time the phoenixin's role in mitigating both morphological and functional testicular derangements associated with MSG consumption. These protective effects were mediated through the ability to restore the HPG hormonal balance and steroidogenic machinery while promoting redox homeostasis, exerting anti-inflammatory and anti-apoptotic effects, and upregulating the SIRT1/AMPK signaling. These findings demonstrated phoenixin's imprint signature in combating the MSG-induced testicular damage and its associated reproductive aberrations and provide compelling evidence for further investigation into its additional mechanisms in testicular protection.

6. Declarations and statements Ethics approval and consent to participate:

We conducted the study protocol according to The Local Committee of Research and Medical Ethics of the Faculty of Medicine, Tanta University.

Availability of data and material:

The corresponding author can provide the datasets used and/or analyzed during the current work upon request.

Competing interests:

The authors declare to have no conflicts of interest.

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