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Protective role of Gramine and N-acetylcysteine against tramadol induced testicular toxicity in rats

Abhijit De[1]*, Sawad Hossain[1], Biswanath Ghosh[1] & Kuntal Samanta[2]

[1]Department of Pharmacology, Bengal School of Technology, West Bengal 712102, India

[2]Department of Pharmacology, Guru Nanak Institute of Pharmaceutical Science and Technology, Panihati, West Bengal 700114, India

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Testicular dysfunction poses a significant health concern, necessitating effective prevention strategies to mitigate its adverse effects. One potential cause of testicular toxicity is the misuse of libido-enhancing drugs, such as tramadol. To investigate protective approaches, this study involved thirty-six adult male Sprague-Dawley rats, divided into five groups. All groups received tramadol hydrochloride at a dose of 75 mg/kg via intraperitoneal injection. Selected groups additionally received oral treatments of Gramine and N-acetylcysteine (NAC) as protective agents over a specified period. Following treatment, comprehensive assessments were conducted. Serum biochemical and hormonal markers measured included nitric oxide, total cholesterol, lactate dehydrogenase (LDH), testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and androgen-binding protein (ABP). Testicular tissues were collected to evaluate sperm parameters: count, motility, and viability and tissue markers such as total protein, cholesterol, and albumin. Oxidative stress indicators, including superoxide dismutase (SOD), glutathione (GSH), and lipid peroxidation (LPO), were also analysed. Histological examinations assessed structural changes within the testes. Results demonstrated that high dose Gramine aided in restoring hormone levels, while NAC significantly (P<0.001) improved hormonal balance. The combined administration of Gramine and NAC yielded the most notable improvements in sperm quality and testicular tissue markers. Additionally, antioxidant defences were effectively normalised. These findings suggest that the combined use of Gramine and NAC holds promise as a therapeutic strategy to counteract tramadol-induced testicular damage.

Keywords: Adiponection receptor, Libido enhancer drug, N-Acetylcysteine, Oxidative stress, Testicular dysfunction

Testicular impairment remains one of the most frequently reported organ-specific dysfunctions, often stemming from a broad spectrum of physiological disturbances. These disturbances encompass endocrine and neurological imbalances, as well as distinct cellular-level alterations[1]. Such cellular disruptions commonly manifest as distorted sperm morphology, accompanied by a notable decline in sperm count, motility, and overall viability[2]. Collectively, these abnormalities significantly contribute to male infertility, a growing concern associated with the excessive or inappropriate use of certain pharmaceutical agents[3].

A prominent example of such a drug is Tramadol hydrochloride, a centrally acting analgesic with weak μ-opioid receptor agonist activity. It is primarily prescribed for managing moderate to severe pain, especially in cases where non-opioid analgesics prove insufficient. However, beyond its intended use, tramadol has recently attracted attention for its off-label use as an aphrodisiac, particularly among younger populations seeking to enhance libido[4,5]. Despite its generally perceived safety profile and relatively low potential for dependency or tolerance, tramadol is not without risks. Data from the Uppsala Monitoring Centre′s VigiAccess™ database reports approximately 972 adverse drug reactions linked specifically to the reproductive and endocrine systems. These ADRs include conditions such as testicular pain, prostatitis, prostatomegaly, oligospermia, difficulty in ejaculation, genital swelling, and androgen insufficiency, underscoring the drug′s potential for reproductive toxicity[6]. Extensive preclinical research in murine models further corroborates these findings. Administration of tramadol at varying doses has been shown to cause significant deformation of seminiferous tubules and Leydig

*Correspondence:
Phone: +91 62917 00648
E-mail: abhijit@bstpharmacy.in

cells, coupled with a marked reduction in serum testosterone and prolactin levels[7,8]. Mechanistically, prolonged high-dose tramadol use appears to disrupt the hypothalamic-pituitary-gonadal (HPG) axis by inhibiting the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. This disruption leads to decreased secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, resulting in impaired testosterone synthesis and hindered sperm maturation from primordial germ cells[9]. In light of these concerning findings, our investigation aimed to explore therapeutic strategies capable of counteracting tramadol-induced testicular and endocrine dysfunctions. For this purpose, we selected Gramine, a naturally occurring indole alkaloid first isolated from *Arundo donax L.* Chemically identified as N,N-dimethyl-1H-indole-3-methylamine, Gramine has gained attention due to its diverse pharmacological profile[10]. Recent studies have revealed its role as a potent agonist of the adiponectin receptor AdipoR1, confirmed through fluorescence polarization-based high-throughput screening assays[11]. Beyond this, Gramine exhibits a broad spectrum of biological activities, including antiviral, antibacterial, anti-inflammatory, anticancer, and insecticidal properties[12]. Its traditional use in Chinese medicine spans treatments for toothaches, urinary tract disorders, and certain cardiovascular conditions, further emphasising its therapeutic potential[13].

To enhance and complement the protective effects of Gramine, we incorporated N-acetylcysteine (NAC) into our experimental design. NAC is a well-known antioxidant and precursor to glutathione, widely used in clinical settings for managing chronic pulmonary diseases such as cystic fibrosis[14], and COPD[15]. Numerous studies have demonstrated NAC′s ability to mitigate testicular damage in rodent models, primarily through its capacity to reduce oxidative stress by neutralizing cyclic peroxides and endoperoxides within testicular cells[16,17].

Building upon this solid scientific foundation, the present study was designed to evaluate the individual and combined effects of Gramine and NAC in counteracting tramadol-induced reproductive toxicity. Through this research, we aim to illuminate a potentially innovative and effective therapeutic approach for preserving male reproductive health in the face of opioid-induced testicular damage.

