



Effect of coke as a suspension of tramadol and diazepam on morphological and biochemical changes in the brain, heart and testes of male wistar rats

Abstract

Objectives: The aim of this investigation was to evaluate the effect of coke on the reported toxicity of tramadol and diazepam.

Methodology: A total of 48 animals were used for the experiment and were divided into 6 groups of 8 animals per group. Animals were observed for any physiological changes within 24 h of administration before sacrifice and the blood and tissue collected for biochemical (Lactate Dehydrogenase [LDH], Glutathione Transferase [GT], Total Antioxidant Status [TAS], Glutathione [GSH], Acetylcholinesterase [AChE]) and histological studies.

Results: Tramadol only administered group and tramadol suspended in coke group showed that coke caused a significant increase in TAS ($p < 0.05$), significant decrease in GT activity ($p < 0.05$), and no significant effect on LDH activity. Suspension of diazepam in coke caused a significant decrease in GT activity and TAS as compared to diazepam only administered group ($p < 0.05$), while no significant effect on LDH activity. Suspension of tramadol with coke did not improve tramadol effects on GSH concentration and AChE activity while suspension of diazepam in coke caused a significant increase in AChE in the brain and significant decrease in GSH concentration. Suspension of either drug with coke had no significant effect on AChE and GSH in the testes. Histology study of the testes showed that abnormal seminiferous tubules shape, germ cell layer, absence of maturation stage and lack of spermatozoa caused by both drugs was not different when they are suspended in coke.

Conclusion: Our results showed that suspension of tramadol and diazepam in coke can increase their toxicity on different organs, thus caution need to be taken when ingesting drugs with softdrinks as it may enhance the unwanted effects of the drugs.

Keywords: cardiotoxicity, neurotoxicity, testes, drug metabolism, coke, tramadol, diazepam

Introduction

Drug abuse is a major challenging facing the society. A drug can be defined as any substance that alters metabolic processes in the body leading to change in physiology, behavior of the individual or both. Drug abuse can be defined as high dose intake, unprescription, and non-medicinal uses of drugs. This has led to societal delinquent, such as violent behavior, armed robbery, cultism, victimization, rape. The toxic effect of drug abuse has led to some chronic disorders, such as kidney and liver problem, cardiovascular and neurological disorders [1-3]. The soothing and pain relieving effect of analgesic drugs make them one of the most abusive drugs globally [4]. Tramadol is an analgesic drug that is prescribed for treatment

linked to pain. Its mechanism of action is by preventing the re-uptake of norepinephrine and serotonin as well as having an affinity for the mu-opiate receptor. It is metabolized in the liver and kidney, in the liver, it undergo various phase II metabolic processes, such as demethylation and conjugation with sulphate and glucuronic acid. Study has shown that one of its metabolite (O-desmethyl tramadol) is more active than the parent drug with greater affinity for the mu-opiate receptor and a two-fold increase in analgesic effect [5-7].

The cheap and readily availability of tramadol has made it one of the most abused drug globally. Some suicide and death related reports have been linked to tramadol overdose [8]. Other effect of tramadol overdose includes: consciousness impairment (~30%), seizures (~15%), agitation

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(~10%) and respiratory depression (~5%) [9]. The addictive effect of tramadol also increases its dependence and prevent the stoppage of its usage [10]. Another widely used analgesic drug is diazepam. It is a member of the benzodiazepine family. It is prescribed for the treatment of convulsion, anxiety and muscular coordination. Its mechanism of action involves the enhancement of Gamma Amino Butyric Acid (GABA), a neurotransmitter by binding to benzodiazepine site on the GABA receptor [11]. This cause central nervous depression. The over-the-counter availability of diazepam also makes it one of the most abused drugs globally. Overdose of diazepam has been linked to brain and liver damage. Physiological defects includes, ante retrograde amnesia, hyperactivity, excessive depression, addiction and dependence, mental disorders [12,13].

