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An *in vivo* and *in silico* predictive study on the toxicological and modulatory effects of abused substances on sperm quality and testicular function in Wistar rats

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Summary

Introduction: Some compounds like Opioids that are commonly used may affect the biological system in addition to having a high potential for addiction. **Objective**: This study assessed the effects of commonly misused substances on sperm quality and testicular function in Wistar rats. **Material and Methods**: Twenty-five Wistar rats weighing an average of 120 ± 0.1 g were randomly assigned to five treatment groups and were orally administered with water for the control, carbonated sugar drink, 150, 300, and 300 mg/kg body weight doses of menthol, monosodium glutamate, and tramadol respectively. The rats were euthanized 24 hours after the last day of the thirty-day treatment. Biochemical assays were on carried out the plasma

and testicular homogenate. **Results**: There was a significant increase ($p<0.05$) in testosterone, FSH, LH, HDL, TG, phospholipids, glycogen, reduced glutathione concentration, sperm total count; %testicular weight change, and; there was also a significant decrease in the %tail defect, and %non-motile sperm across the treatment groups compared to the control. Contrary, there was a significant increase ($p<0.05$) in the testicular ACP and Na-K ATPases activities but MDA levels decreased significantly across treatment groups. The ouabain-α-ATPase complex's binding energy is comparable to that of the α-ATPase complexes with tramadol, glucose, menthol, and MSG, respectively. **Conclusion**: The improved sperm quality and testicular function show that these compounds were not harmful to the reproductive functions of Wistar rats. The docking analysis corroborated the effects of ATPase activity modulation on sperm motility.

Keywords: Wistar rats, Sperm Motility, Sodium Glutamate, Tramadol, Menthol, Follicle Stimulating Hormone.

Resumen

Un estudio predictivo *in vivo* e *in silico* sobre los efectos toxicológicos y moduladores de las sustancias de abuso sobre la calidad del esperma y la función testicular en ratas Wistar

Introducción: Algunos compuestos como los opioides, que se usan comúnmente, pueden afectar el sistema biológico, además de tener un alto potencial de adicción. **Objetivo:** Este estudio evaluó los efectos de sustancias comúnmente utilizadas indebidamente sobre la calidad del esperma y la función testicular en ratas Wistar. **Material y métodos:** Veinticinco ratas Wistar con un peso promedio de 120 ± 0,1 g fueron asignadas aleatoriamente a cinco grupos de tratamiento y se les administró por vía oral, agua para el control, bebida azucarada carbonatada, dosis de 150, 300 y 300 mg/kg de peso corporal de mentol, glutamato monosódico y tramadol, respectivamente. Las ratas fueron sacrificadas 24 horas después del último día del tratamiento de treinta días. Se realizaron ensayos bioquímicos sobre el plasma y el homogeneizado testicular. **Resultados:** Hubo aumento significativo ($p<0,05$) en testosterona, FSH, LH, HDL, TG, fosfolípidos, glucógeno, reducción de la concentración de glutatión, recuento total de espermatozoides, % de cambio de peso testicular, y también hubo una disminución significativa en el porcentaje de defectos en la cola y en el porcentaje de espermatozoides no móviles en los grupos de tratamiento en comparación con el control. Por el contrario, hubo un aumento significativo ($p<0,05$) en las actividades

testiculares de ACP y Na-K ATPasas, pero los niveles de MDA disminuyeron significativamente en todos los grupos de tratamiento. La energía de unión del complejo ouabaína-α-ATPasa es comparable a la de los complejos α-ATPasa con tramadol, glucosa, mentol y glutamato monosódico, respectivamente. **Conclusión:** La mejora de la calidad del esperma y la función testicular muestran que estos compuestos no fueron perjudiciales para las funciones reproductivas de las ratas Wistar. El análisis de acoplamiento corroboró los efectos de la modulación de la actividad de la ATPasa sobre la motilidad de los espermatozoides.

Palabras clave: ratas Wistar, motilidad de los espermatozoides, glutamato de sodio, tramadol, mentol, hormona folículo estimulante.

Resumo

Um estudo preditivo *in vivo* e *in silico* sobre os efeitos toxicológicos e moduladores de substâncias de abuso na qualidade do esperma e na função testicular em ratos Wistar

Introdução: Alguns compostos como os opioides, comumente utilizados, podem afetar o sistema biológico, além de apresentarem alto potencial de dependência. Objetivo: Este estudo avaliou os efeitos de substâncias comumente mal utilizadas na qualidade do esperma e na função testicular em ratos Wistar. **Material e métodos:** Vinte e cinco ratos Wistar com peso médio de 120 ± 0,1 g foram distribuídos aleatoriamente em cinco grupos de tratamento e receberam por via oral água para controle, bebida gaseificada açucarada, doses de 150, 300 e 300 mg/kg de peso corporal de mentol, glutamato monossódico e tramadol, respectivamente. Os ratos foram sacrificados 24 horas após o último dia do tratamento de trinta dias. Ensaios bioquímicos foram realizados em plasma e homogeneizado testicular. **Resultados:** Houve aumento significativo ($p<0,05$) de testosterona, FSH, LH, HDL, TG, fosfolipídios, glicogênio, redução na concentração de glutationa, contagem total de espermatozoides, % de alteração no peso testicular, e também houve diminuição significativa de a porcentagem de defeitos na cauda e a porcentagem de espermatozoides imóveis nos grupos de tratamento em comparação com o controle. Em contraste, houve um aumento significativo ($p<0,05$) nas atividades testiculares de ACP e Na-K ATPase, mas os níveis de MDA diminuíram significativamente em todos os grupos de tratamento. A energia de ligação do complexo ouabaína-α-ATPase é comparável à dos complexos α-ATPase com tramadol, glicose, mentol e glutamato monossódico, respectivamente. **Conclusão:** A melhoria da qualidade espermática e da função testi-

cular mostram que estes compostos não foram prejudiciais às funções reprodutivas de ratos Wistar. A análise de docking corroborou os efeitos da modulação da atividade da ATPase na motilidade espermática.

Palavras-chave: Ratos Wistar, motilidade espermática, glutamato de sódio, tramadol, mentol, hormônio folículo-estimulante.

Introduction

One of the prevailing public health concerns, prevalent in both affluent and impoverished nations, revolves around substance abuse [1]. Certain substances classified as "new emergent drugs of abuse" are increasingly being misused as alternatives to conventional drugs, sought for their psychoactive properties to induce feelings of boldness and pleasure. Conversely, research suggests that substance abuse can also impact male reproductive health. Men may experience infertility due to the presence of endocrine-disrupting chemicals (EDCs), which mimic natural hormones upon binding to common receptors, thus exerting a biological influence [2]. Conversely, they can directly impact sperm cells, as demonstrated by the effects of 1,3-dinitrobenzene (mDNB) on sperm motility [3]. Consequently, impaired sperm motility can hinder its ability to fertilize an egg [4, 5]. According to computer-assisted sperm analysis (CASA), the lateral head displacement is associated with the efficiency of cervical mucus penetration. At the same time, fertilization rates correlate with both the curvilinear velocity (VCL) and straight-line velocity (VSL) of sperm motility in the distal corpus epididymides and distal cauda epididymides [6]. This underscores a connection between fertility and the reduction in sperm motility induced by toxicants [7].