Materials and Methods

Selection of animal models

To establish a classic testicular impaired model, healthy adult twelve-week-old inbred male albino rats of the Sprague-Dawley strain weighing between 200-250g were procured from Chakraborty Breeder, Kolkata, India. The health status of each animal was properly checked before being assigned to the study. They were kept in not more than six in a single cage, with floor occupancy for each being 70 square inches as per the guidelines. The animal room temperature was maintained at 22±3°C with a controlled relative humidity of around 40%±10%. The 12-hour light-dark cycle was balanced by proper illumination within the room. The animals were fed on standard food pellets with microbiologically tested pathogen free drinking water *ad libitium.* All these conditions were in accordance with the standards prescribed by the Committee for Control and Supervision of Experiments on Animals (*CCSEA*), New Delhi, India. The entire experimental procedure was approved by the Institutional Animal Ethical Committee (IAEC) of Bengal School of Technology, India, with an assigned number 1726/CCSEA/IAEC/2023-022 and conducted in accordance with the Committee′s permission and guidelines for animal care.

Reagents

Gramine (G10806), NAC (A7250), Tramadol hydrocholoride (42965), Hank′s Buffer Balanced Salt Solution (H6648), Eosin Y solution (318906), Eosin blue (32855), and Nigrosin (198285) were purchased from Sigma Aldrich. Tris Buffer Ready-to-use Solution pH 7.8 (77-86-1) was procured from Calbiochem. Ethylene diamine hydrochloride (RM 1279) and sodium hydroxide pellets (RM 467) were provided by Rankem. Standard superoxide dismutase and reduced glutathione were purchased from MP Biochemicals, India. All chemicals and reagents employed were of analytical grade and maintained at labeled storage conditions as provided by the manufacturer.

Drug intervention and experimental design

A total of 36 male animals were selected and segregated randomly into six groups based on body weight within a variation of ±20% as per the OECD guideline. The negative control group received normal saline solution, while the positive control (diseased group) was administered Tramadol hydrochloride intraperitoneally (i.p.) for 21 days. The other four

groups were administered treatment drugs, i.e., Gramine at different dose levels and NAC via oral gavage. They were ingested at least half an hour before inducing tramadol hydrochloride in the treatment groups. All the drugs administered orally were prepared in suspension form with a 1% CMC solution, except tramadol hydrochloride. Group 1- Normal control group, received normal saline solution (2 mL/100 g body weight);

Group 2- Challenged group, received Tramadol Hydrochloride i.p. at a dose of 75 mg/kg body weight[18]; Group 3- Treatment group I, received Gramine (high dose) p.o. at a dose of 55 mg/kg body weight[19]; Group 4- Treatment group II, received Gramine (low dose) p.o. at a dose of 27.5 mg/kg body weight[19]; Group 5- Treatment group III, received NAC p.o. 160 mg/kg body weight[20]; Group 6- Treatment group IV, received Gramine and NAC p.o. at a dose of 55 mg/kg and 160 mg/kg body weight respectively.

Assay of serum biochemical and hormonal parameters

After 21 days of intervention, 2-3 mL of blood were collected from each animal in the designated groups from jugular vein in collecting vials and allowed to rest for at least 30 minutes at room temperature before separation of serum by centrifuging in a cooling centrifuge (Remi CM-8 Plus, Mumbai) at 3,914×*g* for 15 minutes[21]. The supernatant was collected, stored quickly at -80°C for subsequent analysis of various biochemical and hormonal parameters. Serum lactate dehydrogenase (LDH) and total cholesterol assay were performed by UV kinetic method and CHOD-PAP method respectively, using Reckon Assay Kit, India[22]. Serum nitric oxide was determined by the Griess Reagent method using Invitrogen™ assay kit, USA[23]. The kit employs the enzyme nitrate reductase to convert nitrate to nitrite. Nitrite is then identified as a coloured azo dye produced by the Griess process, which absorbs visible light at 540 nm. The remaining serum was further used to perform various sex and gonadotropin hormone parameters. Serum testosterone assay was performed by rat-specific competitive ELISA method using BT Lab Kit, China[24]. Serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) were analysed following rat specific sandwich CLIA method by utilising Elabscience kit, USA[25]. Serum androgen binding protein (ABP) was investigated using rat specific sandwich ELISA method using MyBioSource kit, USA[26].

Weight of reproductive organs, collection of sperm, and analysis of total count, motility, and viability

After performing serum parameters, animals were sacrificed by exposing them in CO_2 chamber, and then the lower abdominal section was dissected using surgical accessories. The testes, along with the epididymis and vas deferens, were isolated immediately by removing the adhering tissues. One of the testes was included for investigating sperm-related parameters, and the other was kept for assessing testicular parameters simultaneously. The testis and epididymis were properly weighed to determine their status as atrophic or hypertrophic. The spermatocytes were collected by gentle mincing of epididymis and vas deferens on a petri dish, prefilled with 2 mL of prewarmed Hank′s Buffer Balanced Salt Solution[27]. The caudal part of the epididymis was further pressed with a plastic forcep to allow the sperm cells to float gently within the buffer solution. 10 µL of solution was withdrawn by a single-channeled micropipette on a Neubauer chamber (Hausser Scientific, USA) and allowed the cells to settle down within the WBC counting squares[28]. The entire system was mounted under a software assisted Trinocular (Olypus H 20i, Japan) system and visualised under 10× magnification[29]. The total count was performed in four assigned squares of the chamber, and the results were expressed in cubic mm/mL of suspension. The sperm motility was measured by carefully placing 20 µL of solution on a clean slide enclosed by a cover slip and observed under 25× magnification with a low light illumination in a Trinocular apparatus. The results were expressed in terms of percent motile or immotile sperm with a degree of rapidly progressive, low progressive, non-progressive motility, or immobility[30]. Each count was done in triplicate, and a total of 100 spermatozoa per count per microscopic field were considered to get the final outcome. For assessing sperm viability, a drop of semen was put on a warmed 40°C slide and mixed with a double volume of the dye mixture (one part 5% Eosin Y solution to four parts 10% nigrosin aqueous solution) using a glass rod. The samples were air dried at room temperature. Thin, fat-free semen smears were created at temperatures as high as 36°C. After drying, the smears were fixed with 96% ethanol. They were then washed with water and counterstained with a 10% blue eosin solution for 20-60 seconds. The slides were then cleaned with water again before being coloured for 3 minutes using gentian pigment. After colouring, the slides were washed and dried, resulting in a clear

backdrop that contrasted well with the dyed spermatozoa. The slides were made and microscopically examined under 25×magnification. The final results were reported in percentage viable spermatocytes in the same manner as performed in measuring motility[31].