Coke is one of the most popular soft drinks consumed. Some of the content includes carbonated water, sweetener, flavoring agent, caffeine and preservatives [14]. Coke has been reported to enhance the absorption and bioavailability of some drugs such as ketoconazole, itraconazole, carbamazepine and ibuprofen [15]. The sweetness of coke also makes it a potential solvent for drug suspension to hide the taste of the drugs on suspected victims of rape, robbery or murder [16,17]. The aim of this experiment is to evaluate the effect of suspension of tramadol and diazepam in coke on brain, heart and testes integrity.

Materials and Methods

Chemicals and Reagents

Reduced glutathione, Acetylthiocholine iodide, Nicotinamide Adenine Dinucleotide (NADH) were purchased from Sigma-Aldrich, Germany. Sodium dihydrogen phosphate, disodium hydrogen phosphate, ethanol, Potassium Chloride Solution (KCl), Formalin, Tris KCl, Sodium Hydroxide (NaOH), Tramadol hydrochloride, Diazepam.

Animals

The study was conducted on thirty-six male Wistar rats weighing between 170 g-220 g. These rats were obtained from a breeding animal house from the department of Biochemistry, University of Benin. They were housed at room temperature in plastic cages and were kept under constant healthy environmental and nutritional

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conditions. They were fed on rat pellets and water ad libitum. The maintenance of the animals and the experimental procedures were in accordance with the guiding principles of animal handling. They were left to acclimatize for 2 weeks prior to start of administration. All protocol and design were conducted according to the guidelines provided by Basic and Clinical Pharmacology and Toxicology policy for experimental and clinical studies [18].

Experimental Design

Thirty six (36) male rats weighing between 140 g-60 g of 10-12 weeks old, were randomly divided into six groups of six rats per group as follows: group I (negative control) administered 1 ml/kg distilled water; group II (Coca cola) administered 1 ml/kg coca cola; group III (tramadol) administered 75 mg/kg tramadol dissolved in distilled water; group IV (tramadol+coke) administered 75 mg/kg tramadol dissolved in coke; group V (diazepam) administered 20 mg/kg diazepam dissolved in distilled water; group VI (diazepam+coke) administered 20 mg/kg diazepam dissolved in coke. The dosage was based on [8].

Serum Biochemistry

Serum Total Antioxidant Status (TAS) and Glutathione Transferase (GT) were assayed spectrophotometrically using commercial kits purchased from Bio Diagnostic Co., according to the manufacturer's protocol.

Acetylcholinesterase Activity

This was estimated in the brain and testes according to the method developed by Elman et al. [19]. This is based on the principle of thiol group reacting with DTNB to form yellow colored thionitrobenzoic acid, which was read at 412 nm.

■ Lactate Dehydrogenase

Cardiac level of LDH was evaluated spectrophotometrically according to the kit manual.

■ Glutathione concentration

GSH content was determined according to Jollow et al. [20]. The reaction is based on the fact that the thiol group of GSH reacts with DTNB to form thionitrobenzoic acid. The supernatant was mixed with 4% sulphosalicylic acid. The mixture was allowed to stand for 5 min and then filtered. Thereafter, 1 ml of filtrate was added to 4.0 ml of the Elman's reagent. The absorbance was read at 412 nm against a reagent blank.

■ Histopathological evaluation by an external assessor not preview to the experimental design

Brain, heart and testes tissues were taken from the eviscerated rats and fixed in 10% formalin for 24 hrs, and then processed to obtain paraffin blocks. Sections of 4 μ m-6 μ m thickness were cut using a microtome and stained with hematoxylin and eosin (H and E) stain by using the method of Stevens and Wilson (Stevens and Wilson) [21].

■ Statistical Analysis

Data were organized, tabulated, and statistically analyzed using the SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. (SPSS Inc., Chicago, USA). For quantitative data, the mean and SD were calculated and were expressed as mean \pm standard deviation and analyzed using Analysis of Variance (ANOVA). For comparison of means of more than two groups, the F-test was used. Statistical significance was taken at a p value of less than 0.05.

