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Testicular and Epididymal Histology of Rats Chronically Administered High Doses of Phosphodiesterase-5 Inhibitors and Tramadol

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Summary: Testicular oxidative stress, endocrine disruption and abnormal spermatogenesis in rats exposed to high doses of phosphodiesterase-5 inhibitors (PDE5i) and opioids, with poor reversibility following withdrawal of treatment had been reported. In this study, we examined the histopathological effects of high doses of sildenafil, tadalafil, tramadol and sildenafil+tramadol on the testes and epididymis of rats. Seventy male rats (180 – 200 g b.w) were assigned to one of five groups (n = 14), namely; A: control (0.2 mL normal saline), B: sildenafil (1 mg/100g b.w), C: tadalafil (1 mg/100g b.w), D: tramadol (2 mg/100g b.w) and E: sildenafil+tramadol group (dose as in groups B and D). The drugs were administered orally for 8 weeks. Seven rats were sacrificed per group while the remaining 7/group continued for 8 weeks without treatment. Histopathological examination was carried out at the end of both phases. After 8 weeks of treatment, mean Johnsen's testicular biopsy score (MJTBS) and Leydig cell count decreased significantly ($p<0.001$) in all treated groups compared with the control. The MJTBS and Leydig cell count decreased significantly in tramadol ($p<0.05$) and sildenafil+tramadol ($p<0.01$) groups compared with tadalafil group. After recovery, MJTBS and Leydig cell count were significantly ($p<0.05$) lower in all the groups compared with the control. Histology of the testes of rats in groups B – E showed reduced germ cell and spermatozoa population in the seminiferous tubules after 8 weeks treatment. Additionally, their epididymis showed decreased spermatozoa density. There was no complete reversibility of histopathological alterations following withdrawal of treatment. High doses of sildenafil, tadalafil, tramadol or sildenafil+tramadol impact negatively on testicular histology with poor reversal following withdrawal of treatment.

Keywords*:* Johnsen's score, Phosphodiesterase-5, Opioids, Sildenafil, Tadalafil, Tramadol

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INTRODUCTION

Phosphodiesterase-5 inhibitors (PDE5i) are sex stimulants that are used in the treatment of erectile dysfunction (Supuran *et al.*, 2006; Aversa *et al.*, 2006). They inhibit the degradation of cyclic guanosine monophosphate (cGMP) via inhibition of the action of the enzyme, PDE5 (Salem *et al.*, 2008). Some examples of PDE5 inhibitors are; sildenafil (common name: Viagra) and tadalafil (common name: Cialis). The opioid, tramadol, which is a centrally acting analgesic agent, is also another sex stimulant that has been recently employed in the treatment of premature ejaculation (PE) via its inhibition of the re-uptake of nor-epinephrine and serotonin, and its binding of parent and M1 metabolite to μ opioid receptors in animals (Bamigbade *et al*., 1997; Grond and Sablotzki, 2004; Salem *et al*., 2008).

These stimulants however, have been widely abused with some people taking far above recommended doses. This abuse is traceable to the people's desire to achieve a stronger and longer lasting erection, delay ejaculation and increase their genital size (Bechara *et al*., 2010; Nna *et al*., 2016a, b). Since sildenafil prolongs erection and tramadol delays ejaculation, a combination therapy (sildenafil+tramadol) has been adopted in Nigeria (Oka *et al*., 2015; Nna *et al*., 2016a). Studies have shown that the abuse of these medications have led to several negative effects on different organs including the liver, kidney, cardiac and smooth muscles, stomach, pituitary gland and testes (Eweka and Eweka, 2011; Tuorkey and Abdul-Aziz, 2012; Nna *et al*., 2015; Nna *et al*., 2016).

