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Research Article

Proton Pump Dysfunction and Upregulation of Caspase-3 Activity via Oxidative-Sensitive Signaling Mediate Rohypnol-Induced Testicular Toxicity

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Rohypnol is a benzodiazepine that is used for its anxiolytic, anticonvulsant, muscle relaxant, and hypnotic properties, but commonly abused. It has been reported to induce sexual dysfunction; however, its effect on testicular integrity and sperm quality is yet to be documented. This study evaluated the impact of Rohypnol on testicular integrity, circulatory testosterone, spermatogenesis, and sperm quality. The likely role of oxidative stress-dependent pathway as well as testicular proton pumps and caspase-3 activity in Rohypnol-induced modulation of male reproductive function was explored. In this study, adult male rats were randomized into six groups of six rats each: the control received 1 ml of distilled water as vehicle, while the low- and high-dose Rohypnol-treated groups received 2 mg/kg and 4 mg/kg of Rohypnol for four weeks. The control-, low-, and high-dose recovery groups received 2 mg/kg and 4 mg/kg of Rohypnol for four weeks, respectively, which was followed by a 4-week drug-free recovery period. Rohypnol treatments led to impaired steroidogenesis and spermatogenesis and low sperm quality. This was accompanied by a rise in the markers of testicular injury, oxidative stress, inflammation, and apoptosis. Also, the activities of testicular enzymatic antioxidants and proton pumps were significantly reduced. Although the impacts of low-dose Rohypnol treatment were partially reversible, those of the high-dose Rohypnol treatment were not. Histopathological findings on the testicular tissues were in tandem with the biochemical alterations. Thus, it is safe to infer that Rohypnol induces testicular damage by the suppression of Na^+/K^+-ATPase and Ca^{2+}-ATPase and the activation of caspase-3 through an oxidative-sensitive signaling pathway.

1. Introduction

Drug abuse remains a global burden with negative impacts on health [1]. The challenges associated with abuse of addictive substances are a huge burden and calls for global attention. Substance abuse is a major worrisome health-related phenomena globally [2, 3]. According to Bakare and Isah [2], one of the leading drugs of abuse is (benzo)-diazepines with a prevalence of 20% among substance abusers.

Rohypnol, also called flunitrazepam, is a benzodiazepine that is known to possess anxiolytic, anticonvulsant, muscle relaxant, and hypnotic properties. Although it is a sedative that has been proven to be effective in the management of insomnia, it is commonly abused due to its addictive property [4]. It is primarily abused as a remedy for stimulants, withdrawal symptoms, gaining a state of oblivion, and enhancing the effect of low quality of other substances of abused [5]. Despite its global misuse, there is a dearth of data on its effect on testicular functions.

Oxidative stress is a consequence of a rise in reactive oxygen species (ROS) generation beyond the intracellular ROS scavenging capacity [6]. Also, oxidative stress is a cause and a consequence of inflammation [7]. ROS-driven oxidative stress-induced damage to the testis and spermatozoa triggers inflammatory response and the release of cytokines, which initially attempt to curb the effects of redox imbalance through increased tissue perfusion and enhanced capillary permeability [8, 9]. A chronic inflammatory state ensues if the assault persists, resulting in overwhelming release of inflammatory cytokines such as IL-1β and TNF-α, [8–10] by the testicular macrophages and possibly the Leydig and Sertoli cells and upregulation of myeloperoxidase activity with neutrophil infiltration [7, 11, 12]. Inflammatory injury to the male genitalia further exacerbates ROS production with resultant overwhelming oxidative stress [13, 14].

Oxidative stress has been reported to upregulate caspases signaling [15], cumulating in the activation of caspase-3, with resultant apoptosis. Studies have suggested the global shutdown of mitochondrial function in oxidative stress, which may lead to apoptosis as a result of gradual decrease in cellular energy supply [15]. In addition, dysfunction of the plasma membrane proton pumps leads to mitochondria dysfunction and excessive ROS generation [16], resulting in apoptosis.

Recently, we reported that Rohypnol induces sexual dysfunction in a rat model via suppression of the hypothalamic-pituitary-testicular axis and elevation of circulatory prolactin [17]; however, its effect on testicular function is yet to be fully explored. Till date, no study has shown whether or not Rohypnol-induced sexual dysfunction is associated with impaired spermatogenesis and sperm quality. Also, it is still unclear whether Rohypnol-induced sexual dysfunction is associated with testicular toxicity. Hence, the present study was designed to investigate the effect of Rohypnol on testicular functions, namely, testosterone biosynthesis and spermatogenesis, including sperm quality. The present study also explored the role of caspase-3 signaling and Na^+-K^+-ATPase and Ca^{2+}-ATPase in Rohypnol-induced male reproductive dysfunction.

2. Materials and Methods

2.1. Animals and Treatment. Thirty six 10-week old littermate male Wistar rats of similar weights (180-200 g) that were bred in the Animal Holdings of the Department of Physiology, Ladoke Akintola University of Technology were kept in standard well-ventilated cages in under natural humidity, temperature, and photoperiod of 12 h light/dark cycle and allowed free access to rat feed (Top Feed, Nigeria) and water. They were acclimatized for two weeks and then randomly distributed into six groups ($n = 6$). The control group received 1 ml of distilled water as vehicle, while the low- and high-dose Rohypnol-treated groups received 2 mg/kg and 4 mg/kg of Rohypnol, respectively, for four weeks. The low- and high-dose recovery groups received 2 mg/kg and 4 mg/kg of Rohypnol, respectively, which was immediately followed by a 4-week drug-free recovery period. Drug administration was once daily, between 8 am and 9 am, by gavage. The doses of Rohypnol used in the study are as previously reported [17]. The doses and administration route used in this study were similar to the human dose and exposure route. The animals were weighed daily, and the doses of drugs administered were per the current weight. This study was carried out in line with the recommendations of the National Institute of Health using the guide for the Care and Handling of Laboratory Animals (NIH Publication No. 80-23; revised 1978) and International Guiding Principles for Biomedical Research folling the CIOMS ethical code for animal experimentation [18]. The study experimental protocol was approved by the Ethics Committee of the Oyo State Ministry of Health, Ibadan, Nigeria (reference number AD/13/479/17). Rohypnol was a donation of the National Drug Law Enforcement Agency (NDLEA), Nigeria.

2.2. Sample Collection. After the experimental period, the animals were fasted overnight and culled under intraperitoneal ketamine (40 mg/kg)/xylazine (4 mg/kg) anesthesia as previously documented [19]. Blood sample was obtained through cardiac puncture. The testes, epididymis, prostate, and seminal vesicles were rapidly excised and weighed after the adhering tissues were removed. The relative organ weight was evaluated as organ weight/final bodyweight × 100%. The initial bodyweight of each rat was deducted from the final bodyweight to obtain the bodyweight gain. A portion of the left testis (1 g) was each homogenized in an appropriate volume of cold phosphate buffer solution (dilution factor: 1 in 5) using a glass homogenizer, and the homogenates were centrifuged at 10,000 g for 15 min at 4°C to obtain the supernatant for biochemical assay. The right testes were fixed in Bouin's solution for histological processing.

2.3. Evaluation of Epididymal Sperm Parameters. The caudal epididymis, separated from the left testis immediately after culling, were minced in Biggers-Whitten-Whittingham capacitation medium and epididymal sperm suspension was evaluated for count, motility, and morphology as previously reported [20].