Results

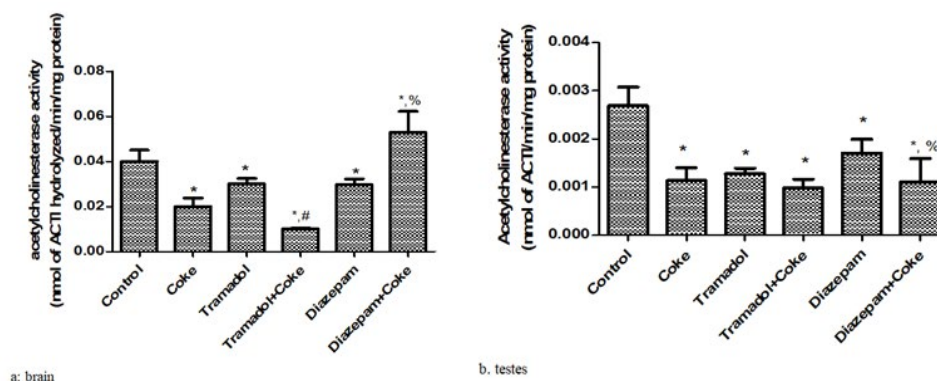
■ Effect of single administration of tramadol and diazepam dissolved in coke on AChE in the brain and testes

Coke, tramadol and diazepam caused a significant reduction in the activity of AChE in the brain as compared to the control. However, dissolving tramadol in coke further caused a significant decrease in the activity of AChE as compared to tramadol only administration. A different result was observed with diazepam, diazepam dissolved in coke caused a significant increase in AChE activity in the brain as compared to other groups ($p < 0.01$). In the testes, coke, tramadol and diazepam caused a significant decrease in the activity of AChE as compared to the control ($p < 0.05$). However, dissolving tramadol and diazepam in coke had no significant effect on AChE activity in the testes **FIGURE 1**.

■ Effect of single administration of diazepam and tramadol dissolved in coke on GSH concentration

In the brain, coke and diazepam caused a significant increase in GSH concentration as compared to control ($p < 0.05$), however, tramadol had no significant difference in GSH concentration when compared to control ($p > 0.05$). While dissolving tramadol in coke had no significant effect on GSH as compared to tramadol only group. Dissolving diazepam in coke caused a significant reduction in GSH concentration as compared to diazepam only group ($p < 0.05$). In the heart, coke caused a significant increase in GSH concentration, tramadol and diazepam caused a significant decrease in GSH concentration as compared to the control ($p < 0.05$). Tramadol dissolved in coke had no significant difference in GSH

FIGURE 1. Activity of acetylcholinesterase in the (a): brain; (b): testes; Results are expressed as mean \pm SD (n=6). * $p < 0.05$ control vs. treatment, % $p < 0.05$ diazepam vs. Diazepam+Coke.