Research indicates that males grappling with opiate addiction tend to exhibit diminished sperm quality. Specifically, alterations in the morphology and motility of sperm are observed, accompanied by a correlation with hypogonadism [8]. Likewise, opioids can impact the functioning of the male reproductive system by decreasing the secretion of gonadotropin-releasing hormone (GnRH) within the hypothalamus of the brain [9]. Moreover, human spermatozoa membranes contain three opioid receptors [10], and endogenous opioid peptides are present in various male reproductive tracts, suggesting a potential role in reproductive processes [11]. Furthermore, sustained opiate administration in male rats has been demonstrated to adversely impact fertility [12]. Opioids can also modify male reproductive function by activating signal transduction through the opioid receptor on the plasma membrane of testicular cells [11]. In addition, studies have indicated that opioids impact male reproduction by decreasing

testosterone levels and increasing concentrations of sex hormone-binding globulin, thereby rendering the hormone inactive [9]. Recently, the emergence of new abused substances has raised significant global concerns due to their potential impact on users, particularly on their reproductive function.

Tramadol, categorized as an opioid drug, is primarily prescribed for the management of acute or chronic severe pain. Its notable feature lies in its heightened affinity for the μ-opioid receptor compared to other opioids [13-16]. Tramadol undergoes conversion by the liver enzyme CYP2D6 into its active metabolite, O-desmethyl tramadol (desmetramadol), responsible for its pharmacological effects. However, in cases where the enzyme is deficient, there might be insufficient production of the active metabolite (desmetramadol) to adequately manage pain [17]. Regulatory bodies like The Joint Committee of World Health Organization (WHO) experts and FAO consider MSG, also known as glutamic acid or its salt, to be safe but addictive. However, despite this, there is a widespread belief among the public that consuming MSG contributes to "Chinese restaurant syndrome," characterized by symptoms such as dizziness, headaches, weakness, numbness, tremors, palpitations, and nausea [18, 19]. Glutamate, a significant aminoacidergic transmitter, serves as an endogenous neurotransmitter involved in various physiological functions. These include trophic roles linked to central nervous system (CNS) ontogenesis, memory and learning processes, and stimulation of dopamine release in the striatum, a critical aspect in addiction [20, 21]. Menthol, a monoterpenoid-class organic compound, can be synthesized artificially or extracted naturally from mints such as corn mint, peppermint, or other mint varieties. The analgesic effects of menthol are attributed to its selective activation of Transient Receptor Potential (TRP) channels [22], Consequently, menthol reduces neural activity that could otherwise stimulate muscles by blocking calcium channels and voltage-sensitive sodium channels [23]. Given the limited literature regarding the impacts of these substances at elevated doses, this study was devised to assess the toxicological and modulatory effects of commonly misused substances on sperm quality and testicular function in Wistar rats. This evaluation was conducted through a combination of experimental and in-silico approaches.

Materials and methods

Procurement of the substances

Menthol, Tramadol, and monosodium glutamate were obtained from Sigma-Aldrich Company Limited, UK

Animal model

The experimental procedures on laboratory animals were done in adherence to the Guideline to the Care and Use of Experimental Animals from the National Institutes of Health (NIH). Institutional approval for the use of the animals was by the Department of Biochemistry Landmark University Animal Care and Use Committee with an Approval number LUAC/BCH/2022. Thirty Wistar rats with an average weight of 120 ± 0.1 g were procured from Animal Holdings Landmark University and were allowed to acclimatize for two weeks before the commencement of the experiment. The animals were kept in wooden cages kept in a well-ventilated animal house and were allowed access to food and water *ad libitium*.

Preparation of the chemical

The drug substances were combined in the following format: menthol with a carbonated sugar drink mixture, monosodium glutamate with a water mixture, and tramadol with a water mixture [24]. Menthol was dissolved in a sugar-carbonated drink, while tramadol and monosodium glutamate were dissolved in water.

Animal grouping, experimental design, and animal sacrifice

The Wistar rats were randomly allocated into five groups (A-E), with five rats assigned to each group:

Group A- Rats are administered 2 ml of water (negative control).

Group B- Rats are administered 2 ml of a carbonated sugar drink (positive control).

Group C- Rats are treated with 150 mg/kg body weight of menthol in 2 ml of carbonated sugar drink (psychoactive mixture) [24].

Group D- Rats are treated with 300 mg/kg body weight of monosodium glutamate in 2 ml of distilled water.

Group E- Rats are treated with 300 mg/kg body weight of 2-[(dimethylamino) methyl]-1-(3-methoxyphenyl) cyclohexanol (tramadol) in 2 ml of distilled water.

Each group received oral administration of the treatment substance daily for a period of 30 days. After a 24-hour interval following the last treatment, the rats were anesthetized using 0.5% halothane and euthanized by severing their aorta with a surgical knife. Blood samples were collected in sample bottles, and the scrotal sac was incised to extract the testes. Serum was separated from clotted blood, and homogenate was obtained from homogenized testicular tissue. Both serum and homogenate samples were then frozen and utilized in biochemical assays.

Methods for the sperm analysis

As soon as the rat was euthanized, the scrotal sac was cut open, and the epididymis was instantly removed, the surrounding fat was removed, and it was then weighed. The sperm per gram caudal epididymis was calculated using the weight of the caudal epididymis. The 2010 WHO standard served as the foundation for the reference utilized for the manual sperm examination [25]. Sperm concentration was measured using the Neubauer hemocytometer and the Epididymal Sperm Concentration was counted following the modified method described by Yokoi and Mayi [26]. Similarly, the Sperm Progressive Motility was evaluated by an earlier method by Sonmez *et al.* [27]. An Olympus light microscope equipped with a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) was adjusted to ×100 magnification to evaluate sperm count and straight-line sperm motility. Similarly, the morphological abnormality was also estimated with an Olympus light microscope, two hundred sperm cells were examined at ×400 magnifications per animal.

Hormonal assay

LH, FSH, and testosterone levels were evaluated using the principle of solid-phase enzyme-linked immunosorbent assay (ELISA) [28].

Biochemical assays

For measuring or estimating the activity of certain biochemical parameters in both serum and testicular samples, a digital UV/VIS spectrometer (Jenway, England) was utilized. Total protein concentration was determined using the method outlined by Gornall *et al.* [29]. The activities of ACP and ALP were assessed following the procedure described by Wright *et al.* [30]. Superoxide dismutase (SOD) activity was estimated by the method detailed by Misra and Fridovich [31], while the reduced glutathione level was determined using the method described by Jollow *et al.* [32]. Measurement of MDA level (a product of lipid peroxidation) was conducted by quantifying thiobarbituric acid reactive substances (TBARS) according to the methods outlined by Satoh [33]. Acetylcholine esterase activity was evaluated by the method described by Ellman *et al.* [34]. The procedure outlined by Ronner *et al.* [35], with modifications as described by Bewaji *et al.* [36] was employed for estimating Na^+/K^+-ATPase activity. Total phospholipids were determined using the method described by Stewart [37]. Glycogen concentrations were assessed following the procedure detailed by Kemp and Van Heijningen [38], while the determination of nitric acid concentrations was conducted according to the method described by Ilavarasan *et al.* [39]. The method described by Gidez *et al.* [40] was adopted for the extraction of HDL from the tissue while the method described by Folch *et al.* [41] was adopted for the extraction of

lipids from the tissue (testes). The evaluation of the HMG-CoA/mevalonate ratio was conducted using the method described by Ramakrishnan and Rao [42].

Molecular docking simulation

The protein structure (ATPase) used in the docking simulation was obtained from the protein data bank (pdb), with the pdb code; 3A3Y (Resolution: 2.80 Å). Water molecules and the bound ligands were deleted from the X-ray crystallographic structure. Molecular docking was performed using AutoDock vina [43] with the help of PyRx [44], a graphical user interface program. The following ligand structures: Ouabain, tramadol, menthol, glucose, and sodium monoglutamate were optimized at the B3LYP/6-31Gd) level of theory before use for docking simulations. The control for the docking experiment was Ouabain (Figure 1), where energies of binding and interactions of other ligands used in the present study were compared. Since 3A3Y.pdb contains various chains with their respective binding sites, we kept the protein structure fixed while sampling the entire protein space with the ligands. The grid dimension used are as follows: center x = -34.6247; y = -55.0409 and z = 61.1613 Å while the size was: size x = 122.871241837; y = 134.560933971 and z = 189.749764137 Å. The exhaustiveness of eight was used throughout the docking experiment. Various interactions were observed for the molecular docking of the studied ligands with 3a3y.pdb file. Figure 1 shows the Ouabain ligand docked into the active site of 3a3y.pdb crystal structure.