2.4. Assessment of Testicular Integrity. Testicular integrity was evaluated as a function of the activities of testicular injury markers [21]. As previously reported, activities of testicular alkaline phosphatase (ALP) (Cat number:5122114, Randox; County Antrim, BT29 4QY, UK), acid phosphatase (ACP) (Cat number: B-31070336; Agape, Knonauerstrasse 54-6330 Cham, Switzerland), gamma-glutamyl transferase (GGT) (Cat number: 31070095; Agape, Knonauerstrasse 54-6330 Cham, Switzerland), lactate dehydrogenase (LDH) (Cat number: 51407002; Agappe Diagnostics Ltd., Knonauerstrasse 54-6330 Cham, Switzerland), and sorbitol dehydrogenase (SDH) (triethanolamine, Cat number: 20150207; Guangdong Guanghua Chemical Factory Co., Ltd., Guangzhou, China; Fructose, Cat number: 57487; Molychem, Mumbai, India; nicotinamide adenine dinucleotide, Cat number: 53849; Central Drug House (p) Ltd., Mumbai, India) were assayed by spectrophotometry per manufacturers' guidelines.

2.5. Determination of Serum Levels of FSH, LH, and Testosterone. Serum concentrations of testosterone (Cat number: B-I-122051006), follicle-stimulating hormone (FSH) (Cat number: B-I-122021801), and luteinizing hormone (LH) (Cat number: B-I-122051002) were determined using ELISA kits (Bio-Inteco, London, UK) per manufacturer's guide.

2.6. Evaluation of Markers of Testicular Oxidative Stress and Antioxidant Systems. The testicular concentrations of malondialdehyde (MDA, the product of lipid peroxidation; Cat number: SG55431503, Loba Chemie Pvt. Ltd., Mumbai, India) [22], xanthine oxidase (XO, marker enzyme of oxidation; sodium hydroxide, Cat number: 20150127, Guangdong Guanghua Chemical Factory Co., Ltd., Guangzhou, China; XO, Cat number: 20150127, Central Drug House (p) Ltd., Mumbai, India; HCl, Cat number: K51071417905, Merck, Darmstadt, Germany) [23], reduced glutathione (GSH; sulphursalicyclic acid, Cat number: 5965833, Loba Chemie Pvt. Ltd., Mumbai, India; 5, 5-dithiobis-(2 nitrobenzene), Cat number: 69793, Central Drug House (p) Ltd., Mumbai, India) [23], superoxide dismutase (SOD, an enzymatic antioxidant; adrenaline, Cat number: 51434, Central Drug House (p) Ltd., Mumbai, India; sodium carbonate, Cat number: 20150127, Guangdong Guanghua Sci-Tech Co., Ltd., Guangzhou, India) [22], catalase (potassium dichromate, Cat number: MKBG1432, Sigma Aldrich, Burlington, MA, USA; acetic acid, Cat number: 64197, Surechem Products Ltd., Ipswich, England) [24], glutathione peroxidase (GPx, an enzymatic antioxidant; Pyrogallol, Cat number: 87661, Loba Chemie Pvt. Ltd., Mumbai, India), and glutathione-S-transferase (GST, an enzymatic antioxidant; glutathione, Cat number: 70188, Loba Chemie Pvt. Ltd., Mumbai, India; 1-chlor-2,4-dinithiobenzene, Cat number: 97007, Loba Chemie Pvt. Ltd., Mumbai, India; potassium phosphate, Cat number: 7778770, Loba Chemie Pvt. Ltd., Mumbai, India) [24] were determined using established colorimetric methods.

2.7. Evaluation of Markers of Inflammation. Testicular nitric oxide (NO; naphthaethyline diamine dihydrochloride, Cat number: A190231605, Loba Chemie Pvt. Ltd., Mumbai, India; sulphanilamide, Cat number: 653741, Surechem Products Ltd., Ipswich, England; phosphoric acid, Cat number: SZBA2160, Sigma Aldrich, Burlington, MA, USA) content and myeloperoxidase (MPO; sulphuric acid, Cat number: 7664939, Central Drug House (p) Ltd., Mumbai, India; TNB, Cat number: 211205, BioInteco, London, UK) activity (a marker of neutrophils infiltration and inflammation) were determined as inflammatory markers using Griess reaction and hydrogen peroxide-dependent oxidation of guaiacol, respectively, by colorimetric methods [25].

2.8. Testicular Proton Pump. Activities of testicular transmembrane proteins (Na^+/K^+-ATPase and Ca^{2+}-ATPase) were determined by spectrophotometry as previously established [26, 27].

2.9. Testicular DNA Fragmentation and Caspase-3 Activity. Testicular DNA fragmentation index (DFI) and caspase-3 activity (Cat number: MB-5945A, Nanjing Mornmed Medical, Nanjing city, Jiangsu province, China) were assayed as markers of DNA integrity and apoptosis [7, 28]. Testicular DNA fragmentation index (DFI) was determined by a spectrophotometric assay using diphenylamine (DPA) methods as previously reported [7]. Activity of testicular caspase-3 was determined using ELISA kits (Elabscience Biotechnology Co., Ltd, Texas, USA) following the manufacturer's guide.

2.10. Histopathological Analysis. The right testes of the rats were fixed in Bouin's solution, dehydrated with ascending ethanol series, and then cleared with toluene. The testes were embedded at room temperature and blocked in paraffin wax incubated in a 60°C incubator overnight. Then, hematoxylin and eosin (H&E) stain was applied to about 5 μm thick paraffin sections of the testes. The sections were examined with light microscopy, and photomicrographs were taken at 100x magnification.

At least 20 seminiferous tubules were evaluated under a light microscope at 200x magnification for the histological scoring. The first seminiferous tubule was selected randomly, while others were observed by moving the section clock wisely. Testicular histoarchitecture was scored as previously documented using Cosentino's scoring system as follows [20, 29]:

(i) Grade 1: normal testicular tissue with an orderly arrangement of germ cells

(ii) Grade 2: loss of cohesion in germ cells, closely packed seminiferous tubules

(iii) Grade 3: disordered and sloughed germ cells with shrunken and pyknotic nuclei impaired borders of the seminiferous tubules

(iv) Grade 4: irregular and damaged seminiferous tubules filled by coagulative necrosis in the germ cells

The mean testicular biopsy score (MTBS), using the Johnsen's scoring system, was employed as an index of spermatogenesis [21, 30]. Seminiferous tubules were evaluated at 400x microscopic field and scored as follows:

(i) 10: complete spermatogenesis with many spermatozoa

(ii) 9: many spermatozoa present but disorganized germinal epithelium

(iii) 8: only a few spermatozoa (<5–10) present

(iv) 7: no spermatozoa but many spermatids present

(v) 6: no spermatozoa and only a few spermatids (<5–10) present

(vi) 5: no spermatozoa or spermatids but several or many spermatocytes present

(vii) 4: only a few spermatocytes (<5) and no spermatids or spermatozoa present

(viii) 3: spermatogonia are the only germ cells present

(ix) 2: no germ cells but Sertoli cells are present

(x) 1: no cells (either germ cell or Sertoli cell) in tubular section

To determine the mean seminiferous tubular diameter (MSTD), mean seminiferous luminal diameter (MSLD), and epithelial height (EH), twenty roundest seminiferous tubules per section were captured using a computerized digital camera attached to a microscope (Omax Digital Microscope, China). Measurements were taken using ImageJ Analyzer software (ImageJ 1.50i, National Institute of Health, USA). Each measurement was taken by two experts who were blinded to the study protocol. The mean value obtained for each testis was used as the measurement for that testis [31, 32].