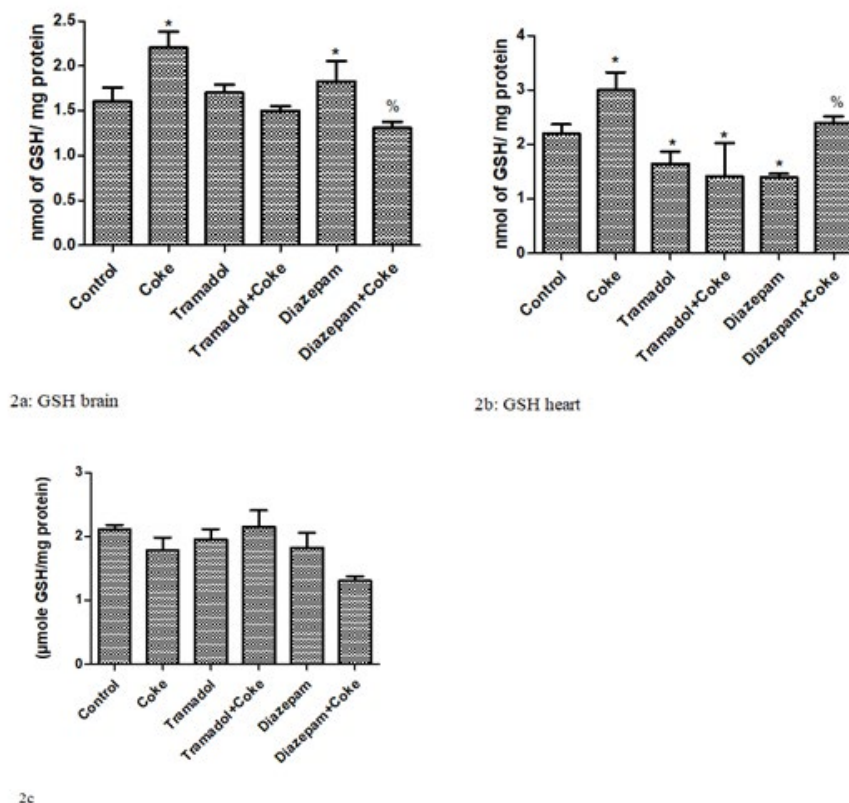


FIGURE 2. Concentration of reduced glutathione in (a): brain; (b): heart and (c): testes; Results are expressed as mean +SD (n=6). *p<0.05 control vs. treatment, % p<0.05 diazepam vs. Diazepam+Coke.

concentration as compared to tramadol only group ($p>0.05$). However, dissolving diazepam in coke caused a significant increase in GSH concentration as compared to diazepam only treatment ($p<0.05$). In the testes, coke, tramadol and diazepam had no significant effect on GSH concentration as compared to the control, neither does dissolving tramadol and diazepam in coke had any significant effect on GSH concentration **FIGURE 2**.

■ Effect of tramadol and diazepam dissolved in coke on serum activity of glutathione transferase, lactate dehydrogenase and total antioxidant status

Coke caused a significant increase in GT activity, while tramadol and diazepam caused a significant decrease in the activity of GT as compared to the control ($p<0.05$). Tramadol and diazepam dissolved in coke caused a further decrease in GT activity when compare to tramadol and diazepam only treated group ($p<0.05$). Coke and diazepam caused a significant decrease in LDH activity ($p<0.05$), while tramadol had no significant effect on serum LDH activity as compared to the control ($p>0.05$). Dissolving tramadol in coke however caused a significant decrease in LDH activity as compared to tramadol only group. Dissolving

diazepam in coke had n caused a significant decrease in serum TAS, while diazepam had no significant effect on serum TAS ($p>0.05$). dissolving tramadol in coke caused significant increase in TAS as compared to tramadol only group, however, dissolving diazepam in coke caused a significant decrease in TAS as compared to diazepam only group ($p<0.05$) **FIGURE 3**, **FIGURE 4** and **FIGURE 5**.

■ Histology

FIGURE 6 showed the Photomicrograph of a testicular section stained by haematoxylin and eosin in control, coke, tramadol, tramadol+coke, diazepam and diazepam+coke treated rats and observed under microscope at $\times 100$ magnification. The interstitial spaces and leydig cells appear normal in all the groups. The control rats was rich in spermatozoa, while the sertoli cells, germ cell layer and the lumen appears normal. The entire treatment group showed abnormal seminefeous tubules shape, germ cell layer, absence of maturation stage and lack of spermatozoa apart from coke only administered group which showed the presence of spermatozoa.

Control: Photomicrograph of a testicular section stained by haematoxylin and eosin showing several normal seminiferous tubules with normal spermatogonia cell and normal

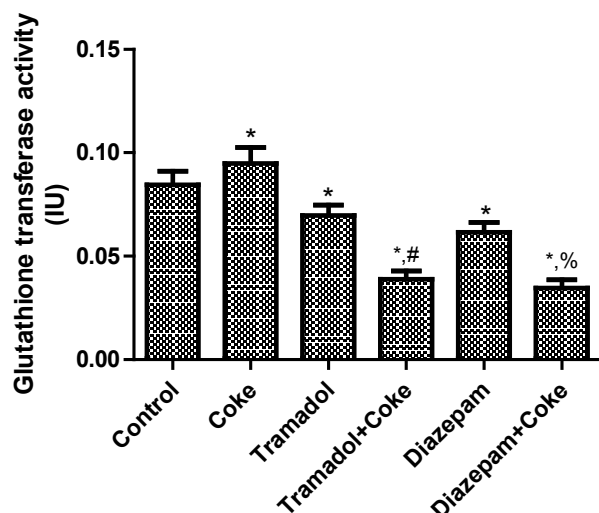


FIGURE 3. Glutathione transferase activity in the serum. Results are expressed as mean \pm SD (n=6). *p<0.05 control vs. treatment, # p<0.05 tramadol vs. Tramadol+Coke, % p<0.05 diazepam vs. Diazepam+Coke.