Analysis of testicular parameters

The isolated testes from different groups of animals were homogenized by suspending in ice-cold Tris-HCl (50 mM)- EDTA buffer (20mM) pH 7.4 using a tissue homogenizer (Remi RQ-127, Mumbai). The homogenized tissue was centrifuged in a cooling centrifuge at slow speed for 10 minutes (Remi CM-8 Plus, Mumbai). The supernatant was collected and included for assessing testicular parameters. Protein level and albumin level were assayed by the traditional Biuret method[32] and the BCG method respectively, using Abcam kit, India[33]. Testicular cholesterol CHOD-PAP method and LDH level by UV kinetic method were determined by using Reckon Assay Kit, India[22].

Assay of free radical scavenging parameters

The homogenate was further used to verify levels of antioxidant enzymes and lipid peroxides generated within tissue. The Superoxide dismutase (SOD) assay by the WST-8 Method[34] and reduced Glutathione (GSH) assay by the Moron Method were performed using appropriate standards[35]. The level of malondialdehyde was determined by a lipid peroxidation assay by the thiobarbituric acid method[36]. Glutathione peroxidase and glutathione reductase levels were analysed using Cayman Kit, Michigan[37,38].

Histopathological studies

Sections of testis were collected from all groups and fixed in 10% neutral buffered formalin. After resting for 24 hours, the sections were dehydrated in ascending concentrations of ethyl alcohol (70-100%) and then prepared using standard procedures for H&E stain as described by Bancroft *et al.*[39]. Prepared slides were observed under an Olympus Trinocular Microscope under 40×magnification, and specific regions were identified for reporting the status of spermatogonia, spermatocytes, Leydig cells, and Sertoli cells[29].

The extent of testicular damage and the efficiency of spermatogenesis were evaluated using Cosentino's scoring system and the average Johnsen testicular biopsy score (MJTBS), respectively. In Cosentino's system, the testis is classified into four categories: Grade I indicates normal testicular tissue, while Grade IV reflects extensive coagulative necrosis of the parenchyma. Regarding the Johnsen score, a score of X indicates normal spermatogenesis; IX shows the presence of several spermatozoa with some disorganization in the germinal epithelium; VIII corresponds to few spermatozoa; scores from VII to II represent various degrees of maturation arrest; and a score of I signifies a complete absence of cells within the seminiferous tubules[40].

The average scores of both Cosentino and Johnsen were calculated for each sample and displayed in graphical form.

Statistical analysis

All the results obtained were thoroughly analysed by applying the statistical method using GraphPad Prism Software 7.0. All the values were presented as Mean ± SEM. Comparisons among groups were done by using two-way ANOVA (Analysis of Variance) followed by Dunnett' Multiple comparison. *P* values less than 0.05 was considered as indicative of significance.

Results

Effect of Gramine and NAC on serum biochemical parameters

Table 1 indicated significant decrease in the level of nitrates and total cholesterol and increase in the level of LDH ($P<0.001$) in tramadol induced group when compared with normal control group. Groups

Table 1 — Effect of Gramine and NAC on serum biochemical parameters

Groups	Nitric Oxide (micromole/L)	Lactate dehydrogenase (LDH) (U/L)	Total cholesterol (mg/dL)
Normal Control	67.93 ± 1.46	98.65 ± 0.62	244.1 ± 6.76
Challenged group	26.09 ± 1.54[a]	198.0 ± 4.60[a]	149.1 ± 0.88[a]
Treatment I	31.23 ± 1.27*	133.7 ± 3.95***	160.0 ± 2.57 **
Treatment II	30.17 ± 1.60	135.2 ± 1.34***	174.6 ± 0.95***
Treatment III	36.68 ± 0.66***	148.6 ± 0.75 ***	164.5 ± 1.55 ***
Treatment IV	38.34 ± 0.53***	155.9 ± 1.32 ***	163.9 ± 0.96***

[*P* values: *$P<0.05$, **$P<0.01$, ***$P<0.001$ when results of each group were compared with challenged group. *P* values: [a]$P<0.001$ when the results of challenged group were compared with normal control group]

receiving high dose and low dose of Gramine showed significant improvement in the level of cholesterol and LDH (P<0.001, P<0.01) as compared with diseased group. No significant result was observed in the level of nitrates in the groups receiving low dose of Gramine. NAC alone and its combination with Gramine expressed highly significant result (P<0.001) in all the biochemical parameters after comparing with the challenged group.

Effect of Gramine and NAC on hormonal parameters

Table 2 showed highly significant decrease in the level of total testosterone, FSH, LH and ABP level (P<0.001) in tramadol induced group as compared with the saline control group. All the treatment groups receiving Gramine at low and high dose along with NAC showed significant improvement in the level of sex hormone, gonatrophins and ABP (P<0.001) when compared with diseased group.