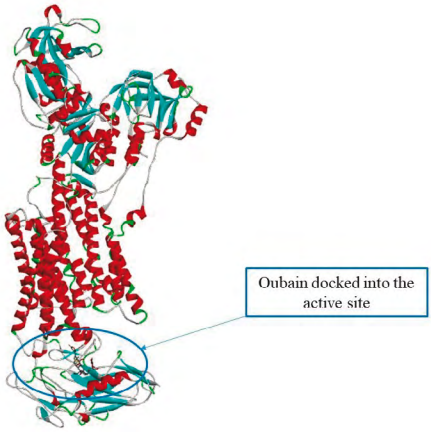


Figure 1. Ouabain ligand in the blue circle, docked in the active site of 3a3y.pdb structure between two chains, as also reported in the original manuscript of the crystal structure.

Histopathology

The standard method for tissue fixation for histological assessment was carried out using the method described by Hewitson and Darby [45]. The testes removed from the euthanized rats were instantly fixed by dropping the tissue in 10 % buffered formalin (pH 7.4) for about 12 h and later embedded in paraffin. The tissue from paraffin was subsequently sectioned (5 mm) with a microtome. Hematoxylin and eosin were used to stain the tissue, which was subsequently mounted in Canada balsam and sectioned. The stained sections were viewed under the light microscope and their histological features were captured using Sony DSC-W35. The examination checked for cell necrosis, degraded cells, cell detachment, and lymphocyte infiltration in the damaged tissues [46].

Data analysis

The results were presented as mean ± SEM. Mean comparison was performed with One Way Analysis of Variance (ANOVA) with Tukey post hoc to assess the significant difference at $p<0.05$. SPSS Statistics 22 (Statistical Package for Social Science) (SPSS Inc., Chicago, IL, USA) was used in all the statistical analyses.

Results

There was a significant increase ($p<0.05$) in testosterone levels across treatment groups compared to the control (Figure 2A). There was a significant increase ($p<0.05$) in the FSH level across the treatment groups compared to the control (Figure 2B). There was a significant decrease ($p<0.05$) in LH level in animals administered carbonated sugar drinks and carbonated sugar drinks & menthol compared to animals in the control group. Contrary, LH levels significantly increased ($p<0.05$) in animals administered MSG and tramadol (Figure 2C).

There was no significant change ($p>0.05$) in sperm volume/weight in the treatment groups compared to the control (Figure 3A). There was no significant change ($p>0.05$) in the sperm concentration count in the treatment groups except in the group administered tramadol which showed a significant increase ($p<0.05$) compared to the control (Figure 3B). Similarly, there was no significant change ($p>0.05$) in the sperm total count in the treatment groups except for the group-administered carbonated sugar drink only which showed a significant decrease ($p<0.05$) compared to the control (Figure 3B). In the % sperm morphology, there was no significant change ($p>0.05$) in the % of the normal sperm cells, % of sperm with head defects, and % of sperm with neck defects in the treatment groups compared to the control (Figure 4A). There was a significant increase ($p<0.05$) in the tail defects in the sperm cells of the animal

groups administered menthol with carbonated sugar drink and MSG while the groups administered tramadol showed a significant decrease ($p<0.05$) in tail defects compared to the control (Figure 4A). Similarly, the sperm %progressive assessment showed no significant change ($p>0.05$) in the %fast and slow-moving sperm cells across treatment groups compared to the control (Figure 4B). Meanwhile, there was a significant increase ($p<0.05$) in the %non-motile sperm cells in the treatment groups except in the group administered tramadol which showed no significant change ($p>0.05$) compared to the control (Figure 4B).

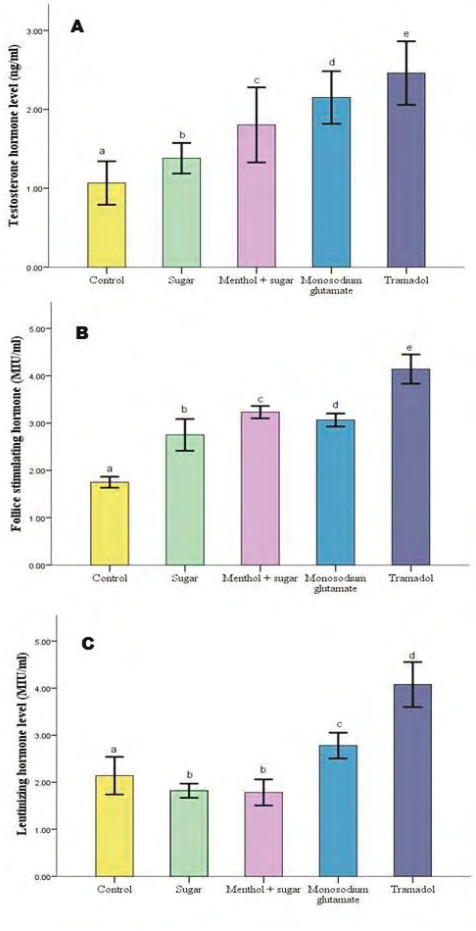
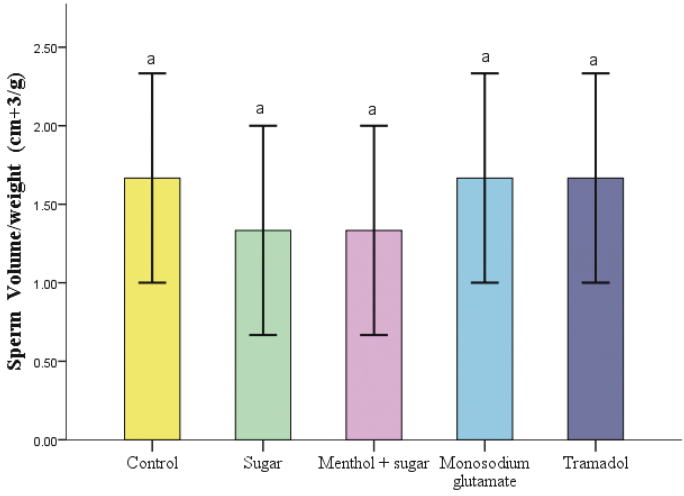


Figure 2. Effects of carbonated sugar drink, menthol and carbonated sugar drink, monosodium glutamate, and tramadol on testosterone level (A), follicle-stimulating hormone level (B), and luteinizing hormone levels (C), on male Wister rats. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

a



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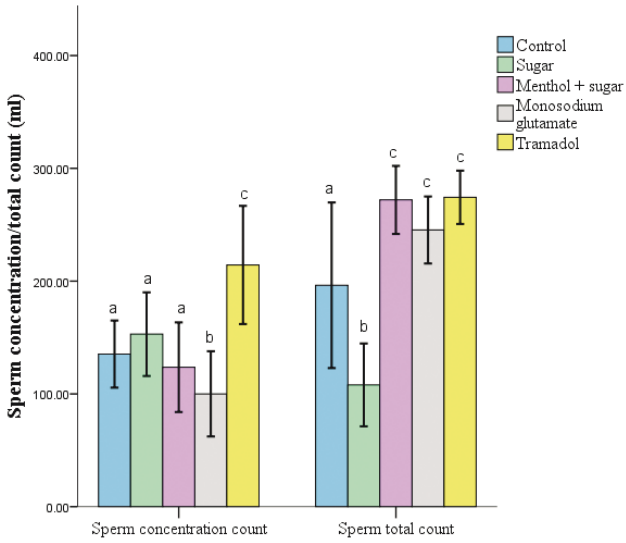