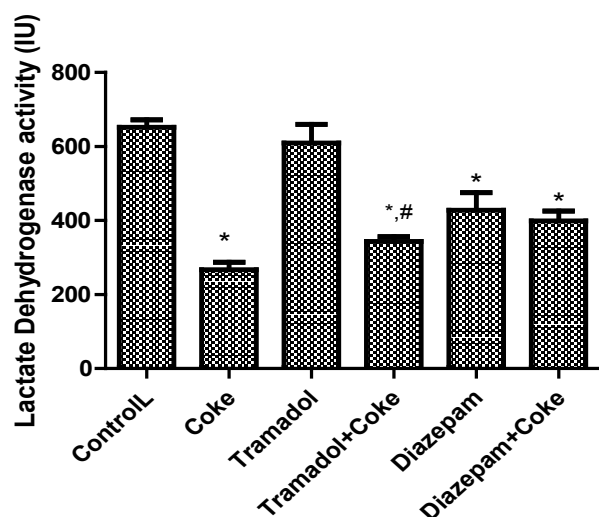


FIGURE 4. Lactate Dehydrogenase (LDH) activity in the serum. Results are expressed as mean \pm SD (n=6). *p<0.05 control vs. treatment, # p<0.05 tramadol vs. Tramadol+Coke, % p<0.05 diazepam vs. Diazepam+Coke.

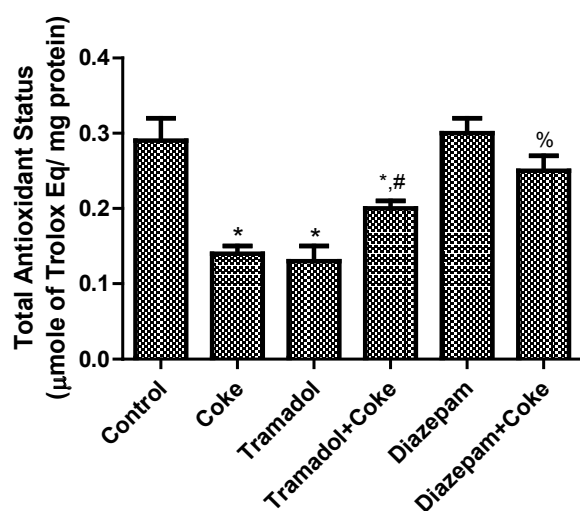


FIGURE 5. Total antioxidant status, activity of glutathione transferase and lactate dehydrogenase in the serum. Results are expressed as mean \pm SD (n=6). *p<0.05 control vs. treatment, # p<0.05 tramadol vs. Tramadol+Coke, % p<0.05 diazepam vs. Diazepam+Coke.