Previous studies reported that sildenafil improved the growth of spermatogenic cells (Shehab *et al*., 2014) and inhibited free radical generation at recommended doses (Akintunde *et al*., 2012). In a study by Yildiz *et al.* (2011), low dose sildenafil did not cause histological alterations in the seminiferous tubules of rats, whereas high dose resulted in degeneration, desquamation, disorganization and reduction in germ cell population along with

interstitial oedema, capillary congestion and hemorrhage. Tadalafil (1.8 mg and 2.6 mg/kg b.w.) caused thinning of the basement membrane, congestion of blood vessels, degeneration of germinal cells, irregular nuclear membrane of Sertoli, spermatogonia and Leydig cells, and loss of acrosomal cap by round spermatids (Hashish, 2015). Opioids on the other hand have been reported to increase sperm DNA damage via testicular oxidative stress (Pasqualotto *et al.*, 2000; Safarinejad *et al.*, 2013). El-Ghawet (2015) reported that tramadol (40 mg/kg b.w.) caused disorganization of the seminiferous tubules with decreased spermatogenic cells. In our previous study, we reported that high doses of separate and combined administration of PDE5i and opioid caused testicular oxidative stress-induced reproductive toxicity with poor reversal following their withdrawal (Nna and Osim, 2016).

Although several studies have documented the histopathological effects of PDE5i and opioid on male reproductive system, there is still a gap as to the effects of treatment and subsequent withdrawal of these medications on the testes and epididymis. The present study was therefore carried out to investigate the histopathological effects of high doses of sildenafil, tadalafil, tramadol and the combination therapy (sildenafil+tramadol) on the testes and epididymis of albino Wistar rats. Withdrawal effect of these drugs were also assessed.

MATERIALS AND METHODS

Drugs

Sildenafil citrate (Maxheal Laboratories Pvt Ltd, India), tadalafil (Pfizer, India) and tramadol hydrochloride (Glow Pharma Pvt Ltd, India) were purchased from Unipervit Pharmacy, Calabar, Nigeria.

Laboratory Animals

Seventy male Wistar rats (8 – 10 weeks old, weighing 180 – 200 g) were used for this study. The rats were purchased from the Department of Agriculture, Faculty of Science, and kept in the Department of Physiology, University of Calabar, Nigeria. They were housed in animal cages, with wood dust as bedding. They were allowed *ad libitum* access to water and feed, and exposed to 12/12 h light/dark cycle. The rats were allowed 7 days for habituation before drug administration commenced. The rats were housed following the principles for animal care as recommended in Helsinki's 1964 declaration. The study protocol was approved by the Animal Ethics Committee of the University of Calabar, Nigeria.

Experimental Design and Drug Administration

The male animals were divided into 5 groups (n = 14) thus; control (0.2 mL normal saline), sildenafil treated group (1 mg/100g b.w.), tadalafil treated group (1 mg/100g b.w.), tramadol (2 mg/100g b.w.) and sildenafil + tramadol treated group (dosed as in sildenafil and tramadol treated groups). The doses were selected from our previous studies (Nna *et al.*, 2015; Nna and Osim, 2016; Nna *et al.*, 2016a). The drugs were administered *per os*, thrice a week. After 8 weeks of treatment, 7 rats were sacrificed per group under chloroform anaesthesia and the right testis and epididymis collected for analysis. The remaining 7 rats/group continued for another 8 weeks recovery period before they were sacrificed and right testis and epididymis also collected for analysis.

Histopathological Studies

Histopathological assessment of the testis and epididymis was done using haematoxylin and eosin (H & E) technique. Briefly, the right testis and epididymis of each rat were carefully harvested and fixed in Bouin solution, dehydrated, and embedded in paraffin blocks. Tissue blocks were sectioned, stained using H & E, and viewed using a light microscope. Sections of the testicular tissue were examined for number of Leydig cells in 20 random intertubular regions (an area surrounded by three seminiferous tubules) using a light microscope at a magnification of x400 as used by Mohamed *et al.* (2011). Mean Johnsen's testicular biopsy score (MJTBS) was assessed in 10 seminiferous tubules using a previously described method (Johnsen, 1970) as used by Aksu *et al.* (2017). The grading for MJTBS is shown in table 1.

Table 1: Johnsen's mean testicular biopsy score (MJTBS) (Glander *et al.*, 2000)

Score	Description
1	Tubular sclerosis, no seminiferous epithelial cells.
2	Only Sertoli cell, no germ cells.
3	Only spermatogonia.
4	No spermatids, arrest of spermatogenesis at the primary spermatocyte stage.
5	Many spermatocytes, but no spermatids.
6	No late spermatids, arrest of spermatogenesis at the spermatid stage.
7	Many early spermatids, but no late spermatids.
8	Few late spermatids.
9	Disorganised tubular epithelium with many late spermatids.
10	Full spermatogenesis

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Data analysis was done using Graph Pad prism 7.0 (Graph Pad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used, followed by Tukey test (post-hoc test). Data from the treatment phase were compared with that of the recovery phase using student's t-test. Values of $p<0.05$ were considered to be statistically significant.