Spermatogenic index, a pointer of spermatogenesis, was determined using the four-point system [21, 32]. The number of the cell layers, cell types, and the presence of late spermatids in the seminiferous tubules were scored as follows:

(1) Only spermatogonia present

(2) Spermatogonia and spermatocytes present

(3) Spermatogonia, spermatocytes and round (early) spermatids present with <5 late spermatids per tubule

(4) Spermatogonia, spermatocytes, and round spermatids present with up to 25 late spermatids per tubule

Two hundred seminiferous tubules were examined per testis, and the spermatogenic index was determined as total score of the 200 seminiferous tubules examined divided by the number of seminiferous tubules examined (200).

2.11. Statistical Analysis. One-way analysis of variance (ANOVA) was used to compare the mean values of parameters across the groups, followed by Tukey's post hoc test for multiple pair-wise comparisons. GraphPad Prism for Windows (versions 5.01, GraphPad Software, Inc.) was used for statistical analysis. Data are presented as mean ± SD of six replicates. Significance was considered at the level of $P < 0.05$.

3. Results

3.1. Body and Organ Weight Change. The noted changes in the body weights and reproductive organ weights of the experimental animals are presented in Table 1. Rats in the low- and high-dose Rohypnol-treated groups had a significant reduction in the mean body weight gain when compared with the control, although the body weight gains observed in the low- and high-dose Rohypnol-treated groups were comparable. The observed weight gain pattern did not improve following Rohypnol withdrawal. In addition, although the relative weights of the testes and seminal vesicles of the Rohypnol-treated animals were comparable to the control rats, Rohypnol led to significant reductions in the absolute weights of the testes and seminal vesicles. Interestingly, these changes in the testes and seminal vesicles were significantly reversed by Rohypnol withdrawal. Furthermore, Rohypnol led to significant decrease in the absolute and relative weights of the epididymides and prostate. Even though the observed weight change of the prostate was completely reversed to normal following Rohypnol withdrawal, the noted weight change of the epididymides was only reversed in the low-dose treatment group but not in the high-dose treatment group.

3.2. Circulatory FSH, LH, and Testostrerone. Rohypnol treatments significantly reduced serum concentrations of testosterone, LH, and FSH when compared to the control group, although the circulatory levels of these hormones were significantly higher in the high-dose Rohypnol-treated rats compared to the low-dose Rohypnol-treated rats. When compared with the control following Rohypnol withdrawal, Rohypnol-induced alteration in the level of FSH was reversed, but the alterations in serum LH and testosterone were not reversed (Figure 1).

3.3. Epididymal Sperm Parameters. The effects of Rohypnol administration on sperm parameters are shown in Tables 2 and 3. Although, Rohypnol administration did not cause a significant change in sperm count, it caused a significant and an irreversible reduction in sperm motility and viability. In addition, Rohypnol treatments led to a significant and an irreversible increase in the percentage of abnormal sperm morphology, respectively. Furthermore, Rohypnol treatments caused different anomalies of the sperm form, which were not completely reversibly following Rohypnol withdrawal.

3.4. Markers of Testicular Integrity. Testicular ALP, ACP, and GGT were significantly increased with Rohypnol treatments compared to the control, although high-dose Rohypnol treatment caused a significantly higher rise in these testicular marker enzymes when compared with the low-dose treatment. The effect of low-dose treatment, but not high-dose treatment of Rohypnol, on testicular GGT was reversible following Rohypnol withdrawal compared to the control. Interestingly, the effects of Rohypnol on testicular ALP and ACP were irreversible even with Rohypnol withdrawal. In addition, Rohypnol treatments caused a significant rise in testicular LDH and decline in testicular SDH compared with the control. Also, when compared with the age-matched control, the effect of Rohypnol on testicular LDH and SDH was only reversible in the low-dose Rohypnol-treated rats (Figure 2).

3.5. Markers of Oxidative Stress, Enzymatic Antioxidants, and Inflammation. Administration of Rohypnol, at low and high doses, caused a significant rise in testicular MDA compared to the control. Also, Rohypnol treatments significantly decreased the testicular concentration of GSH and the activities of GPx and GST. Rohypnol-induced alterations in testicular MDA and glutathione system were significantly reversed to near normal in the low-dose treatment group, but not in the high-dose treatment group, when compared to the control. The same pattern of effects was observed in respect to the activities of testicular SOD and catalase; however, Rohypnol-induced suppression of testicular SOD and catalase activity was not reversible (Table 4).

Table 1: Effect of Rohypnol on the weights of reproductive organs.

Parameters	Treatment period			Recovery period		
	Control	Low-dose Rohypnol	High-dose Rohypnol	Control recovery	Low-dose Rohypnol recovery	High-dose Rohypnol recovery
Body weight gain (g)	14.07 ± 0.90	9.80 ± 0.72*	7.73 ± 0.64*	30.27 ± 1.10	20.10 ± 1.15*	18.95 ± 0.07*
Paired testis						
Ab. weight (g)	2.51 ± 0.07	2.29 ± 0.072*	2.15 ± 0.15*	2.52 ± 0.21	2.33 ± 0.25	2.31 ± 0.31
R. weight (g)	1.23 ± 0.09	1.19 ± 0.22	1.08 ± 0.10	1.22 ± 0.07	1.13 ± 0.12	1.02 ± 0.09
Epididymis						
Ab. weight (g)	1.59 ± 0.07	1.11 ± 0.12*	1.07 ± 0.22*	1.61 ± 0.08	1.30 ± 0.04	1.19 ± 0.03*
R. weight (g)	0.78 ± 0.04	0.56 ± 0.11*	0.55 ± 0.07*	0.77 ± 0.05	0.57 ± 0.03	0.53 ± 0.08*
Prostate gland						
Ab. weight (g)	0.39 ± 0.04	0.23 ± 0.07*	0.23 ± 0.02*	0.37 ± 0.04	0.27 ± 0.03	0.26 ± 0.02
R. weight (g)	0.19 ± 0.02	0.12 ± 0.01*	0.12 ± 0.04*	0.20 ± 0.03	0.12 ± 0.01	0.12 ± 0.02
Seminal vesicle						
Ab. weight (g)	0.90 ± 0.23	0.76 ± 0.18*	0.71 ± 0.15*	0.91 ± 0.22	0.64 ± 0.14	0.60 ± 0.17
R. weight (g)	0.45 ± 0.13	0.37 ± 0.08	0.37 ± 0.10	0.46 ± 0.11	0.28 ± 0.06	0.27 ± 0.08

Ab.: absolute; R.: relative. Data are presented as mean ± SD of 6 replicates; *$P < 0.05$ vs. age-matched control.