Figure 3. Effects of carbonated sugar drink, menthol+ carbonated sugar drink, monosodium glutamate, and tramadol on sperm volume/weight (A) and sperm concentration/total count (B), on Wister rats. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

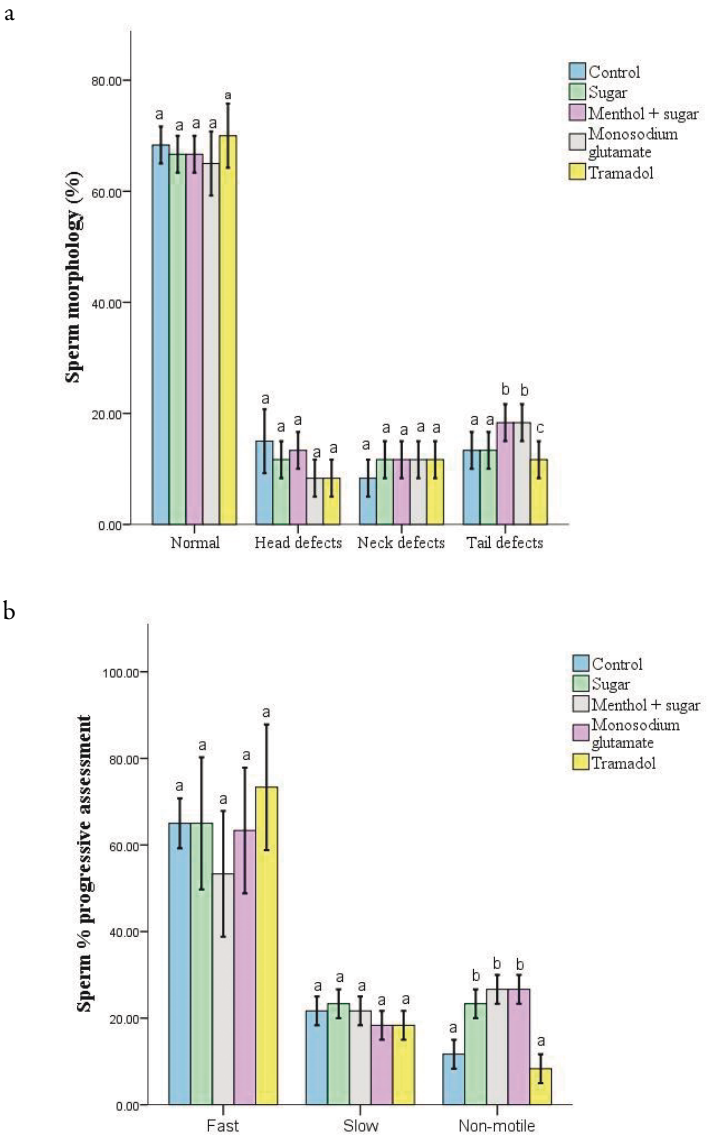

Figure 4. Effects of carbonated sugar drink, menthol+ carbonated sugar drink, monosodium glutamate, and tramadol on the total count concentration sperm morphology (A) and sperm % progressive assessment (B), in male Wister rats. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

There was a significant increase ($p<0.05$) in the total cholesterol concentration in the testis of rats administered carbonated sugar drink and menthol with carbonated sugar drink but there was a significant decrease ($p<0.05$) in the testis of the group administered MSG compared to the control (Figure 5A). Contrary, there was no significant change ($p>0.05$) in the cholesterol concentration in the testis of the rat treated with tramadol compared to the control (Figure 5A). Furthermore, the plasma HDL concentration increased significantly ($p<0.05$) across the treatment groups compared to the control (Figure 5B). Similarly, the treatment groups showed a significant increase ($p<0.05$) in the testicular triglyceride concentration compared to the control (Figure 5C). There was a significant increase ($p<0.05$) in phospholipid concentration in all the treatment groups except in the group administered carbonated sugar drink only which decreased significantly ($p<0.05$) when compared to the control (Figure 5D).

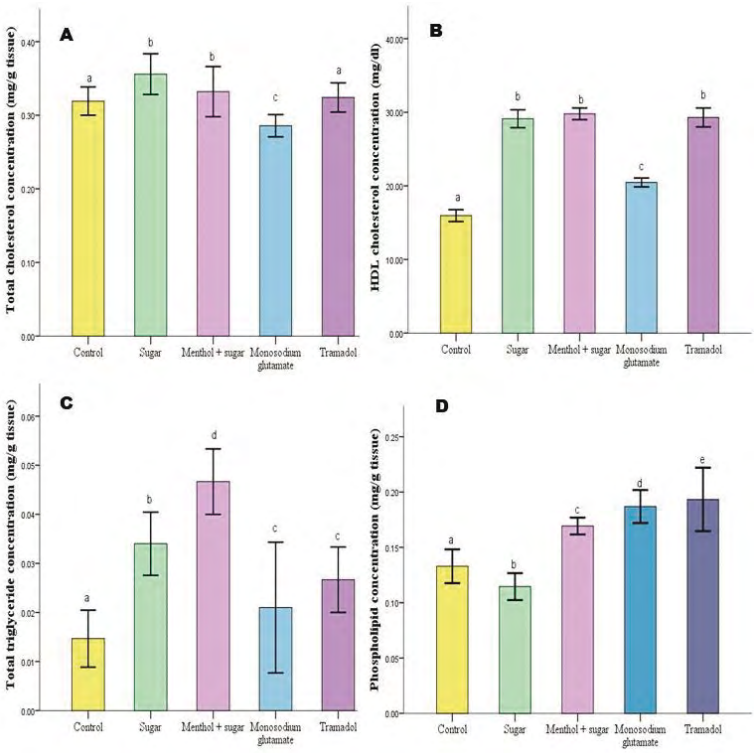

Figure 5. Effects of carbonated sugar drink, menthol+ carbonated sugar drink, monosodium glutamate, and tramadol on the total cholesterol concentration (A), plasma HDL cholesterol concentration (B), total triglyceride concentration (C), and phospholipid concentration (D), in the testis of rats. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

There was a significant decrease ($p<0.05$) in the catalase activity in the testis of the rats across the treatment groups compared to the control (Figure 6A). Similarly, there was a significant increase ($p<0.05$) in SOD activity in the treatment groups except for the group treated with MSG which showed a significant decrease ($p<0.05$) in the activity of SOD compared to the control (Figure 6B). There was a significant increase ($p<0.05$) in MDA concentration in the group administered menthol with carbonated sugar drink while there was a significant decrease ($p<0.05$) in MDA in the group treated with MSG compared to the control (Figure 6C). There was no significant change ($p>0.05$) in the MDA level in the testis of the rat administered carbonated sugar drink only and tramadol compared to the control (Figure 5C). Reduced glutathione levels in the testis of the treated rats increased significantly ($p<0.05$) in all the groups except in the group administered MSG which showed a significant decrease ($p<0.05$) compared to the control (Figure 6D).