RESULTS

Mean Johnsen's testicular biopsy score (MJTBS) in the different experimental groups after (A) 8 weeks treatment period and (B) 8 weeks recovery period

Figure 1A shows MJTBS for the control (8.0 ± 0.6), sildenafil (4.3 ± 0.8), tadalafil (5.0 ± 0.6), tramadol (3.7 ± 0.8) and sildenafil + tramadol (3.2 ± 0.8) group after 8 weeks treatment period. MJTBS was significantly ($p<0.001$) reduced in all treated groups compared with the control. MJTBS was also significantly reduced in tramadol ($p<0.05$) and sildenafil + tramadol ($p<0.01$) groups compared with tadalafil group. After 8 weeks recovery period, MJTBS was significantly ($p<0.001$) reduced in sildenafil (5.8 ± 0.8), tramadol (4.8 ± 0.8) and sildenafil + tramadol (5.7 ± 0.8) recovery groups compared with the control (8.0 ± 0.6). MJTBS was also significantly ($p<0.001$) decreased in tramadol recovery group compared with tadalafil recovery group (6.8 ± 0.8) and not significantly different ($p>0.05$) between tadalafil recovery and control group (Figure 1B).

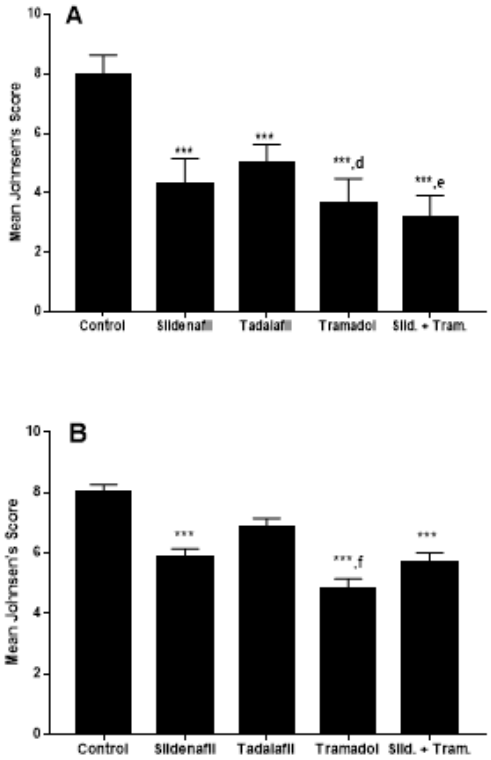

Figure 1: Comparison of mean Johnsen's score between the different experimental groups after (A) 8 weeks treatment period, and (B) 8 weeks recovery period. Values are mean ± SD, n = 7. ***$p<0.001$ vs control; d = $p<0.05$, e = $p<0.01$, f = $p<0.001$ vs tadalafil.

Leydig cell count in the different experimental groups after (A) 8 weeks treatment period and (B) 8 weeks recovery period

Figure 2A shows the Leydig cell count for control (208 ± 11.0), sildenafil (141 ± 14.0), tadalafil (160 ± 7.1), tramadol (130 ± 7.1) and sildenafil + tramadol (128 ± 9.7) group after 8 weeks treatment period. Leydig cell count was significantly ($p<0.001$) lower in all treated groups compared with the control. However, Leydig cell count was significantly ($p<0.05$) higher in tadalafil group compared with sildenafil group, and significantly ($p<0.001$) lower in tramadol and sildenafil + tramadol groups compared with tadalafil group. After 8 weeks recovery period, Leydig cell count was significantly ($p<0.001$) lower in all recovery groups (178 ± 12.1, 183 ± 8.8, 172 ± 10.4 and 160 ± 11.1, for sildenafil, tadalafil, tramadol and sildenafil + tramadol recovery group, respectively) compared with control (217 ± 17.9) but was not significantly different among the recovery groups (Figure 2B).