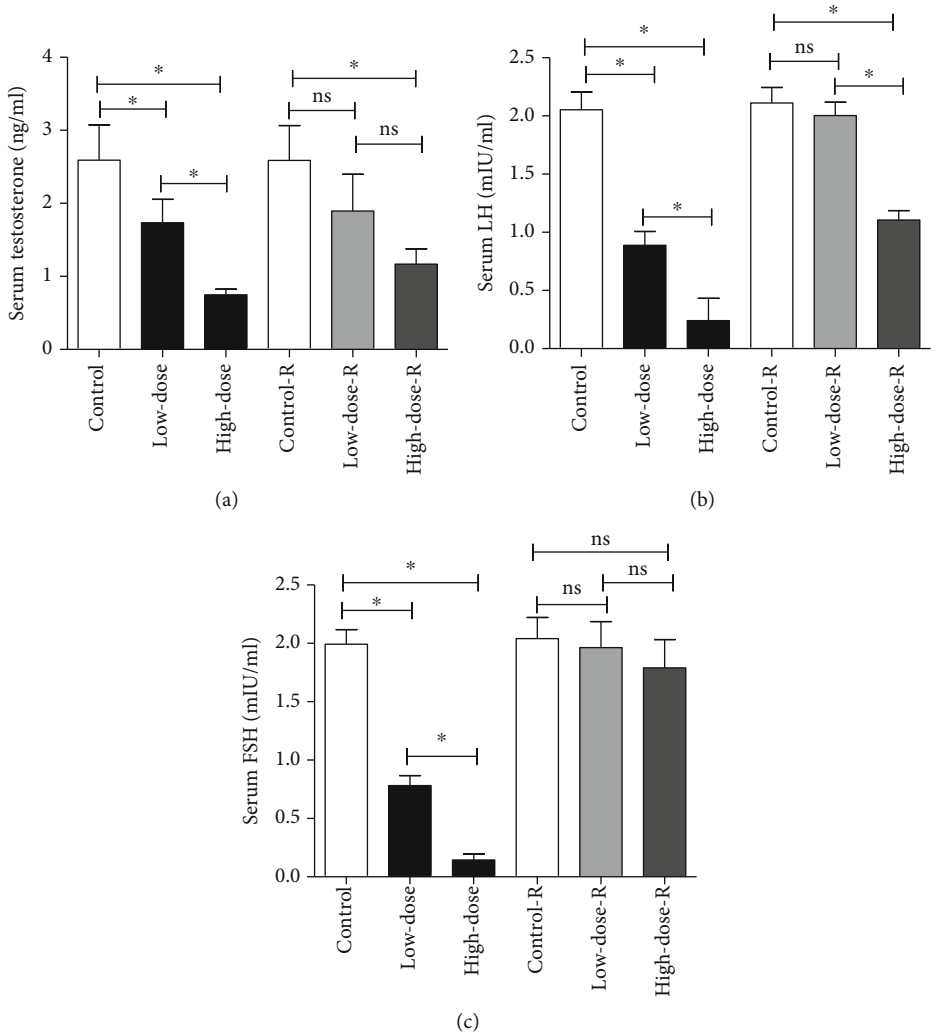

Figure 1: Effect of Rohypnol on circulatory testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH). R: recovery; ns: not significant. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$.

Table 2: Effect of Rohypnol on epididymal sperm parameters.

Parameters	Treatment period			Recovery period		
	Control	Low-dose Rohypnol	High-dose Rohypnol	Control recovery	Low-dose Rohypnol recovery	High-dose Rohypnol recovery
Sperm count (μm)	12.93 ± 1.47	10.23 ± 0.67	10.12 ± 1.38	12.13 ± 1.31	10.30 ± 0.96	9.30 ± 0.27
Sperm motility (%)	81.00 ± 6.54	68.17 ± 3.76*	59.67 ± 3.26*	80.33 ± 5.95	70.83 ± 2.63*	69.67 ± 6.21*
Sperm viability (%)	87.50 ± 4.32	77.17 ± 4.83*	69.00 ± 6.06*#	86.17 ± 4.16	78.67 ± 3.07*	75.67 ± 3.93*
Abnormal sperm morphology (%)	8.00 ± 2.82	16.17 ± 2.71*	19.17 ± 2.29*	8.33 ± 2.50	14.50 ± 2.73*	17.50 ± 2.07*

Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$ vs. age-matched control; $^{\#}P < 0.05$ vs. age-matched low-dose Rohypnol.

Data on inflammatory markers as depicted in Figure 3 indicate that Rohypnol administration significantly increased testicular level of NO and MPO activities when compared to the control. Rohypnol-induced changes in testicular NO content and MPO activity were only reversible in the low-dose treatment group but not in the high-dose treatment group when compared with the age-matched control following Rohypnol withdrawal.

3.6. Testicular Proton Pumps. Rohypnol exposure as revealed in Figure 4 significantly depressed Na^+/K^+ and Ca^{2+} pumps. Although the effects of low-dose and high-dose Rohypnol on Na^+/K^+ pump were similar after 6 weeks; when compared with the age-matched control after a 6-week recovery period, Rohypnol-induced decline in the Na^+/K^+ pump activity was reversible in the low-dose Rohypnol-treated group but not in the high-dose Rohypnol-treated group. On the other hand,

Table 3: Effect of Rohypnol on various epididymal sperm form anomalies.

Parameters	Treatment period			Recovery period		
	Control	Low-dose Rohypnol	High-dose Rohypnol	Control recovery	Low-dose Rohypnol recovery	High-dose Rohypnol recovery
Head anomaly						
Headless tail	5.75 ± 0.50	5.50 ± 0.57	6.25 ± 0.50	5.25 ± 0.50	6.50 ± 0.50*	6.40 ± 0.54*
Midpiece anomaly						
Curved midpiece	9.80 ± 0.44	9.20 ± 0.45	9.00 ± 0.70	9.20 ± 0.83	9.00 ± 0.81	9.60 ± 0.54
Bent midpiece	8.60 ± 0.54	10.20 ± 1.09*	9.40 ± 0.54	8.75 ± 0.50	10.25 ± 0.95	8.75 ± 0.50
Tail anomaly						
Tailless head	4.60 ± 0.54	4.75 ± 0.50	4.60 ± 0.54	3.80 ± 0.83	4.60 ± 0.54	2.50 ± 0.57*#
Rudimentary tail	3.60 ± 0.54	5.50 ± 0.58*	6.75 ± 0.50*	3.50 ± 0.57	3.50 ± 0.57	7.33 ± 0.58*#
Bent tail	5.80 ± 0.44	6.80 ± 0.45*	7.20 ± 0.41*	5.40 ± 0.54	6.66 ± 0.57*	5.55 ± 0.57
Curved tail	8.60 ± 0.54	8.75 ± 0.50	8.75 ± 0.50	8.80 ± 0.83	8.25 ± 0.50	8.75 ± 0.50
Looped tail	1.20 ± 0.45	2.80 ± 0.83*	1.40 ± 0.54#	1.40 ± 0.55	2.25 ± 0.50	1.40 ± 0.54

Data are presented as mean ± SD of 6 replicates; *$P < 0.05$ vs. age-matched control; #$P < 0.05$ vs. age-matched low-dose Rohypnol.