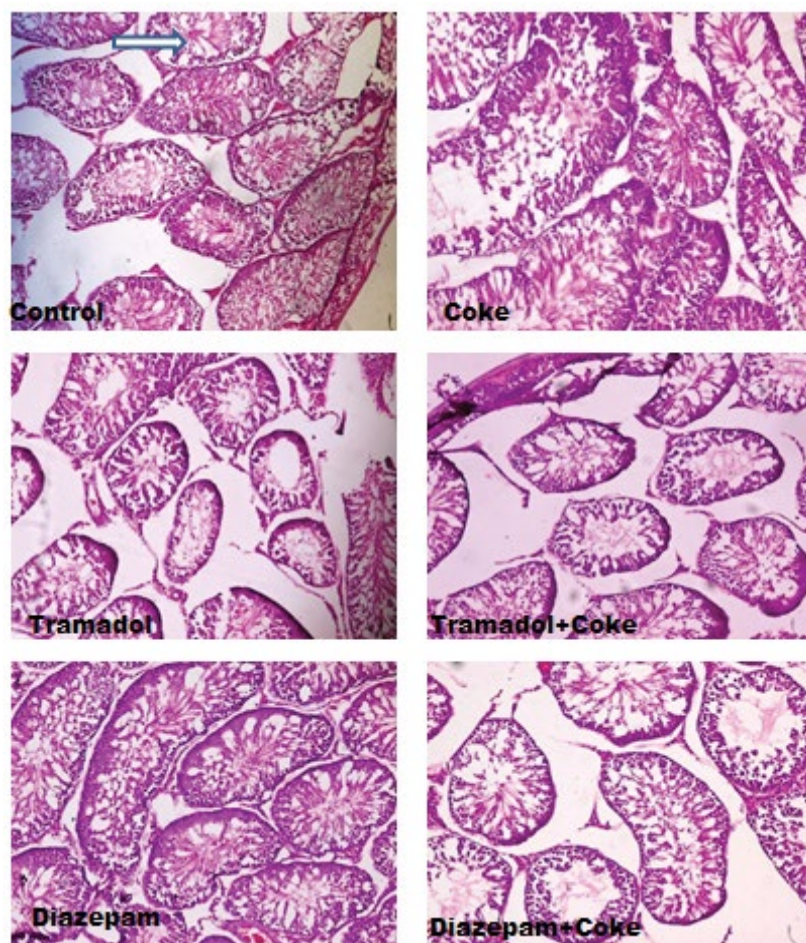


FIGURE 6. Hematoxylin-Eosin (HE)-stained sections in testes

sertoli cells and showing normal germ cell layer with normal maturation stages, the lumen appear normal with presence of spermatozoa. The interstitial spaces and leydig cells appear normal.

Coke: Photomicrograph of a testicular section stained by haematoxylin and eosin showing some seminiferous tubules with bizzare shapes and vacuolations, some show no germ cell layer nor maturation stages, some normal tubules show lumen appearing normal with presence of spermatozoa. The interstitial spaces and leydig cells appear normal.

Tramadol: Photomicrograph of a testicular section stained by haematoxylin and eosin showing severely atrophic seminiferous tubules with thickened collagenic laminae, majority of seminiferous tubules had double cell layers indicative of cessation of spermatogenesis, the lumen appear widened without spermatozoa. The interstitial spaces and leydig cells appear normal.

Tramadol+Coke: Photomicrograph of a testicular section stained by haematoxylin and eosin showing some normal seminiferous

tubules with normal maturation stages, there are others with maturation arrest and their lumen appear wide and lack spermatozoa. The interstitial spaces and leydig cells appear normal.

Diazepam: Photomicrograph of a testicular section stained by haematoxylin and eosin showing some normal seminiferous tubules with normal maturation stages, there are others with maturation arrest and vacuolation; their lumen appear wide and lack spermatozoa and some show sloughed germ cells within the lumen. The interstitial spaces and leydig cells appear normal.

Diazepam+Coke: Photomicrograph of a testicular section stained by haematoxylin and eosin showing few normal seminiferous tubules with normal maturation stages, there are others with bizzare shapes and maturation arrest ; their lumen appear wide and lack spermatozoa and some show sloughed germ cells within the lumen. The interstitial spaces and leydig cells appear normal.

■ Heart

FIGURE 7 showed the Photomicrograph of a section of the heart, stained by haematoxylin