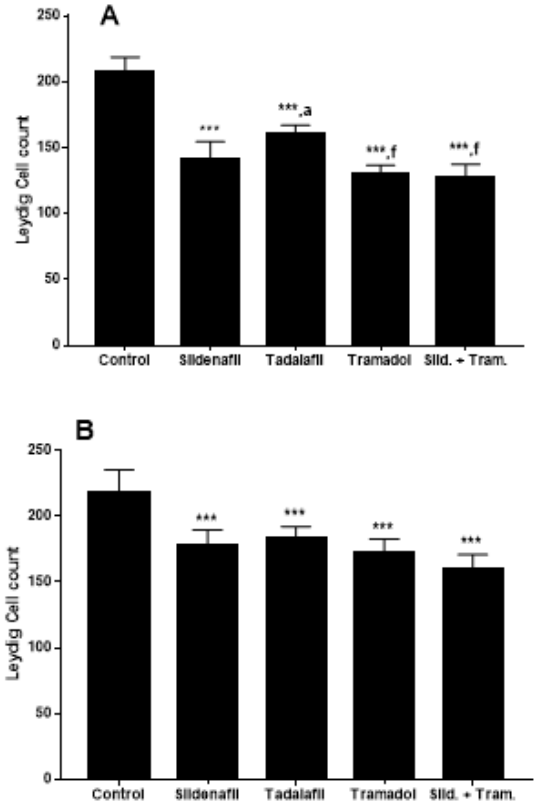

Figure 2: Comparison of Leydig cell count between the different experimental groups after (A) 8 weeks treatment period, and (B) 8 weeks recovery period. Values are mean ± SD, n = 7. ***$p<0.001$ vs control; a = $p<0.05$ vs sildenafil; f = $p<0.001$ vs tadalafil.

Mean Johnsen's testicular biopsy score and Leydig cell count between the treatment and recovery groups

Although, the mean Johnsen's testicular biopsy scores (figure 1) and the Leydig cell counts (figure 2) increased in the recovery groups compared to the treated groups they were still significantly lowered than the controls after the 8 weeks recovery period.

Histology of testes after 8 weeks treatment period

Figure 3 (A, B, C, D & E) shows photomicrograph of the testes of rats in the different experimental groups after 8 weeks of treatment with the different interventions. Histopathological examination of the testes revealed normal seminiferous tubules in the

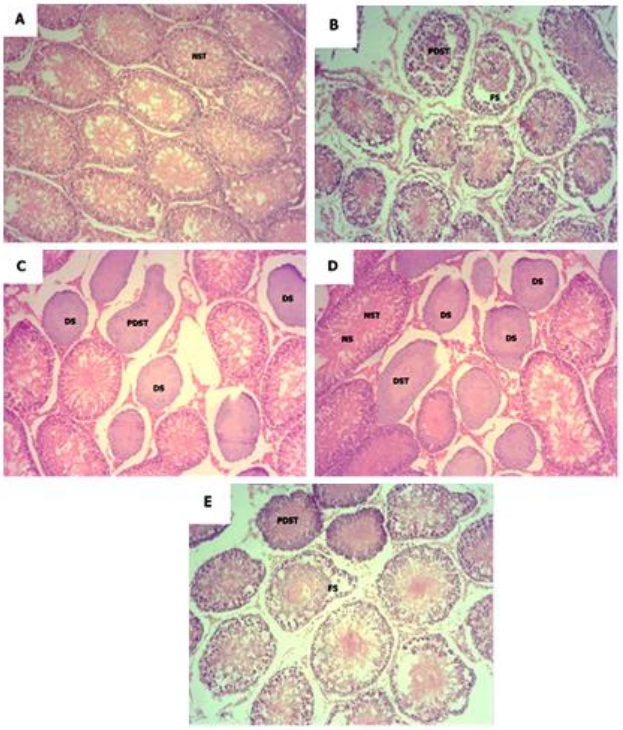

Figure 3 Photomicrograph of the testes of rats in the different experimental groups after 8 weeks of treatment with the different interventions X 400. (A) Control group, showing NST (normal seminiferous tubules); (B) Sildenafil – treated group, showing PDST (poorly differentiated seminiferous tubule) and FS (fewer spermatozoa population); (C) Tadalafil – treated group, showing PDST (poorly differentiated seminiferous tubule) and DS (damaged spermatozoa); (D) Tramadol – treated group, showing DS (damaged spermatozoa) and DST (damaged seminiferous tubule); (E) Sildenafil + tramadol – treated group, showing PDST (poorly differentiated seminiferous tubule) and FS (fewer spermatozoa population).