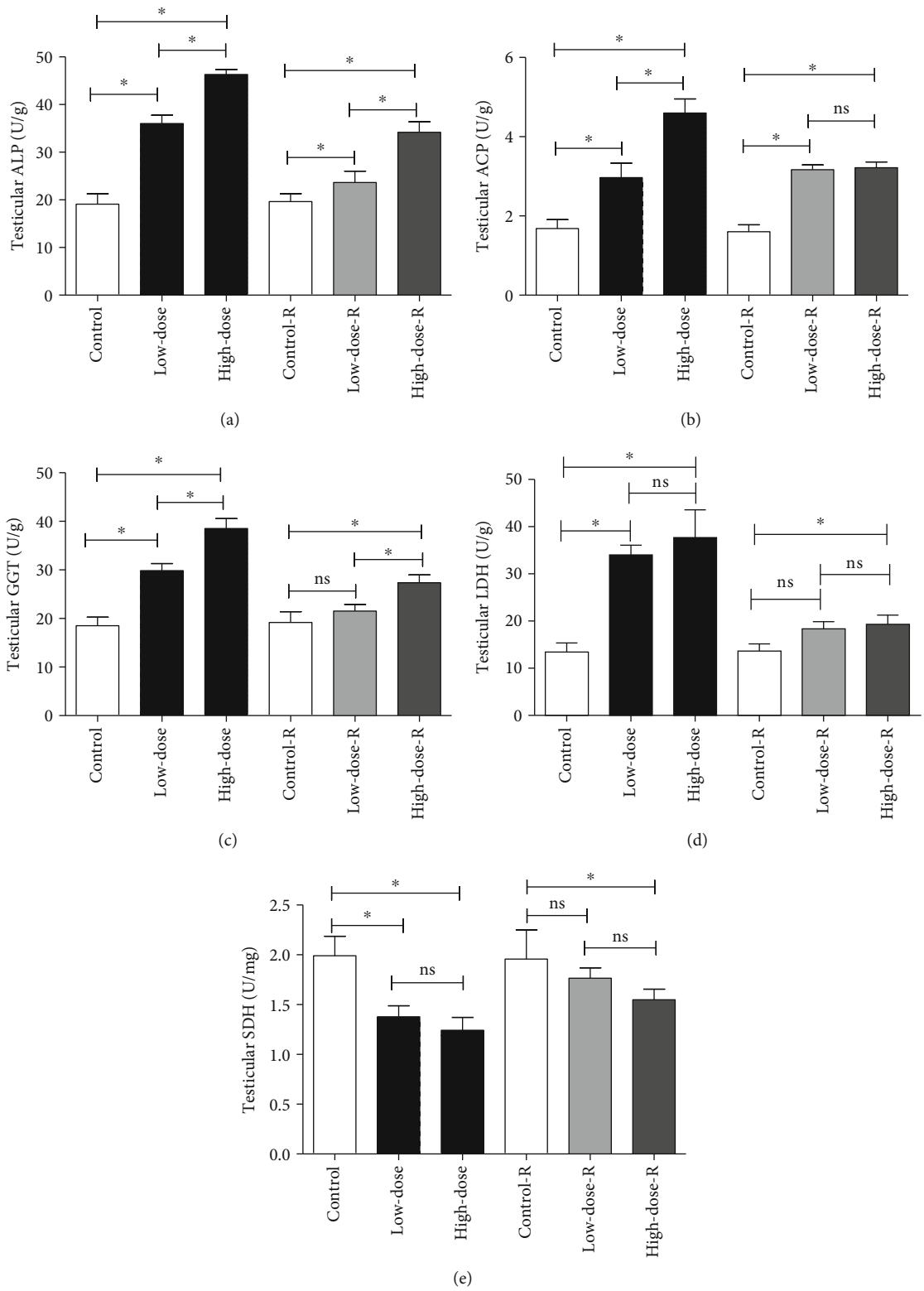

Figure 2: Effect of Rohypnol on markers of testicular integrity. R: recovery; ALP: alkaline phosphatase; ACP: acidic phosphatase; GGT: gamma glutamyl transferase; LDH: lactate dehydrogenase; SDH: sorbitol dehydrogenase; ns: not significant. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$.

Rohypnol-induced decline in the Ca^{2+} pump activity was not reversible in the low- and high-dose Rohypnol-treated groups.

3.7. Testicular DNA Fragmentation and Apoptosis. Rohypnol treatments, at low and high doses, led to significant increase in testicular DNA fragmentation as depicted by DNA

Table 4: Effect of Rohypnol on markers of oxidative stress in the testis.

Parameters	Treatment period			Recovery period		
	Control	Low-dose Rohypnol	High-dose Rohypnol	Control recovery	Low-dose Rohypnol recovery	High-dose Rohypnol recovery
MDA (Umol/g)	1.40 ± 0.18	6.03 ± 0.71*	6.58 ± 0.55*	1.43 ± 0.12	2.10 ± 0.60	3.36 ± 0.68*#
GSH (uM)	3.94 ± 0.43	2.58 ± 0.27*	1.36 ± 0.32*#	3.96 ± 0.42	3.54 ± 0.37	2.74 ± 0.23*#
SOD (U/mg)	164.4 ± 13.16	95.80 ± 9.70*	72.60 ± 14.38*#	164.6 ± 11.87	83.00 ± 11.07*	69.60 ± 5.36*
Catalase (U/mg)	6.92 ± 0.50	4.30 ± 0.57*	2.24 ± 0.43*#	6.84 ± 0.37	3.62 ± 0.67*	2.87 ± 0.48*
GPx (U/mg)	5.44 ± 0.48	4.14 ± 0.25*	1.94 ± 0.16*#	5.48 ± 0.49	4.52 ± 0.31	3.02 ± 0.25*#
GST (U/mg)	8.80 ± 1.16	6.30 ± 0.60*	5.10 ± 0.77*	9.08 ± 1.09	8.26 ± 0.78	6.52 ± 0.46*#

MDA: malondialdehyde; SOD: superoxide dismutase; GSH: reduced glutathione; GPx: glutathione peroxidase; GST: glutathione-S-transferase. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$ vs. age-matched control; $^{\#}P < 0.05$ vs. age-matched low-dose Rohypnol.

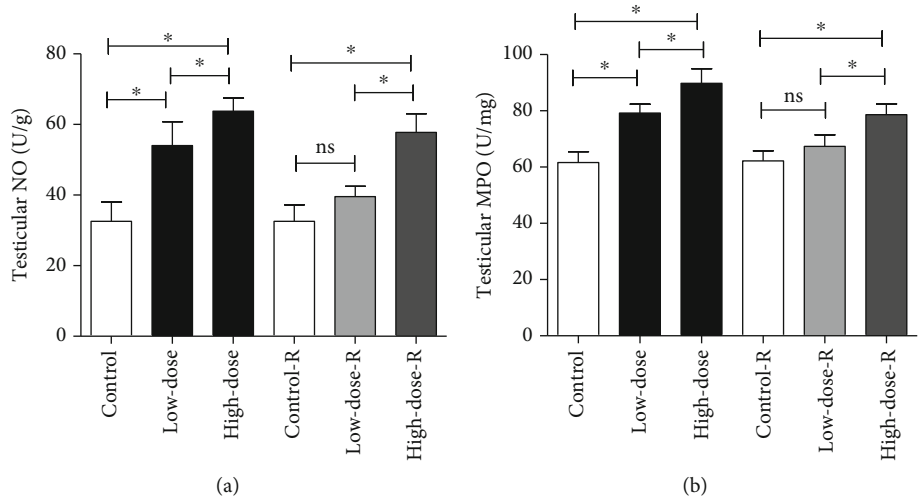

Figure 3: Effect of Rohypnol on testicular nitric oxide (NO) and myeloperoxidase (MPO) activity. R: recovery; ns: not significant. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$.