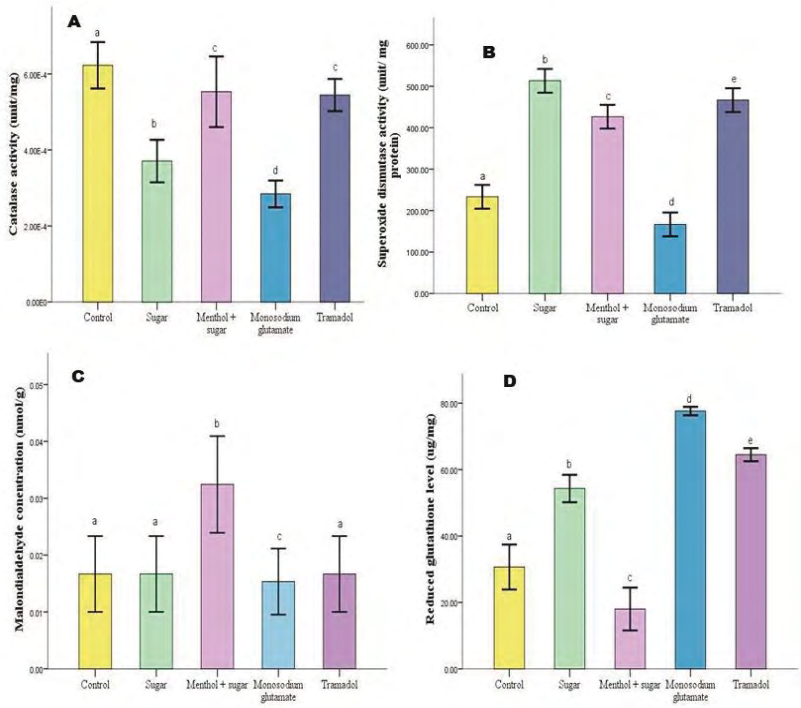

Figure 6. Effects of carbonated sugar drink, menthol+ carbonated sugar drink, monosodium glutamate, and tramadol on catalase activity (A), superoxide dismutase activity (B), malondialdehyde concentration (C), and reduced glutathione concentration (D), in the testis. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

There was a significant increase ($p<0.05$) in the nitric oxide level in the testis of the rats in the treated groups except for the group treated with tramadol which showed a significant decrease ($p<0.05$) in NO level compared to the control (Figure 7A). There was a significant increase ($p<0.05$) in the glycogen concentration in the treatment groups compared to the control (Figure 7B). There was a significant increase ($p<0.05$) in the % testicular weight change compared to the control (Figure 7C). The groups treated with menthol with carbonated sugar drinks and tramadol showed a significant decrease in the % organ body weight while the groups administered MSG and carbonated sugar drinks only showed an increase in organ body ratio compared to the control (Figure 7D).

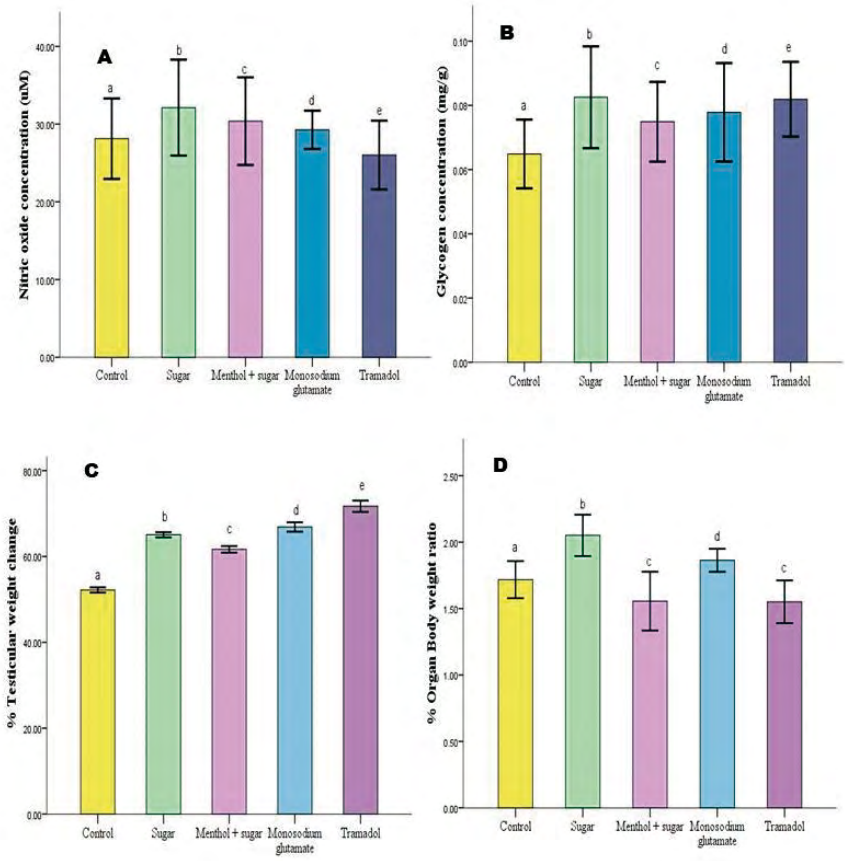

Figure 7. Effects of carbonated sugar drink, menthol + carbonated sugar drink, monosodium glutamate, and tramadol on nitric oxide concentration (A), glycogen concentration (B), % testicular weight change (C), and % organ body weight (D), of the testis. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

There was a significant decrease ($p<0.05$) in the HMG-CoA/mevalonate ratio in the treatment groups except for the group administered carbonated sugar drink which showed a significant increase ($p<0.05$) compared to the control (Figure 8A). There was no significant change ($p>0.05$) in Na-K ATPase activity in the treatment groups except in the group administered tramadol which showed a significant increase ($p<0.05$) compared to the control group (Figure 8B). There was a significant increase ($p>0.05$) in the acetylcholine esterase activity across the treatment groups compared to the control, however, the enzyme activity decreased significantly ($p<0.05$) in the group administered a combination of carbonated sugar drink and menthol (Figure 8C).

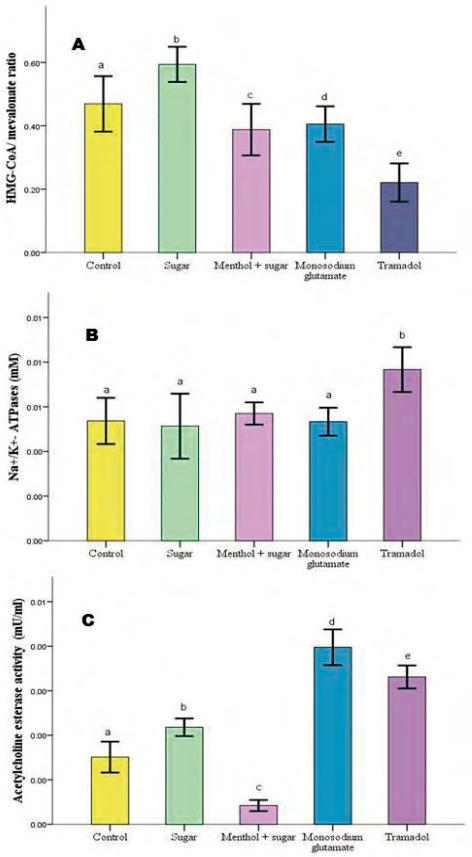

Figure 8. Effects of carbonated sugar drink, menthol+ carbonated sugar drink, monosodium glutamate, and tramadol on HMG-CoA mevalonate ratio (A), Na^+/K^+- ATPases activity (B), and acetylcholine esterase activity (C) in the testis. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

There was a significant increase ($p<0.05$) in the total protein concentration in the treatment groups except for the group administered MSG which showed a significant decrease ($p<0.05$) compared to the control (Figure 9A). There was a significant increase ($p<0.05$) in the acid phosphatase activity compared to the control (Figure 9B). There was a significant decrease ($p<0.05$) in alkaline phosphatase activity in the groups administered menthol with carbonated sugar drink and monosodium glutamate while the other treatment groups did not show any significant ($p>0.05$) change (Figure 9B).