Effect of Gramine and NAC on weight of reproductive organs

Table 3 indicated significant changes in the testicular and epididymal weight (P<0.001) in diseased animals as compared with normal animals. The administration of high dose of Gramine and its combination with NAC in animals showed significant (P<0.001) alterations in organ weight when compared with the diseased animals. Animals receiving low dose of Gramine showed moderate significant (P<0.01) and non-significant improvements in epididymal weight respectively when compared with diseased animals.

Effect of Gramine and NAC on sperm parameters

Table 4 depicted the significant decrease (P<0.001) in the level of sperm count, motility and viability of tramadol induced group when compared with normal control group. The entire treatment group with Gramine and NAC with its combination showed significant restoration (P<0.01, P<0.001) in the level of sperm parameters as compared with the tramadol induced group.

Effect of Gramine and NAC on testicular parameters

Table 5 showed significant alteration (P<0.001) in the level of testicular protein, albumin, LDH, cholesterol of tramadol induced group when compared to normal animals. After administration of high and low dose of Gramine along with NAC in treatment groups, showed a highly significant results (P<0.001) in all the testicular levels as compared to tramadol induced group.

Table 2 — Effect of Gramine and NAC on hormonal parameters

Groups	Testosterone (ng/mL)	Follicle-stimulating hormone (FSH) (ng/mL)	Luteinizing hormone (LH) (ng/mL)	Androgen-binding protein (ABP) (ng/mL)
Normal Control	1.91 ± 0.03	1.45 ± 0.03	2.46 ± 0.03	0.96 ± 0.06
Challenged group	0.75 ±0.02 a	0.50 ± 0.02 a	1.16 ± 0.03 a	0.17 ± 0.03 a
Treatment I	0.95 ± 0.03***	0.80 ± 0.03***	1.20 ± 0.03***	0.20 ± 0.03***
Treatment II	0.86 ± 0.03***	1.28± 0.03***	1.86 ± 0.02***	0.25 ± 0.03***
Treatment III	1.25 ± 0.03 ***	1.07 ± 0.04***	1.62 ± 0.03***	0.58 ± 0.04***
Treatment IV	1.36 ± 0.02 ***	1.13 ± 0.02***	1.71 ± 0.03***	0.69 ± 0.04***

[P values: *P<0.05, **P<0.01, ***P<0.001 when results of each group were compared with challenged group. P values: [a]P<0.001 when the results of challenged group were compared with normal control group]

Table 3 — Effect of Gramine and NAC on weight of reproductive organs

Groups	Testicular weight (g)	Epididymal weight (g)
Normal Control	3.37 ± 0.13	0.68 ± 0.03
Challenged group	4.19 ± 0.41 a	0.93 ± 0.02 a
Treatment I	3.83 ± 0.28***	0.78 ± 0.01 ***
Treatment II	4.02 ± 0.18**	0.86 ± 0.03
Treatment III	3.28 ± 0.22***	0.73 ± 0.01***
Treatment IV	3.33 ± 0.31***	0.72 ± 0.01***

[P values: *P<0.05, **P<0.01, ***P<0.001 when results of each group were compared with challenged group. P values: [a]P<0.001 when the results of challenged group were compared with normal control group]

Table 4 — Effect of Gramine and NAC on sperm parameters

Groups	Sperm count (cubic, mm/mL)	Sperm motility (%)	Sperm viability (%)
Normal Control	6.32 ± 9.05	83.33 ± 1.11	69.17 ± 1.10
Challenged group	2.84 ± 3.20 a	35.17 ± 1.42 a	40.33 ± 2.04 a
Treatment I	3.54± 4.37***	52.50 ± 0.99***	55.67 ± 0.95***
Treatment II	3.04 ± 2.76***	42.33 ± 1.08**	46.17 ± 0.90
Treatment III	4.67 ± 6.09***	63.17 ± 0.65***	61.50 ± 1.17***
Treatment IV	4.99 ± 57.60***	68.83 ± 0.79***	63.00 ± 0.57***

[P values: *P<0.05, **P<0.01, ***P<0.001 when results of each group were compared with challenged group. P values: [a]P<0.001 when the results of challenged group were compared with normal control group]

Effect of Gramine and NAC on oxidative stress and antioxidant enzyme parameters

Table 6 clearly showed that the level of antioxidant enzymes such as SOD, reduced glutathione, glutathione peroxidase, glutathione reductase and rate of lipid peroxidation in tramadol received group were significantly affected (P<0.001) when compared with control group. NAC along and its combination with Gramine showed highly significant improvement (P<0.001) in the levels of these enzymes and rate of lipid peroxidation when compared with tramadol exposed group. Group receiving high dose of Gramine showed less significant value in case of reduced GSH (P<0.01), glutathione reductase (P<0.05) and lipid peroxidation (P<0.01) level as compared to diseased group, except in case of SOD and glutathione peroxidase where the results were non-significant. Low dose Gramine treated group only showed significant improvement in level of reduced glutathione (P<0.05) and glutathione reductase (P<0.01) but no significant response in level of SOD, glutathione peroxidase and lipid peroxidation.

Histopathological findings

Fig. 1 depicted the histopathological status of the testicular cells present within the lumen of seminiferous

Table 5 — Effect of Gramine and NAC on testicular parameters

Groups	Albumin (gm/dL)	Total Protein (gm/dL)	LDH (U/L)	Total Cholesterol (mg/dL)
Normal Control	1.71 ± 0.038	1.64 ± 0.009	665.2 ± 2.15	27.64 ± 0.67
Challenged group	1.20± 0.026 a	4.25 ± 0.039 a	318.2 ± 0.79 a	93.33 ± 0.88 a
Treatment I	1.46 ± 0.011***	5.16 ± 0.010***	470.2 ± 2.44***	50.50 ± 0.76***
Treatment II	1.35 ± 0.012***	4.90 ± 0.015***	441.7 ± 1.28***	57.00 ± 0.63***
Treatment III	1.53 ± 0.010***	5.31 ± 0.014***	488.2 ± 1.95***	45.00 ± 0.68***
Treatment IV	1.64 ± 0.009***	5.28 ± 0.165***	510.8 ± 0.87***	38.17 ± 0.54***