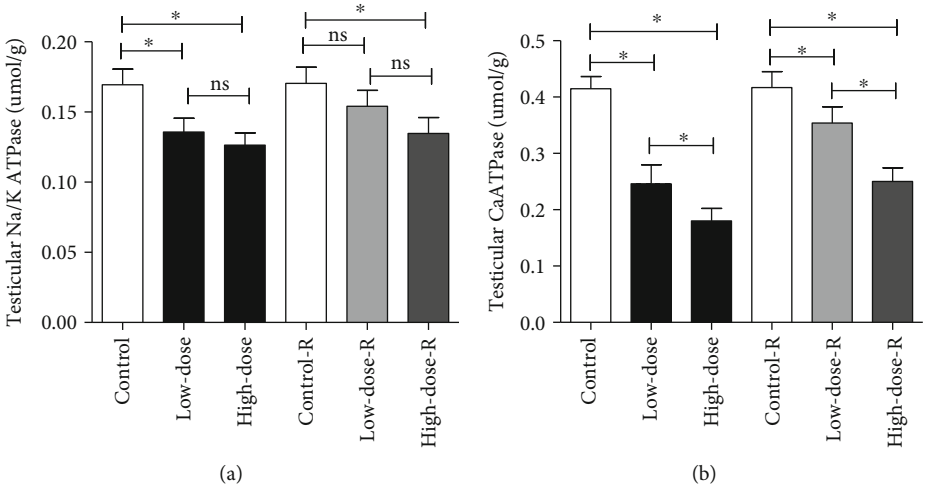

Figure 4: Effect of Rohypnol on testicular Na/K-ATPase and Ca-ATPase activity. R: recovery; ns: not significant. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$.

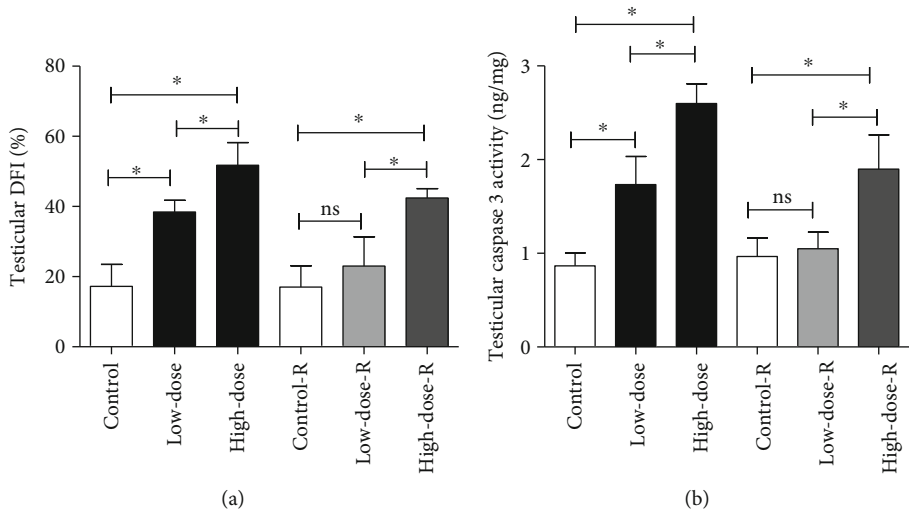

Figure 5: Effect of Rohypnol on testicular caspase-3 and DNA fragmentation index (DFI). R: recovery; ns: not significant. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$.

fragmentation index, and testicular caspase-3 activity when compared with the control. The effect of low-dose Rohypnol, but not high-dose Rohypnol, on testicular DNA fragmentation and caspase-3 activity was reversible (Figure 5).

3.8. Histopathological Findings. The representative photomicrographs of the testicular tissue samples as observed using light microscopy are presented in Figure 6. The testicular tissues obtained from the control rats showed no obvious pathological changes. On the other hand, testicular tissues from animals treated with low-dose Rohypnol had abnormal histoarchitecture such as atrophy, distension of the interstitium, reduced spermatozoa in the lumen of the seminiferous tubules, and reduced epithelial height, while testicular tissues from animals treated with high-dose Rohypnol had abnormal morphology such as loosely packed seminiferous tubules and damaged seminiferous tubules in addition to those observed in the low-dose Rohypnol-treated animals. Rohypnol withdrawal during the recovery period significantly improved testicular cytoarchitecture.

The effects of Rohypnol on spermatogenesis, as suggested by histopathological spermatogenic indices, are shown in Table 5. Administration of Rohypnol significantly reduced the seminiferous tubular diameter and epithelial height but increased the seminiferous luminal diameter. The observed changes in the seminiferous tubular diameter, seminiferous luminal diameter, and epithelial height were significantly more pronounced in the high-dose Rohypnol-treated rats compared to the low-dose Rohypnol-treated rats and were not reversible when compared with the control. Johnsen's biopsy score, an index of spermatogenesis, was significantly reduced following 6 weeks of high-dose Rohypnol treatment when compared with the control and low-dose Rohypnol treatment. The high-dose Rohypnol-induced alteration in Johnsen's biopsy score was reversed by Rohypnol withdrawal. Also, high-dose Rohypnol treatment significantly increased Cosentino's score (a marker of testicular damage) and reduced spermatogenic index (a marker of spermatogenesis) when compared to the control and low-dose Rohypnol treatment. The observed changes in the Cosentino's score and spermatogenic index were also not reversible following Rohypnol withdrawal.

4. Discussion

Rohypnol is one of the common drugs of abuse, and it has been shown to induce hyperprolactinemia and results in male reproductive dysfunction [17]. A previous study from our laboratory showed that Rohypnol lead to sexual dysfunction through the suppression of hypothalamic-pituitary-testicular axis, resulting in low circulatory testosterone [17]. The testis is made up of highly active cells that require energy, which is generated as ATP by the mitochondria through oxidative phosphorylation, to drive their functions. During oxidative phosphorylation, incomplete reduction of oxygen by an electron generates ROS [33, 34], which leaks out of the mitochondria and act as signaling molecules and secondary messengers that regulate intracellular pathways and various physiological processes such as capacitation, hyperactivation, acrosome reaction, and spermoocyte fusion [35]. Thus, mitochondrial dysfunction leads to excess ROS generation that exceeds the buffering capacity of the cellular antioxidants, resulting in oxidative stress [6]. Interestingly, the testes and spermatozoa are rich in polyunsaturated fatty acids, which make them highly prone to ROS attack. Although Rohypnol induces sexual dysfunction, till date, no study has documented its impact on testicular functions.