The histology of the testis across the treatment groups showed normal histomorphology with a typical seminiferous tubule containing different types of germ cells; spermatogonia lying on the basement membrane with other cells proliferating in a centripetal direction when compared to the control (Figure 10).

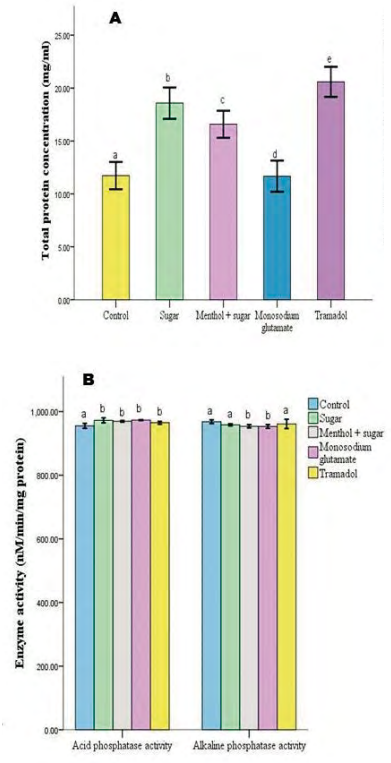

Figure 9. Effects of carbonated sugar drink, menthol+ carbonated sugar drink, monosodium glutamate, and tramadol on total protein concentration (A) and acid/alkaline phosphatase activity (B) in the testis. Data are presented as mean ± SEM; bars with a different superscript are significantly different at a confidence interval of $p<0.05$.

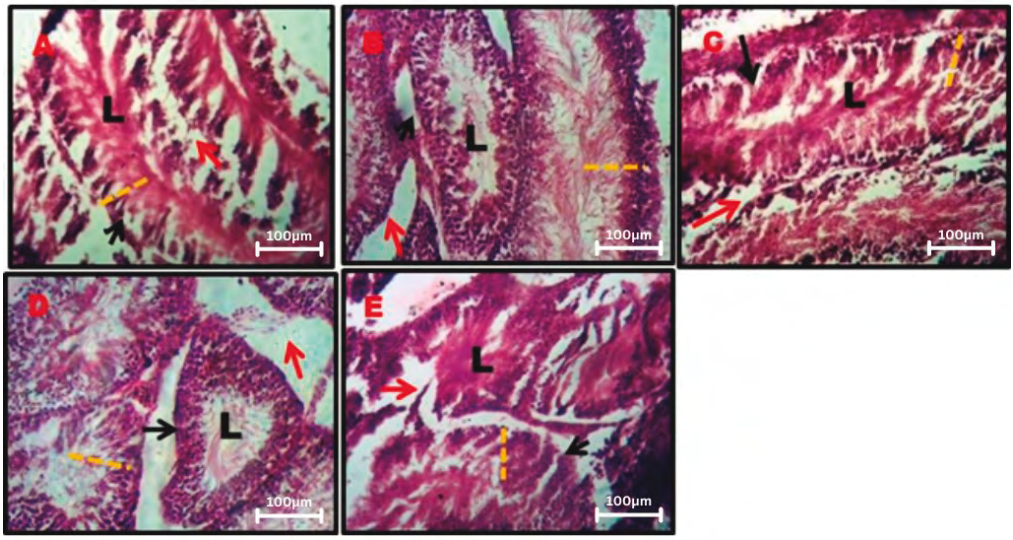

Figure 10. Photomicrograph of the testis (**H and E x100**): **Control:** The histomorphology is normal; normally, the seminiferous tubule includes several types of germ cells; at the membrane basement, other cells are lined up and grow in a centripetal orientation. Erosion at the basement membrane is afferent (A). **Carbonated sugar drink:** The histomorphology is normal; normally, the seminiferous tubule includes several types of germ cells; at the membrane basement, other cells are lined up and grow in a centripetal orientation (B). **Menthol + carbonated sugar drink:** The histomorphology is normal; normally, the seminiferous tubule includes several types of germ cells; at the membrane basement, other cells are lined up and grow in a centripetal orientation (C). **Monosodium glutamate:** The histomorphology is normal; normally, the seminiferous tubule includes several types of germ cells; at the membrane basement, other cells are lined up and grow in a centripetal orientation (D). **Tramadol:** The histomorphology is normal; normally, the seminiferous tubule includes several types of germ cells; at the membrane basement, other cells are lined up and grow in a centripetal orientation (E). (Black arrow: Basement membrane, L: lumen, Dotted line: Seminiferous epithelium).

In the course of the docking process, nine conformers were generated and the conformers with the highest binding affinities, that is, those with the highest negative values are presented in Table 1. From the docking results, the ligand with the best binding affinity is the control (Ouabain) with a value of -8.5 kcal/mol. This is not surprising since many hydroxyl moieties are used for binding at the residue. The one with the

lowest binding energy is due to monosodium glutamate (MSG) with a value of -5.1 kcal/mol. Thus, various interactions were observed for the molecular docking of the studied ligands with 3a3y.pdb file. From Figure 11A, Oubain showed only hydrogen bonding interactions; conventional and carbon. The conventional H-bond is however moderate and occurs over a distance of 2.69 Å with His B: 80, while the C-H bonds are weak and occur over a distance of 3.63 Å with Ser B: 105 and Asp A: 897. This H-bonding is greatly important in mediating the stability of a protein-ligand complex. The dominant interactions are strong conventional hydrogen bonding and moderate carbon-hydrogen bonding interactions. Interestingly, other chemical interactions played out in the Protein-Tramadol complex, as can be seen in (Figure 11B). The interactions of tramadol with the amino acid residues of the pocket are observed to be purely hydrophobic over long ranges. The strongest is the Pi-Sigma interaction and is with Val26 of chain G. The Pi-orbital mediated interaction, Pi-Pi Stacked interaction, is mainly due to chain A with the aromatic ring of tramadol. Mixed hydrogen bonding and hydrophobic interactions (mainly Alkyl and Pi-Alky) interactions were observed in the Protein-Menthol complex (Figure 11C). Hydrogen bonding interaction was observed to be strong over a distance of 2.02 Å between the hydroxyl hydrogen and Gln203 of chain B. Just like Ouabain, the interaction of 3a3y.pdb protein with Glucose is between oxygen and hydroxyl groups in the ligand structure and the amino acid residues (Figure 11D). The interaction of 3a3y.pdb protein with MSG ligand is observed to be mainly hydrogen bonding. Intramolecular interaction between Glu21 and Lys377 was also observed (Figure 11E).