[P values: *P<0.05, **P<0.01, ***P<0.001 when results of each group were compared with challenged group. P values: [a]P<0.001 when the results of challenged group were compared with normal control group]

Table 6 — Effect of Gramine and NAC on oxidative stress and antioxidant enzyme parameters

Groups	Lipid peroxidation (nmol/L)	Superoxide dismutase (EU/dL)	Reduced Glutathione (µg/mL)	Glutathione Peroxidase (nmol NADPH/min/mg protein)	Glutathione Reductase (nmol NADPH/min/mg protein)
Normal Control	24.57 ± 0.52	25.98 ± 0.32	45.75 ± 0.37	0.042 ± 0.08	22.84 ± 0.31
Challenged group	46.33 ± 0.27 a	10.97 ± 0.30 a	18.40 ± 0.40 a	0.017 ± 0.09 a	13.38 ± 0.40 a
Treatment I	44.14 ± 0.43**	11.40 ± 0.24	20.52 ± 0.32**	0.019 ± 0.08	15.31 ± 0.48*
Treatment II	44.89 ± 0.71	11.18 ± 0.19	19.92 ± 0.39*	0.018 ± 0.04	15.36 ± 0.26**
Treatment III	32.86 ± 0.28***	19.62 ± 0.26***	33.73 ± 0.41***	0.034 ± 0.09***	19.41 ± 0.30***
Treatment IV	31.92 ± 0.41***	19.58 ± 0.19***	34.79 ± 0.57***	0.037 ± 0.06***	20.56 ± 0.21***

[P values: *P<0.05, **P<0.01, ***P<0.001 when results of each group were compared with challenged group. P values: [a]P<0.001 when the results of challenged group were compared with normal control group]

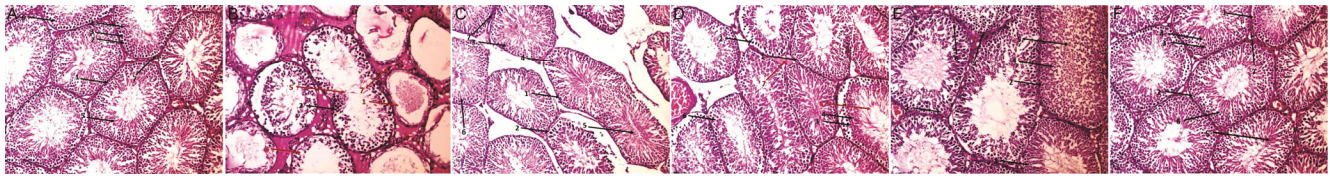

Fig. 1 — Histopathological Status of a section of rat testes stained by H&E at 40×. The black arrow represents the normal cellular structure while the red arrow represents the damaged or absence of cells from the particular section. Arrow (1,2,3,4,5,6,7) representing mostly normal histological architecture of spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, spermatozoa, Sertoli cells and Leydig cells, respectively. Normal saline treated group showing normal architecture of cells within the lumen of seminiferous tubules and at the junction of cells (A). Tramadol exposed group showing marked absence of spermatogonia cells, spermatids and Sertoli cells with diminished secondary spermatocyte and spermatozoa within the lumen of seminiferous tubules along with Leydig cells. Primary spermatocytes seemed to be normal (B). High dose of Gramine showing moderate improvement in the architecture of all the cells within lumen of seminiferous tubules with marked progression within Sertoli and Leydig cells (C). Low dose of Gramine showing degenerative changes in the status of spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids, with marked absence in spermatocytes within the lumen of seminiferous tubules. Absence of Leydig cells were also observed (D). NAC treated group showing significant improvement in the status of all the testicular cells specially spermatogonia to spermatocytes (E). Gramine and Gramine treated group showing the most significant improvement in the status of testicular cells with clearly visible Leydig and Sertoli cells. Numerous spermatocytes were appeared within the lumen of seminiferous tubules (F).

tubules when observed at 40×. The modifications and alterations in the architecture of spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa of different treatment group were reported. Changes within the status of Sertoli and Leydig cells were also marked. Fig. 2 & 3 represented the statistical analysis of the Cosentino′s and Johnsen′s score assigned to each group respectively.

Discussion

The current investigation meticulously examined the potential modulatory effects of Gramine, both as a standalone treatment and in combination with N-acetylcysteine (NAC), on tramadol-induced testicular dysfunction in male rats. Building upon a substantial body of prior research, which has conclusively demonstrated the deleterious impact of various dosages of tramadol on multiple facets of male reproductive health, our study sought to elucidate whether Gramine could confer protective or restorative effects against such damage. Previous studies have documented that tramadol negatively influences hormonal profiles[7]; biochemical parameters, oxidative stress markers, and directly inflicts morphological damage upon testicular tissue[8,40]. Consistent with these established findings, our experimental data revealed significant disturbances across these parameters following tramadol administration, underscoring its profound disruptive influence on testicular function.