In the present study, Rohypnol induced alteration in testicular morphology and histoarchitecture with significant reduction in testicular weight compared to the control. This was accompanied by a significant reduction in the body weight gain and weights of the epididymides, prostate, and seminal vesicles. In addition, Rohypnol exposure led to atrophy of the seminiferous tubules, loosely packed seminiferous tubules, distension of the testicular interstitium, decrease in the number of spermatozoa in the lumen of the seminiferous tubules

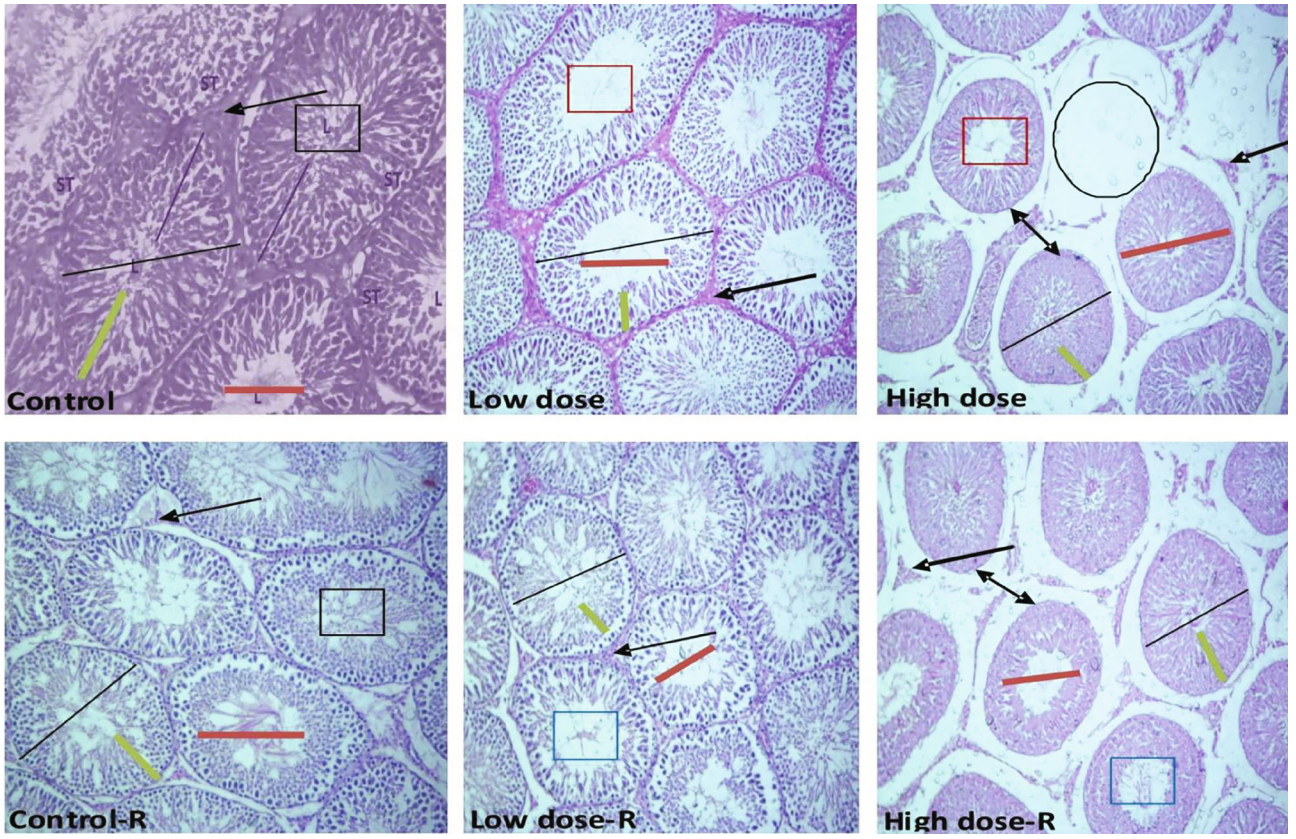

Figure 6: Photomicrograph of the testicular tissues of rats exposed to low and high doses of Rohypnol compared with the control (H&E stains). The control and control recovery animals shows preserved seminiferous tubules with normal Leydig cells (arrow). There is varying degree of maturation of germ cells across the germ cell layer (green line) and normal mature sperm cells within the seminiferous lumen (black box). The low-dose Rohypnol-treated animals show reduced seminiferous tubular diameter (black line), widened seminiferous lumen (red line), and reduced epithelial height (green line). There are varying degrees of maturation of germ cells across the germ cell layer (green line) and scanty mature sperm cells within the seminiferous lumen (red box). The testis of the low-dose recovery group appears similar to that of the low-dose Rohypnol-treated, but with improved number of mature sperm cells within the seminiferous lumen (blue box). Also, the testis of the high-dose Rohypnol-treated group appears similar to those of the low-dose Rohypnol-treated animals; however, they also show loosely packed seminiferous tubules (double arrow), damaged seminiferous tubules (circle), and scanty mature sperm cells within the seminiferous lumen (red box). The high-dose recovery group shows a similar testicular cytoarchitecture with that of the high-dose Rohypnol-treated group, but with improved number of mature sperm cells within the seminiferous lumen (blue box). R: recovery.

Table 5: Effect of Rohypnol on testicular cytoarchitecture and indices of spermatogenesis.

Parameters	Treatment period			Recovery period		
	Control	Low-dose Rohypnol	High-dose Rohypnol	Control recovery	Low-dose Rohypnol recovery	High-dose Rohypnol recovery
MSTD (μm)	206.0 ± 3.60	184.30 ± 2.08*	166.30 ± 1.52$^{*}_{\#}$	202.00 ± 3.60	186.00 ± 5.56*	179.30 ± 1.52*
MSLD (μm)	87.33 ± 6.42	127.30 ± 6.65*	125.00 ± 6.24*	89.00 ± 6.24	114.30 ± 4.04*	112.70 ± 4.51*
Epithelial height (μm)	121.70 ± 3.51	60.00 ± 2.00*	36.67 ± 1.52$^{*\#}$	114.00 ± 6.55	71.67 ± 3.05*	66.33 ± 2.51*
Histoarchitecture/Cosentino's score (arbitrary unit)	1.00 ± 0.00	1.66 ± 0.51	3.33 ± 1.21$^{*\#}$	1.00 ± 0.00	1.33 ± 0.51	3.33 ± 0.52$^{*\#}$
Biopsy/Johnson's score (arbitrary unit)	9.67 ± 0.51	9.00 ± 0.89	2.83 ± 1.47$^{*\#}$	9.50 ± 0.83	9.50 ± 0.84	8.50 ± 1.04
Spermatogenic index (arbitrary unit)	3.83 ± 0.41	3.66 ± 0.52	2.16 ± 0.75$^{*\#}$	3.83 ± 0.40	3.83 ± 0.41	2.66 ± 0.51$^{*\#}$

MSTD: mean seminiferous tubular diameter; MSLD: mean seminiferous luminal diameter. Data are presented as mean ± SD of 6 replicates; $^{*}P < 0.05$ vs. age-matched control; $^{\#}P < 0.05$ vs. age-matched low-dose Rohypnol.

as well as widening of the lumen, and reduced epithelial height. Our findings corroborate earlier reports that drugs of abuse damage the testis, resulting in distorted testicular histoarchitecture [7, 10, 36, 37]. Also, testicular damage has been associated with increased diameter of the seminiferous tubule lumen and deterioration of sperm quality and quantity [38]. The increase in the diameter of the seminiferous tubular lumen despite the observed atrophy of the tubule is possibly due to the impaired spermatogenesis and reduced epithelial height following Rohypnol exposure [39]. The observed Rohypnol-induced alterations in testicular histomorphology were only partially ameliorated upon cessation of Rohypnol in animals exposed to the low dose but not in those exposed to the high dose. This conforms to earlier reports on codeine and tramadol, similar drugs of abuse [10, 37].