Table 1. The binding free energy values of the ligands

Ligand	Binding affinity (kcal/mol)
Ouabain	-8.5
Tramadol	-6.9
Glucose	-6.0
Menthol	-5.9
Monosodium glutamate	-5.1

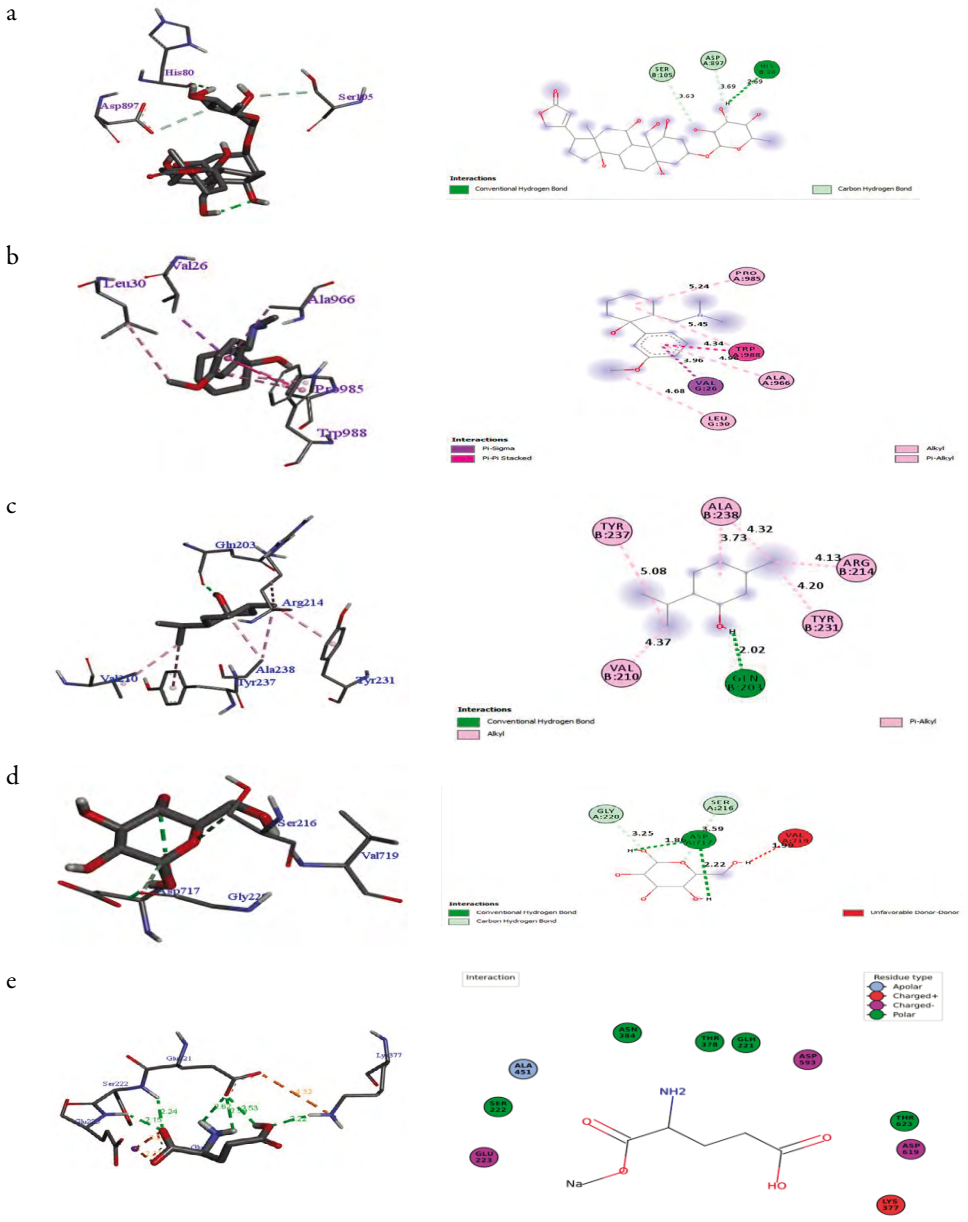

Figure 11. Interaction diagrams of Ouabain ligand (the reference inhibitor) (A), Tramadol ligand (B), Menthol ligand (C), glucose (D), and sodium monoglutamate (E), with the amino acid residues of the protein pocket (ATPase) respectively. The bond distances are given Angstrom (Å).

Discussion

Reproductive endocrine hormones trigger a series of biochemical processes in specialized testicular cells, crucial for spermatogenesis and the generation of high-quality sperm cells. Consequently, the health and integrity of testicular cells play a pivotal role in this process. Variations in the frequency of stages within the cycle of the seminiferous epithelium serve as indicators to assess the effects of treatments on spermatogenesis [47]. Sertoli cells serve as nurturing cells within the testis, creating a luminal environment essential for providing energy for spermatogenesis. Additionally, they offer crucial nutritional support for the growth and maturation of male germ cells into sperm cells [48, 49]. Furthermore, lactate is secreted by Sertoli cells as a by-product of lipid, amino acid, and glycogen metabolism [50, 51]. Similarly, When FSH binds to the Sertoli cells, it activates adenylyl cyclase and increases cytosolic cAMP, which then activates cAMP-dependent kinases and resulting in the phosphorylation of several unidentified proteins in the cell [52, 53]. When the enzymes are activated, it produces steroid, nuclear RNA, and protein synthesis, as well as steroid and protein secretion [54]. Furthermore, in the basal compartment of the testes are located Leydig cells which produce steroid hormones, primarily testosterone. The circulating luteinizing hormones (LH) secreted by the pituitary gland stimulate testosterone production in the Leydig cells [55]. Testosterone controls spermatogenesis by regulating the adhesion of round spermatids to Sertoli cells and the release of spermatids from the testis [56]. Because the generation of these hormones was normal, the chemicals did not interfere with hormone production or harm the testicular cells that serve as the source of these hormones. The interstitial germ cell required for spermatogenesis and other testicular machinery is housed in the testes. As a result, the rise in testes weight seen in rats fed these compounds may be explained by the hypertrophy or hyperplasia of the interstitial cells, without a change in the ratio of testicular to germ cells or Sertoli cells, as a result of the substance's impact [57]. Cell hypertrophy could be linked to sugar's high caloric content and an increase in food intake brought on by MSG's regulation of the brain's center for hunger. The decrease in organ body weight observed in menthol with carbonated sugar drinks and tramadol, on the other hand, may be due to an increase in metabolic activity. Additionally, these drugs may affect biochemical processes that affect the activity of germ cells or testicular cells, which could ultimately affect the quality of sperm production. The total sperm count is equal to the number of sperm in the entire ejaculate (sperm concentration times semen volume), where sperm concentration is the number of sperm per unit volume (milliliter) of semen [58]. Though no one test or sperm characteristic can predict male fertility or infertility with 100% certainty since no one sperm feature or function reflects the capacity of spermatozoa to complete the intricate processes that will result in pregnancy [59]. Due to the

descriptive nature of various sperm parameters, some studies have found a connection between IVF and motility [60], concentration [61], and normal/abnormal morphology because some of the sperm parameters are descriptive [62]. However, the current investigation demonstrated that none of the semen quality indicators were adversely affected by the medications used in the experiment; rather, it appeared that numerous semen metrics were enhanced [63], which may have been caused by the cumulative effect of chemicals with an endogenous source. An electrochemical gradient created by the electrogenic translocation of three sodium ions and two potassium ions out of the cell, mediated by the heteromeric integral membrane protein known as Na K-ATPase, resulting in the breakdown of ATP to ADP, which powers the cell [64-66]. The discovery of an α-4 isoform of Na, K-ATPase in the central region of the flagellum of spermatozoa pointed to a physiological function associated with the particular activity of these cells. This unique Na, K-ATPase isoform was crucial for sperm cell motility since blocking the α-4 isoform stops sperm from moving [67]. Because of this, the increased ATPase activity observed in this study may be proof that tramadol has the potential to increase sperm fertility. Additionally, it has been discovered that the cholinergic system is present in mammalian testicular cells like Sertoli cells and germ cells, both of which include functioning Ach receptors that are associated with sperm motility [68, 69]. The current study's elevated activity of Na K-ATPase and Ach helped to explain why there were so many sperms with fast motility and so few with slow motility or none at all.