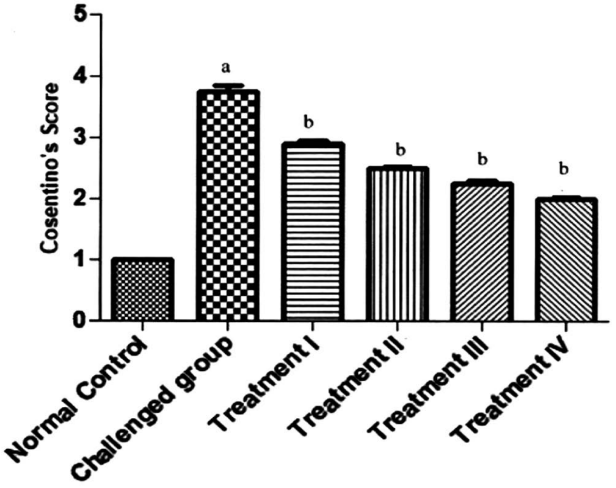

Fig. 2 — Consentino′s Score. [Bars represent the mean± SD (n = 6/group), significant difference vs. a respective groups viz Normal Control vs Challenged group (a) $P<0.05$, Challenged group vs Treatment I (b) $P<0.05$, Treatment II, Treatment III & Treatment IV]

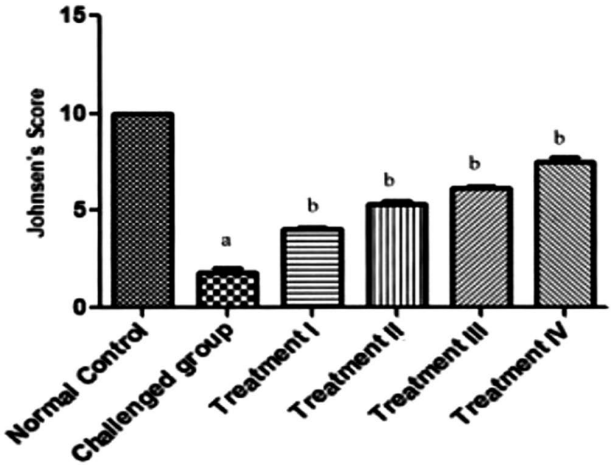

Fig. 3 — Johnsen′s Score. [Bars represent the mean± SD (n = 6/group), significant difference vs. a respective groups viz Normal Control vs Challenged group (a) $P<0.05$, Challenged group vs Treatment I (b) $P<0.05$, Treatment II, Treatment III & Treatment IV]

In an effort to assess the therapeutic potential of Gramine, we administered it at different doses, both alone and concomitantly with NAC, and observed noteworthy ameliorative effects. Of particular interest was the observation that Gramine administration led to improvements in serum cholesterol levels and lactate dehydrogenase (LDH) activity, with these effects exhibiting a dose-dependent pattern. LDH, notably abundant in testicular tissue as the isoenzyme LDHx, is widely recognised as a sensitive biomarker for tissue injury and pathology. Elevated LDH levels often correlate with cellular damage, necrosis, or inflammation within the tissue. The normalisation of LDH levels following treatment suggests that Gramine may exert a protective influence on testicular tissue, possibly attenuating tramadol-induced cellular injury. However, it is important to note that the current study primarily relied on systemic biochemical markers; to definitively confirm tissue-level protection, further investigations involving detailed histopathological examinations of testicular architecture would be essential. Such tissue-specific analyses would provide more concrete evidence of cellular preservation or regeneration attributable to the treatment[41].

Regarding cholesterol, this lipid molecule plays a dual and complex role in male reproductive physiology. On one hand, it serves as a vital precursor for testosterone biosynthesis within Leydig cells, which is crucial for the maintenance of normal male reproductive functions. The observed positive modulation of cholesterol levels in both serum and

testicular tissue following Gramine and NAC treatments suggests a potential enhancement of steroidogenic activity. This could translate into increased testosterone synthesis and a partial restoration of Leydig cell functionality compromised by tramadol. On the other hand, cholesterol accumulation within sperm cell membranes, particularly in the acrosome, can be detrimental to fertility. Elevated membrane cholesterol content is known to impair sperm capacitation, a necessary process for successful fertilization. Therefore, while systemic or testicular cholesterol normalisation might be beneficial for maintaining adequate testosterone levels and overall testicular health, it could concurrently pose risks to sperm functionality. This apparent contradiction underscores the necessity for comprehensive studies that evaluate not only biochemical parameters but also sperm quality, motility, capacitation status, and fertilization potential following such treatments. Only through such multifaceted assessments can the net effect on male fertility be accurately ascertained. In addition to lipid and enzymatic markers, we evaluated serum testosterone levels, which are often suppressed following opioid exposure, including tramadol. Our findings indicated a trend toward increased testosterone concentrations upon administration of Gramine and NAC, aligning with the biochemical and tissue-specific data suggesting a partial recovery of Leydig cell function. This hormonal rebound is promising, as testosterone is integral to spermatogenesis, libido, and overall reproductive health. Nonetheless, it is imperative to recognise that hormonal restoration alone does not necessarily equate to functional fertility. To establish whether these biochemical improvements translate into tangible reproductive benefits, future studies should incorporate comprehensive fertility assessments, such as mating trials, sperm count evaluations, and assessments of sperm motility and morphology.

Furthermore, the role of nitric oxide (NO) within the testicular milieu was also examined. NO, synthesised predominantly via endothelial nitric oxide synthase (eNOS), is a key mediator of vascular tone and blood flow regulation within the testes. Adequate testicular perfusion is essential for optimal spermatogenesis and testicular health. Our data demonstrated that Gramine and NAC modulated nitrate levels, which serve as stable indicators of NO production. These findings suggest that the treatments might enhance testicular blood flow and oxygenation, thereby supporting spermatogenic processes. However, NO's role in testicular physiology is complex and dual-faceted. While physiological levels of NO are beneficial and necessary for normal testicular function, excessive or dysregulated NO production can lead to nitrosative stress, contributing to cellular damage and apoptosis[42]. Consequently, the observed modulation of nitrate levels warrants cautious interpretation. Maintaining a delicate balance in NO levels is critical; an optimal concentration could promote testicular health, whereas dysregulation might negate potential benefits or exacerbate injury. Further detailed investigations into NO dynamics, including measurements of nitrosative stress markers and eNOS activity, are necessary to delineate the precise role of NO modulation by Gramine and NAC in this context.