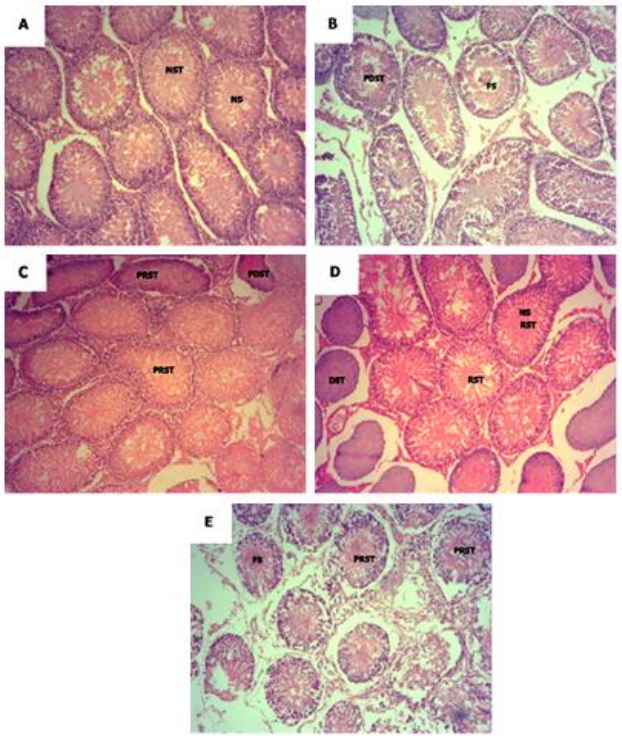

Figure 4: Photomicrograph of testes of rats in the different experimental groups after 8 weeks recovery period X 400. (A) Control group, showing NST (normal seminiferous tubules) and NS (normal spermatozoa population); (B) Sildenafil recovery group, showing PDST (poorly differentiated seminiferous tubule) and FS (fewer spermatozoa population); (C) Tadalafil recovery group, showing PDS (poorly differentiated seminiferous tubule) PRST (poorly recovered seminiferous tubules) and LSD (low spermatozoa density); (D) Tramadol recovery group, showing DST (damaged seminiferous tubule) and some RST (recovered seminiferous tubule); (E) Sildenafil + tramadol recovery group, showing PRST (poorly recovered seminiferous tubule) and FS (fewer spermatozoa population).

control group. The sildenafil treated group showed poorly differentiated seminiferous tubules and fewer spermatozoa population. Seminiferous tubules were also poorly differentiated with damaged spermatozoa seen in the tadalafil treated group. Damaged seminiferous tubules and damaged spermatozoa were observed in tramadol treated group. The sildenafil + tramadol treated group showed poorly differentiated seminiferous tubules and fewer spermatozoa population.

Histology of testes after 8 weeks recovery period

Figure 4 (A, B, C, D & E) shows photomicrograph of the testes of rats in the different experimental groups after 8 weeks recovery period. Seminiferous tubules and spermatozoa population were normal in the control group. The sildenafil recovery group showed poorly differentiated seminiferous tubules and fewer spermatozoa population. Low spermatozoa density, poorly differentiated seminiferous tubules and some poorly recovered seminiferous tubules were observed in the tadalafil recovery group. The tramadol recovery group showed damaged seminiferous tubules and some recovered seminiferous tubules. The sildenafil + tramadol recovery group showed poorly recovered seminiferous tubules and fewer spermatozoa population.

Histology of the epididymis after 8 weeks treatment period

Figure 5 (A, B, C, D & E) shows photomicrograph of the epididymis of rats in the different experimental groups after 8 weeks of treatment with the different interventions. Normal spermatozoa density and intact basement membrane were observed in the control group. The sildenafil treated group showed low spermatozoa density and broken basement membrane. In the tadalafil treated group, intact basement membrane, low spermatozoa density and some areas with normal spermatozoa density were observed. The tramadol treated group presented an epididymis with low spermatozoa density and broken basement