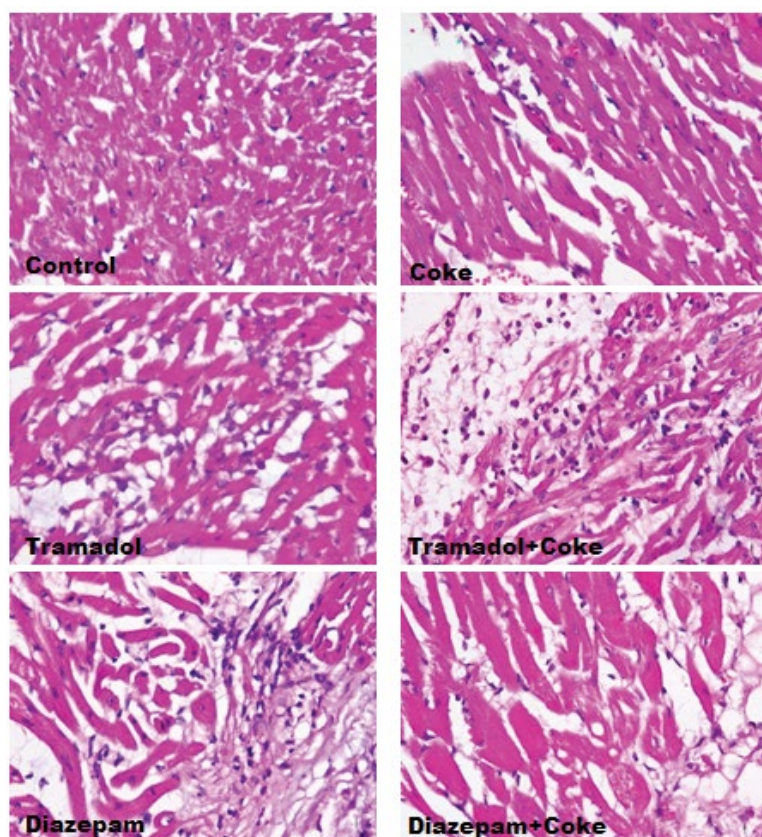


FIGURE 7. Hematoxylin-Eosin (HE)-stained section in rat's heart

and eosin in control, coke, tramadol, tramadol+coke, diazepam and diazepam+coke treated rats and observed under microscope at x100 magnification. Rats in the control and coke groups showed normal cardiac muscle without any pathological lesion. There was mild inflammation, moderate fat deposit and degeneration of cardiac muscles in other groups (tramadol, tramadol+coke, diazepam and diazepam+coke).

Control: Photomicrograph of a heart section stained by Haematoxylin and Eosin showing normal heart tissue with normal epicardial and myocardial layer, the cardiac muscles generally are normal, no pathological lesion seen in the heart tissue.

Coke: Photomicrograph of a heart section stained by haematoxylin and eosin showing normal heart tissue with normal epicardial and myocardial layer, the cardiac muscles generally are normal, no pathological lesion seen in the heart tissue.

Tramadol: Photomicrograph of a heart section stained by haematoxylin and eosin showing epicardial layer with moderate infiltration of inflammatory cells there is moderate fat deposit and degeneration of cardiac muscles seen. **Tramadol+Coke:** Photomicrograph of

a heart section stained by haematoxylin and eosin showing subepicardial adipose tissues with moderate infiltration of inflammatory cells and the infiltration also mildly involve the pericardial layer, there is dilation of the valves and severe congestion noted.