The hypothalamus, serving as the principal regulator for the secretion and inhibition of gonadotropins, appears to be modulated by our investigational compound. It is well documented that tramadol markedly suppresses the secretion of FSH and LH hormones integral to the feedback mechanisms governing spermatogenesis within the seminiferous tubules and the facilitation of testosterone release from Leydig cells[40]. The hypothesis-generating premise posits that augmenting the levels of these gonadotropins via pharmacological intervention may elucidate their pivotal role in activating the hypothalamic-pituitary-gonadal axis[13]. It is established that the investigational drug functions as a potent agonist of adiponectin receptor 1 (AdipoR1), which is expressed in rat testis, skeletal muscle, and adipose tissue[43]. Activation of AdipoR1 by adiponectin induces a surge in kisspeptin protein levels, which directly activates the mitogen-activated protein kinase (MAPK) pathway and precipitates depolarisation through calcium influx in a concentration-dependent manner. This mechanism plausibly stimulates the hypothalamus to secrete gonadotropin-releasing hormone (GnRH), counteracting the inhibitory effects exerted by tramadol[44]. As an AdipoR1 agonist, our compound is hypothesised to modulate kisspeptin levels, a critical regulator of sex hormone homeostasis. The observed elevations in LH, FSH, and testosterone levels substantiate this hypothesis-generating framework regarding the drug′s pathway of action. Furthermore, the enhancement of androgen-binding protein (ABP)

levels through the drug and its combination provides positive evidence that increased testosterone availability within the testis may facilitate spermatogenesis by promoting androgenic action and elevating testicular sex hormone concentrations.

Tramadol exposure was intricately associated with a significant downregulation of protein expression within testicular tissues, concomitant with compelling evidence of heightened cellular apoptosis. Among the myriad proteins affected, albumin a pivotal plasma protein primarily involved in osmotic regulation, molecular transport, and maintaining fluid balance exhibited pronounced depletion. This observation underscores the extensive metabolic disturbances precipitated by tramadol. The mechanistic basis of this reduction may be attributable to the reactive metabolites of tramadol, particularly O-desmethyltramadol, which is extensively metabolised via the cytochrome P450 enzyme system, notably CYP2D6[45]. These metabolites are known to covalently bind to lipid membranes, thereby disrupting membrane integrity and compromising the structural and cytoplasmic stability of proteins within testicular cells. While the subsequent restoration of protein levels following administration of Gramine and NAC suggests a reparative or protective effect on the lipoprotein bilayer and intracellular protein stability, the precise molecular pathways and signaling cascades underlying this protective mechanism remain elusive and warrant further detailed investigation through molecular and biochemical studies.

N-Acetylcysteine (NAC), renowned for its capacity to modulate amino acid metabolism and to disrupt the formation of reactive tramadol metabolites, likely plays a central role in preserving proteostasis within the testicular milieu. Its antioxidant properties, coupled with its ability to replenish intracellular glutathione levels, may contribute significantly to maintaining protein integrity under toxic insult. However, the extent to which NAC acts independently versus synergistically with Gramine to sustain or restore testicular proteomic balance remains an open question. Elucidating this requires comprehensive proteomic profiling, coupled with mechanistic assays designed to delineate specific pathways involved in protein synthesis, folding, and degradation within the testicular tissue under treatment conditions.

The observed elevation in luteinizing hormone (LH) levels upon administration of Gramine and NAC may partially account for the notable improvements seen in sperm parameters, especially motility and sperm count. Prior research has documented NAC′s efficacy in enhancing sperm quality, including improvements in motility, viability, and morphology; however, the combined application with Gramine appears to produce a synergistic benefit that is particularly promising. Notably, our data indicated a marked enhancement in progressive sperm motility following the combination treatment, suggesting a potential enhancement of functional sperm competence. These biochemical and motility improvements were further corroborated by histopathological evidence, which revealed notable preservation of seminiferous tubule architecture and spermatogenic cell lineages. Nonetheless, it is critical to acknowledge that while histological findings provide valuable insights into tissue integrity, they do not constitute definitive evidence of functional fertility. To substantiate the impact of this treatment on fertility outcomes, future studies should incorporate functional reproductive assays such as *in vitro* fertilization (IVF) rates, sperm-oocyte binding assays, and assessments of acrosome reaction efficiency.

Furthermore, the normalisation of nitrate levels observed under treatment conditions may reflect an improvement in testicular perfusion, potentially contributing to the observed enhancements in sperm viability and overall testicular function. Nitric oxide (NO), synthesised primarily via endothelial nitric oxide synthase (eNOS), plays a dual role in testicular physiology: it acts as a vasodilator facilitating blood flow and oxygen delivery, but excessive or dysregulated NO production can lead to nitrosative stress, resulting in cellular damage. Consequently, while the observed elevation in nitrate levels could suggest improved vascular function and tissue oxygenation, it also warrants cautious interpretation. Maintaining a balanced NO level is critical, as both deficiency and excess pose risks to testicular health. Excessive NO can react with superoxide anions to form peroxynitrite, a potent reactive nitrogen species capable of inducing lipid peroxidation, protein nitration, and DNA damage. Therefore, further investigations measuring specific markers of nitrosative stress, eNOS activity, and the balance between pro- and anti-nitrosative factors are necessary to fully elucidate the role of NO modulation in the context of tramadol toxicity and therapeutic intervention.

Similarly, the observed increase in tissue cholesterol content, while potentially conferring protective effects against physiological stress and

supporting membrane fluidity, may also have adverse implications for sperm function if levels become excessive. Cholesterol plays a critical role in maintaining membrane integrity; however, elevated cholesterol content within sperm membranes, particularly in the acrosomal region, has been associated with impaired capacitation, reduced acrosome reaction, and diminished fertilization potential. The complex relationship between cholesterol homeostasis, membrane dynamics, and sperm-oocyte fusion underscores the necessity for further studies examining the precise impact of altered cholesterol levels on sperm functional competence, membrane fluidity, and overall fertilization efficacy.