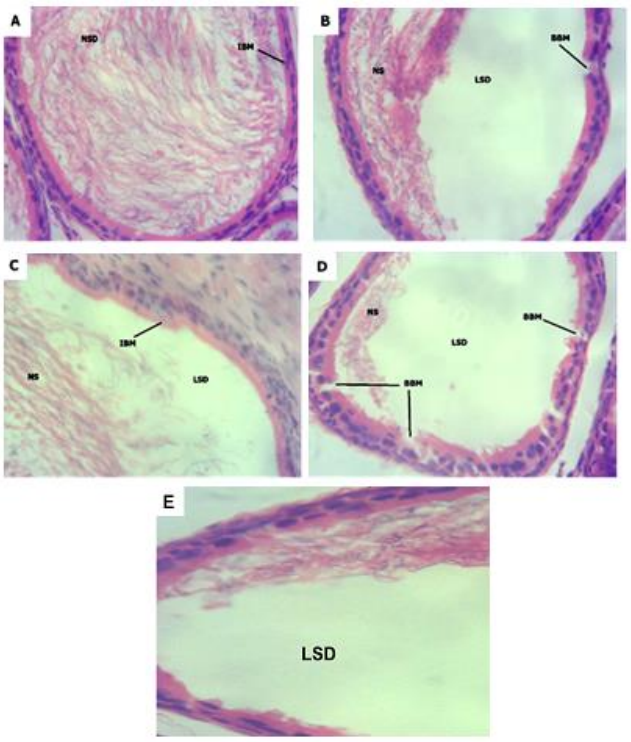

Figure 5: Photomicrograph of epididymis of the different experimental groups after 8 weeks of treatment X 400. (A) Control group, showing NSD (normal spermatozoa density) and IBM (intact basement membrane); (B) Sildenafil – treated group, showing LSD (low spermatozoa density) and BBM (broken basement membrane); (C) Tadalafil – treated group, showing IBM (intact basement membrane) LSD (low spermatozoa density) and some areas with NSD (normal sperm density); (D) Tramadol – treated group, showing LSD (low spermatozoa density) and BBM (broken basement membrane); (E) Sildenafil + tramadol – treated group, showing a large area of LSD (low spermatozoa density).

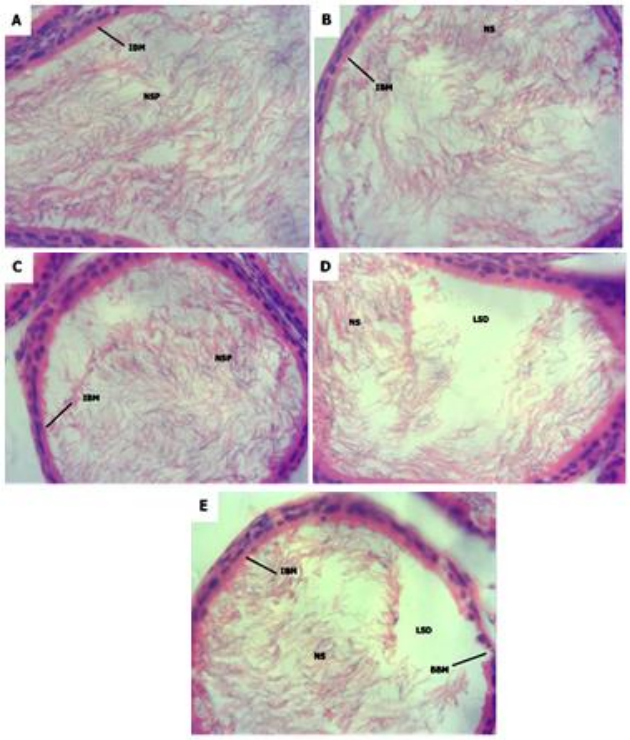

Figure 6: Photomicrograph of epididymis of the different experimental groups after 8 weeks recovery period X 400. (A) Control group, showing NSP (normal spermatozoa population) and IBM (intact basement membrane); (B) Sildenafil recovery group, showing NSP (normal spermatozoa population) and IBM (intact basement membrane); (C) Tadalafil recovery group, showing NSP (normal spermatozoa population) and IBM (intact basement membrane); (D) Tramadol recovery group, showing LSD (low spermatozoa density) and some areas with NSD (normal spermatozoa density); (E) Sildenafil + tramadol recovery group, showing BBM (broken basement membrane) and areas with LSD (low spermatozoa density).

membrane. A large area of low spermatozoa density was seen in the sildenafil + tramadol treated group.