High-density lipoprotein (HDL) serves as the principal supplier of cholesterol for steroidogenic tissue [70]. The interstitial tissue housing Leydig cells contains steroidogenic tissue equipped with specialized high-density lipoprotein-binding sites [71]. The elevation of HDL observed in this study corroborated findings indicating a correlation between heightened serum HDL levels and increased hepatic production, attributed to the demand from steroidogenic tissue [72, 73]. Evidence from both clinical and experimental sources underscores the significance of lipid metabolism in governing testicular physiology and male fertility [74]. Likewise, the function of steroidogenesis in the testis relies on either the *de novo* synthesis of cholesterol in Leydig cells or the stored cholesterol ester [75]. Cholesterol is a precursor of steroid hormones that are essential for sperm production [76, 77]. Furthermore, HMG-CoA reductase activity is required for interstitial tissue and Leydig cell homeostasis [78, 79]. Similarly, a series of biochemical reactions leads to the production of hydroxy-methyl glutaryl-CoA (HMG-CoA), which in turn leads to the production of mevalonate, which in turn leads to the production of cholesterol. The decrease in the HMG-CoA/mevalonate ratio correlates with increased activity of the reductase, which may be responsible for increased cholesterol production, steroidogenesis, and improved sperm quality, as demonstrated in this study [80]. Glycogen is found in the seminiferous tubules and

Sertoli cells, and its abundance varies at different stages of spermatogenesis. Glycogen is most likely important in the maturation of germinal cells. Despite being the primary source of ATP and sperm activity, failure of glucose uptake into the cell will result in the cell switching to glycogen metabolism as an alternative source of glucose [81-83]. The increased glycogen in this study could indicate intact testicular energy metabolism, which is required for spermatogenesis. NO is an inflammatory mediator that is produced in normal testis and has been found to regulate Leydig cell function and spermatogenic development in previous studies [84, 85]. The increased NO production and steroidogenesis in this study indicated that these emerging psychoactive substances had no negative effects on interstitial cell and spermatogenic activity [86, 87]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major phospholipids that serve as a building block of the cell membrane in the developing testis during sperm cell development [88-90]. Phospholipids are involved in sperm membrane permeability and fluidity [91-93], acrosomal reactions [94], and sperm motility [95]. The increased phospholipid level reported in this study could be linked to improved sperm quality hence these substances did alter the biochemical process for either phospholipid production or spermatogenesis. *Triacylglycerides* (TAG), membrane remodeling, and synthesis are indicators of male germ cell differentiation [96]. The final enzymatic step in TAG biosynthesis involves the joining of a long-chain fatty acid to a diacylglycerol molecule, which is mediated by the enzyme acyl-CoA: diacylglycerol acyltransferase (DGAT), which is found in most tissues' cells [97]. Similarly, the increased TAG production may be related to testicular cell differentiation vital in sperm production which is also an indication of the un-interruption of this process in the teste by these substances. Proteins present in the testis and epididymis play vital roles in regulating spermatogenesis, sperm maturation, and the differentiation of testicular cells [98]. The enhanced sperm quality and improved testicular function observed in this study may be linked to elevated total protein concentrations induced by these substances, supporting their non-toxic effects on male reproductive physiology. The abundance of long-chain and very long-chain unsaturated fatty acids in germ cell and sperm cell membranes renders them susceptible to lipid peroxidation, which is the primary cause of cellular damage [99, 100]. This damage entails alterations in germ cell membrane structure and sperm function, leading to cytopathological modifications and cell death [101-103]. The reduction in the lipid peroxide biomarker (MDA) observed in this study further underscores the non-toxic effects on both testicular and sperm cells. Additionally, lipid peroxidation, typically induced by toxicants generating ROS/free radicals, prompts the activation of antioxidant enzymes such as catalase and SOD to neutralize them. Therefore, the decreased activities of these endogenous antioxidant enzymes might be attributed to a reduced generation of radicals by the substances administered, providing further evidence for the observed low concentra-

tion of MDA in this study. Similarly, the levels of GSH indicated that these substances may not have an oxidative effect on testicular germ cells.

ACP is an appropriate biomarker for assessing prostate function [104]. ACP in semen is synthesized and secreted by the prostate [105], which is regulated by androgens [106], therefore decrease in ACP activity in plasma is an inflammatory effect on the prostate [107, 108]. The increase in the activity of ACP in this study also was an expression of an enhancement of the function of the prostate which was an indication of the safety of testicular function. Alkaline phosphatase (ALP) is found in seminal fluid and is thought to play a role in the glycolytic and fructose formation pathways in sperm [109]. Therefore in some mammals, ALP in sperm is produced in the testes or a testicular accessory organ (prostate, epididymis) [110, 111], while in some other species where the majority of semen plasma is alkaline phosphatase (SPAP), activity originates from the epididymis and testicle and can serve as an ejaculatory biomarker to distinguish azoospermia or oligospermia from ejaculatory failure [112]. The reported activity of testicular ALP in this study indicates that none of these substances could be deleterious on spermatogenesis or testicular function. The cellular architecture of the testis was normal, with no distortion or degeneration, supporting our claim that these substances which were administered at the highest daily/therapeutic did not disrupt reproductive efficiency in male rats. The last phase of the loss of testicular function, as characterized clinically, is testicular cell degeneration [113]. Reduced sperm production may also be linked to higher rates of germ cell degeneration [114, 115]. Therefore, the enhanced testicular and sperm quality in this study is indicative that these substances are not deleterious to the testicular cells.

Moreover, molecular docking facilitates a clearer comprehension of the molecular mechanisms underlying biochemical processes. The insights obtained through docking are poised to enhance the outcomes of atomic-level experiments [116-118]. Ouabain, known as a potent Na^+/K^+-ATPase inhibitor [119], exhibited a lower binding energy, supporting its stronger inhibitory effect on ATPase activity. Conversely, the higher binding energy observed with tramadol and glucose might be indicative of activity activation of Na^+/K^+-ATPase. The observed sperm progressive assessment in this study could be attributed to the activation of Na^+/K^+-ATPase activity during the acrosome reaction. The process of acrosome reaction (AR) in sperm cells involves fusion and vesiculation of the outer acrosomal membrane, which interfaces with the cell plasma membrane. This reaction is crucial for fertilization both in vivo and in vitro and necessitates preceding changes in the sperm known collectively as capacitation [1, 2]. The binding energies observed with tramadol and glucose may suggest their potential to induce sperm cell capacitation through the activity of Na^+,K^+-ATPase, with result-

ing K^+ influx being vital for mammalian sperm AR [3]. This study has demonstrated that ATPase activity serves as one of the signaling pathways in the regulation of sperm motility, capacitation, and acrosome reaction. Additional, molecular docking data in this study confirmed the previously described involvement of α-ATPase activity in sperm motility [67].

Conclusion

The development of healthy sperm cells relies on the intricate interplay between biochemical processes and hormonal effects within intact interstitial cells. Disruption of hormonal function or oxidative damage to the testicular cell membrane can impede reproductive functions or sperm integrity. Therefore, maintaining intact testicular cells and optimal levels of reproductive hormones may have contributed to the improved sperm quality observed in this study. The substances administered did not exhibit detrimental effects on testicular cells or reproductive function in male rats. Additionally, the in-silico study predicted that the enhanced sperm motility observed could be attributed to the effects of these substances on sperm cell ATPase activity.

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Data availability

Data will be made available on request.

Acknowledgments

Not Applicable

CRediT authorship contribution statement

Nwonuma Charles: Conceptualization, Data curation, Writing review & editing, Formal analysis, Investigation, Methodology, **Adedoyin, Adeola:** Investigation, Writing original draft, Methodology, **Inemesit. Asokwa:** Resources, Methodology. **Okeniyi Funmilayo:** Supervision, Writing review & editing. **Onyemaka Melody**: Project administration, Supervision, **Osemwegie, Omorefosa :** Writing review & editing, Ojo Adeleke: Formal analysis, Investigation, **Irokanulo Emenike:** Resources, Formal analysis **Adah, Deborah :** Formal analysis, Investigation **Alejolowo, Omokolade :** Data curation, Methodology.

Conflicts of interest

Authors declare that they have no conflicts of interest

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