Our data also showed reduction in sperm motility and viability and increased abnormal sperm morphology in rats treated with Rohypnol compared to the control. Although the sperm count was not altered, this finding clearly shows the toxic effect of Rohypnol on sperm cells. The unaffected sperm count is likely due to the exposure period. It has been established that spermatogenesis takes 50 to 57 days in rats [10, 40]; however, the present exposure lasted 6 weeks (42 days) and did not target spermatogenesis per se but the toxic effect of Rohypnol. This could explain why the effect of Rohypnol on spermatogenesis may not reflect on the sperm count after 42 days. Though there are no data available in the literature to compare this finding with, it aligns with previous reports on the impact of some drugs of abuse such as codeine [28], cannabis [41], and tramadol [42] on sperm quality. Furthermore, the present data show that the impact of Rohypnol on sperm morphology is more on the tail (required for motility) and midpiece (where 80% of the mitochondria are located, which is also required for energy generation for motility) [43]. This may, at least in part, explain the observed Rohypnol-induced sperm dysmotility.

The Rohypnol-induced low sperm quality observed in the current study was associated with reduced circulatory LH, FSH, and testosterone. This is indicative of a likely suppression of the hypothalamic-pituitary-testicular axis and in consonance with our previous study [17]. The role of the mitochondria is not limited to energy production for spermatozoa; it is also involved in the maintenance of optimal steroidogenesis and male fertility [44–47]. Hence, Rohypnol-induced impairment of steroidogenesis may be a consequence of mitochondria dysfunction. The function of the testis includes spermatogenesis and steroidogenesis; therefore, Rohypnol-induced suppression of spermatogenesis and steroidogenesis could infer that Rohypnol induced testicular toxicity. Surprisingly, our data also revealed that the impact of Rohypnol on steroidogenesis, but not sperm quality, was only reversible in the low dose-treated animals after Rohypnol cessation.

Activities of testicular ALP, ACP, GGT, LDH, and SDH are markers of testicular integrity and indices of Sertoli cell function [7, 48]. The altered activities of these testicular injury markers are a reflection of the toxic effect of Rohypnol on the testes, which was only reversibly in the low-dose Rohypnol-treated animals. The rise in testicular ALP and ACP activities in Rohypnol-treated animals may be secondary to the release of these nonspecific phosphatases from the lysosomes of the degenerating cells and rapid catabolism of the testicular cells [7, 49], which may also expound the observed Rohypnol-induced testicular atrophy and reduced testicular weight [7, 50]. LDH catalyzes the interconversion of lactate and pyruvate [7] in favor of lactate generation in an anaerobic state [22, 51]. The rise in LDH and GGT in Rohypnol-treated rats may be compensatory in an attempt to meet the metabolic demand of the developing germ cells, which prefer lactate as the energy substrate [52]. Also, SDH converts sorbitol, an aldose reductase-driven glucose product, to fructose [21]. Rohypnol-led decline in testicular SDH activity may infer that Rohypnol suppressed testicular substrate (glucose) utilization and energy generation, which could contribute to testicular dysfunction.

Rohypnol-induced increase in testicular MDA and decline in testicular GSH and activities of SOD, catalase, GPx, and GST in this study are suggestive of Rohypnol-induced oxidative stress in the testes of the rats. These findings are in agreement with earlier reports on the induction of oxidative injury by drugs of abuse like codeine [7, 10] and tramadol [42]. It is plausible to infer that Rohypnol depletes the testicular antioxidant enzymes, resulting in a redox dyshomeostasis and oxidative stress. This likely explains the observed compromise in the integrity of the testicular structure and functions. Remarkably, Rohypnol-induced oxidative stress was accompanied by marked testicular inflammatory response, evident by a rise in testicular NO and MPO activites, suggesting the role of oxidative stress and inflammation in Rohypnol-induced testicular damage. The nexus between oxidative stress, inflammation, and male fertility has continued to gain more attention [8, 9, 53, 54]. It is possible that Rohypnol-induced ROS-driven oxidative stress to the testes triggers inflammatory response and the release of cytokines, which upregulates NO and MPO activities, resulting in increased tissue perfusion and enhanced capillary permeability [7–9] and consequent inflammatory injury to the testes with persistent exposure to Rohypnol, with partial recovery after Rohypnol cessation in the low-dose Rohypnol-treated animals. We also hypothesized that Rohypnol-induced decline in the activities of enzymatic antioxidants and increase in inflammatory markers may be due to downregulation of the nuclear factor erythroid 2-related factor 2 (Nrf2) and upregulation of the nuclear factor-kappa B (NF-κB) signaling, respectively [55].

Rohypnol-induced dysfunction of testicular Na^+/K^+-ATPase and Ca^{2+}-ATPase in the present study may be a consequence of oxidoinflammatory response [26]. Impairment of these proton pumps depresses the generation and maintenance of electrochemical gradient across the cell membrane of the testes and vital organelles such as the mitochondria, resulting in metabolic dysfunction and loss of cell volume [56]. Thrilling enough, oxidative stress, inflammation, and Na^+/K^+-ATPase and Ca^{2+}-ATPase dysfunction are triggers of caspase signaling [26], which culminate in the activation of caspase-3. Rohypnol-led caspase-3 activation possibly cleaves inhibitor of caspase-activated DNAse I (ICAD) to produce CAD [57, 58], which degrades

chromosomal DNA and results in DNA fragmentation and apoptosis [59]. Findings from this study demonstrated that the testicular damage caused by Rohypnol is irreversible in the high-dose treatment group, but partially reversible in the low-dose group.

5. Conclusion

We conclude that Rohypnol induces testicular dysfunction through the activation of oxidative-sensitive signaling pathways. This causes loss of structural and functional integrity of the testis. Rohypnol cessation, however, partially reversed these alterations when used in low dose but not in high dose. This report, to the best of our knowledge, is the first to document the effect of Rohypnol and its cessation, as a drug of abuse, on testicular integrity. It is recommended that other possible mechanisms that could mediate Rohypnol-induced reproductive toxicity be explored. The impact of Rohypnol, as a substance of abuse, on other organs should also be evaluated.

Data Availability

Data will be made available by the corresponding author upon reasonable request.

Conflicts of Interest

The authors do not have any conflict of interest with any individuals or organizations with respect to the data presented.

Authors' Contributions

Conceptualization and design were provided by Ajayi AF and Akhigbe RE Data curation was done by Ajayi AF, Oluwole DT, Akhigbe RE, Hamed MA, and Ajayi LO. Formal analysis w performed by Oluwole DT, Akhigbe RE, and Hamed MA. Investigation was conducted by Oluwole DT, Akhigbe RE, Hamed MA, and Ajayi LO. Methodology was provided by Ajayi AF, Akhigbe RE, and Hamed MA. Project administration was done by Ajayi AF, Oluwole DT, Akhigbe RE, Hamed MA, and Ajayi LO. Resources were acquired by Ajayi AF, Oluwole DT, Akhigbe RE, Hamed MA, and Ajayi LO. Software was acquired by Akhigbe RE and Hamed MA. Supervision was done by Ajayi AF. Validation was conducted by Ajayi AF and Akhigbe RE. Writing—original draft was done by Akhigbe RE. Writing—review and editing was done by Ajayi AF, Oluwole DT, Akhigbe RE, Hamed MA, and Ajayi LO.

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