Histology of the epididymis after 8 weeks recovery period

Figure 6 (A, B, C, D & E) shows Photomicrograph of epididymis of the different experimental groups after 8 weeks recovery period. The control, sildenafil recovery and tadalafil recovery groups showed normal spermatozoa density and intact basement membrane. The tramadol recovery group showed low spermatozoa density and some areas with normal spermatozoa density. The sildnenafil + tramadol recovery group showed low spermatozoa density and broken basement membrane.

DISCUSSION

Phosphodiesterase type 5 inhibitors (PDE5i) and the opioid, tramadol, have been reported to prolong erection and delay ejaculation, respectively. We previously reported a number of deleterious effects associated with high doses of these drugs (Nna *et al.*, 2015; Nna and Osim, 2016; Nna *et al.*, 2016a). The present study investigated the histopathological effects of high doses of sildenafil, tadalafil, tramadol and the combination therapy on the testes and epididymis of albino Wistar rats.

Mean Johnsen's score was significantly reduced in all treated groups compared with the control. This indicates that the individual drugs and the combination therapy affected spermatogenesis negatively. In this study, Leydig count was significantly reduced in all treated groups compared with the control, and significantly higher in tadalafil treated group compared with sildenafil treated group. It was also significantly lower in tramadol and sildenafil + tramadol treated groups compared with tadalafil treated group. From these results, tadalafil seems to have a less severe negative effect compared with the other medications. We previously reported that high doses of sildenafil, tadalafil, tramadol or sildenafil + tramadol caused significant testicular oxidative stress (Nna and Osim, 2016) and endocrine disruption (Nna *et al.*, 2016a). Therefore, the observed reduction in Leydig cell count in this study maybe attributable to oxidative stress. The reduction in Leydig cell count in

the treated groups is an indication that spermatogenesis was negatively affected. This was confirmed by the reduced Johnsen's score. In our previous study, we reported reduced sperm count following chronic administration of PDE5 inhibitors and opioids (Nna and Osim, 2016). Mean Johnsen's testicular biopsy score is a more specific measure of spermatogenesis. It has been reported that testicular secretion of androgens depends not only on the activity of the interstitial Leydig cells, but also on the number of these cells present in the testis (Teerd *et al.*, 2007). We also previously reported a reduced circulating levels of FSH and LH, which further compounds the Leydig cells' problem since they will not be efficiently stimulated by the circulating FSH and LH.

From this study, the effect of tramadol on the seminiferous tubules is similar to that reported by El-Ghawet (2015) that high doses of sildenafil, tadalafil, tramadol and sildenafil + tramadol affected spermatogenesis negatively by destroying the seminiferous tubules and spermatozoa. The present results validate our previous study which showed that sildenafil, tadalafil, tramadol and sildenafil + tramadol reduced testicular antioxidant enzyme activities, reduced sperm count and viability, and increase the number of abnormal spermatozoa (Nna and Osim, 2016). Al-Fartosi (2009) also reported that long-term administration of sildenafil citrate (50 mg and 100 mg) significantly decreased sperm count and increased sperm malformations. Histology of the epididymis of the tadalafil group showed intact basement membrane contrary to Hashish (2015), who reported that the basement membrane of the epididymis of rats became thin following treatment with 1.8 mg and 2.6 mg/kg b.w of tadalafil. The epididymis is the first site of storage of spermatozoa for maturation after spermatogenesis in the seminiferous tubules. The reduced sperm density in the epididymis of the treated groups may be attributed to the reduced spermatogenesis in the testis owing to the numerous damaged seminiferous tubules.

Consistent with our previous reports on the effects of PDE5i and tramadol on testicular oxidative stress (Nna and Osim, 2016) and endocrine disruption (Nna *et al.*, 2016a), this present study shows that none of the treated groups recorded complete recovery from the negative effects of the various treatments following withdrawal.

CONCLUSION

High doses of sildenafil, tadalafil, tramadol and the combination therapy (sildenafil+tramadol) cause severe histopathological deficits in the testes and epididymis of rats with poor reversal following withdrawal of treatment. These histopathological alterations have a marked negative effect on spermatogenesis.

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