The histopathological alterations induced by tramadol, characterised by hypertrophy and atrophy of testicular cells, reinforce the severity of its cytotoxic effects. Cellular hypertrophy may represent a compensatory response aimed at preserving functional capacity in the face of injury, whereas atrophy could be indicative of protein catabolism, apoptotic processes, or degenerative changes. The observed distortions in seminiferous tubule morphology, including disrupted cellular architecture and compromised spermatogenic lineage, raise significant concerns regarding the long-term implications for spermatogenesis and overall testicular homeostasis. Such structural damage not only hampers sperm production but also impairs the supportive functions of Sertoli and Leydig cells, which are essential for maintaining the testicular microenvironment.

From an oxidative stress perspective, the study further consolidates the critical role of free radical generation in tramadol-induced testicular toxicity. Tramadol markedly suppressed the activity levels of key antioxidant enzymes, including superoxide dismutase (SOD) and reduced glutathione (GSH), aligning with prior reports of oxidative imbalance[46]. The resultant accumulation of reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide exacerbates lipid peroxidation, leading to membrane destabilisation, mitochondrial dysfunction, and apoptosis within testicular cells. The oxidative insult compromises cellular integrity and function, ultimately impairing spermatogenesis and testicular health. While Gramine administered at higher doses demonstrated modest efficacy in restoring antioxidant enzyme levels, its combination with NAC produced a markedly enhanced protective effect. The synergistic action was particularly evident in the restoration of

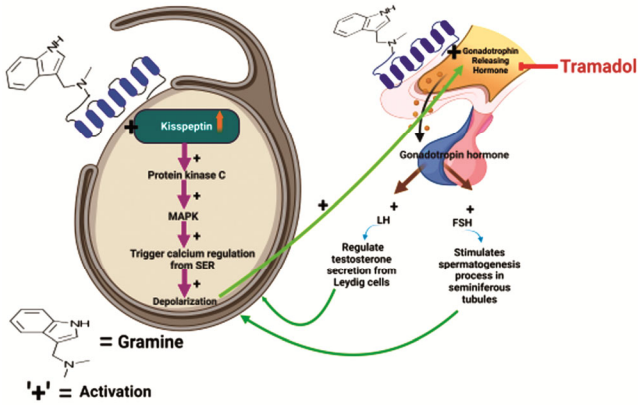

Fig. 4 — Schematic diagram representing the possible mode of action of Gramine in improving tramadol induced testicular impairment.

SOD and GSH levels, reflecting an improved cellular redox state[47]. NAC′s capacity to replenish intracellular glutathione, coupled with its free radical scavenging activity, likely contributed significantly to this observed synergy. Moreover, the activity of glutathione reductase (GR) a critical enzyme responsible for recycling oxidized glutathione back to its reduced form was significantly improved under combination therapy, further supporting the re-establishment of intracellular antioxidant defenses. Nonetheless, it is important to recognise that not all antioxidant parameters were uniformly restored, and some variability in response suggests that additional mechanisms, possibly including upregulation of other enzymatic systems or non-enzymatic antioxidants, might be involved in the complex redox regulation within testicular tissue. Furthermore, the activity of glutathione peroxidase (GPx), an essential enzyme for detoxifying lipid hydroperoxides and hydrogen peroxide, was markedly suppressed by tramadol exposure and only partially restored following treatment. This incomplete recovery indicates potential limitations of Gramine and NAC in fully reversing oxidative damage, particularly within the lipid-rich environment of testicular tissue, which is highly susceptible to peroxidative injury. The persistence of oxidative stress markers despite treatment underscores the need for comprehensive antioxidative strategies, possibly involving additional agents targeting lipid peroxidation pathways, to achieve more complete protection of testicular cellular integrity and function.

Conclusion

This investigation conclusively demonstrates that both Gramine and N-acetylcysteine (NAC), whether

administered independently or synergistically, confer substantial protective effects against tramadol-induced testicular toxicity in rat models. Tramadol exposure precipitated profound disruptions across hormonal regulation, biochemical parameters, oxidative stress indices, sperm parameters, and histopathological architecture, underscoring its deleterious impact on male reproductive function. Higher dosages of Gramine effectively mitigated certain adverse outcomes by elevating serum cholesterol, lactate dehydrogenase activity, and testicular antioxidant enzymatic defences. NAC, renowned for its antioxidative capacity and glutathione replenishment, potentiated these protective effects, particularly when combined with Gramine, resulting in marked restoration of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and androgen-binding protein (ABP) levels indicators of reestablished hypothalamic-pituitary-gonadal axis functionality. Additionally, sperm count, motility, and viability were significantly improved, correlating with preservation of seminiferous tubule integrity and spermatogenic cellular architecture. The observed elevation of nitrate and cholesterol levels suggested enhanced testicular perfusion and steroidogenic activity, though maintaining homeostatic balance remains critical to prevent functional impairments. Histopathological evaluations corroborated biochemical findings, revealing diminished cellular degeneration and restored tissue organisation. Collectively, these findings underscore the therapeutic potential of combined Gramine and NAC administration in safeguarding reproductive health against opioid-induced toxicity, warranting further elucidation through molecular, mechanistic, and long-term fertility assessments. The possible mechanism of action of Gramine is postulated and depicted in Fig. 4.

Ethical statement

The guidelines of Committee for Control and Supervision of Experiments on Animals (*CCSEA*), New Delhi, India of Govt. of India were followed and the experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) with the approval number of 1726/CCSEA/IAEC/2023-022.

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Conflict of interest

The authors have no conflict of interest to declare.

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