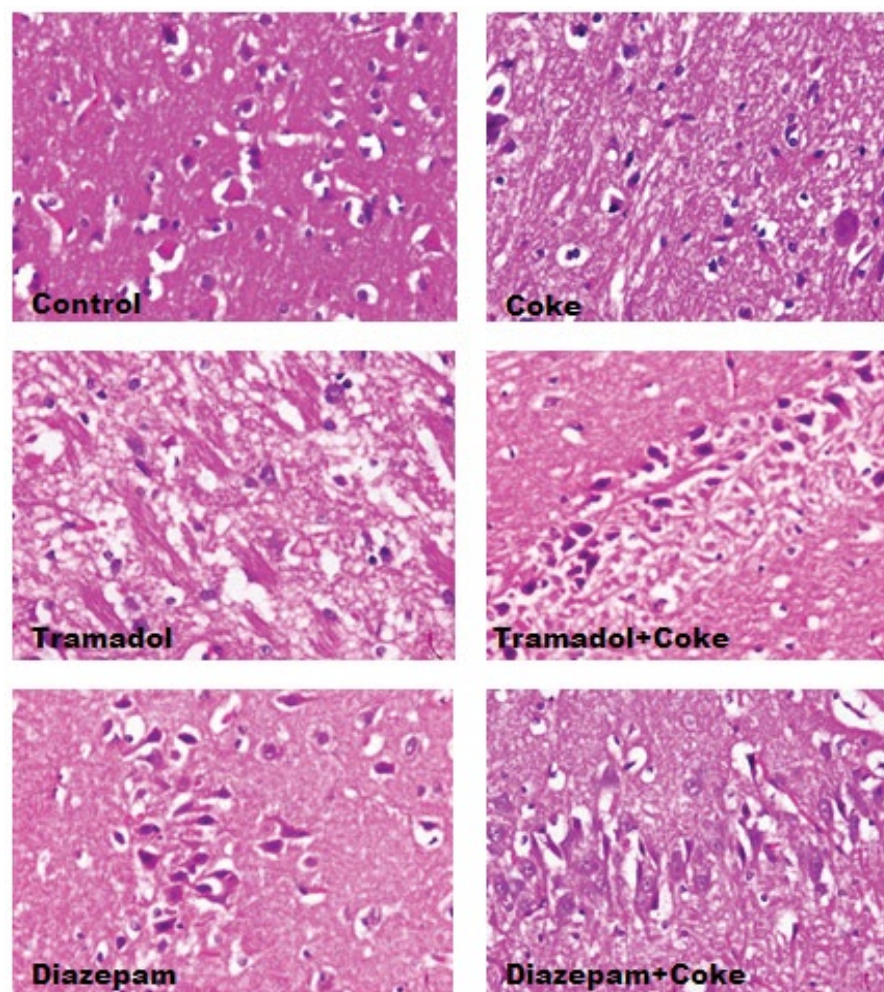
Diazepam: Photomicrograph of a heart section stained by haematoxylin and eosin showing mild subepicardial adipose tissues with very mild infiltration of inflammatory cells, the epicardial and myocardial layer appear normal.

Diazepam+Coke: Photomicrograph of a heart section stained by haematoxylin and eosin showing subepicardial adipose tissues with moderate infiltration of inflammatory cells and the infiltration also mildly involve the pericardial layer, there is dilation of the valves and severe congestion noted.

■ Brain

FIGURE 8 showed the Photomicrograph of a section of the hippocampus region of the brain, stained by haematoxylin and eosin in control, coke, tramadol, tramadol+coke, diazepam and diazepam+coke treated rats and observed under microscope at x100 magnification. The hippocampus from rats in the control and coke group showed normal organization of the cornu ammonius and neuronal cells. The

FIGURE 8. Hematoxylin Eosin (HE) stained section of rat's brain



rats in tramadol and diazepam administered groups showed abnormal CA, degeneration and depletion of neuronal cells in the hippocampus. Suspension of tramadol and diazepam in coke further exacerbate the toxicity of the drugs.

Control: Photomicrograph of the hippocampus region of the brain section stained by hematoxylin and eosin showing hippocampus with normal structural organization of the cornu ammonis including CA1, CA2 AND CA3, the neuronal cells seen are normal and not dispersed.

Coke: Photomicrograph of a brain section stained by hematoxylin and eosin showing hippocampus with normal structural organization of the cornu ammonis including CA1, CA2 AND CA3, and the neuronal cells seen are normal and not dispersed.

Tramadol: Photomicrograph of a brain section stained by hematoxylin and eosin showing hippocampus with poor structural organization of the cornu ammonis, CA2 show focal area of degeneration and depletion of neuronal cells and CA3 shows mildly dispersed cells.

Tramadol+Coke: Photomicrograph of a brain section stained by hematoxylin and eosin showing hippocampus with moderate structural organization of the cornu ammonis, the CA2 show depletion of neuronal cells and the structure not seen in compact form.

Diazepam: Photomicrograph of a brain section stained by hematoxylin and eosin showing hippocampus with moderate structural organization of the cornu ammonis, the CA2 show depletion of neuronal cells and the structure not seen in compact form.

Diazepam+Coke: Photomicrograph of a brain section stained by hematoxylin and eosin showing hippocampus with moderately normal structural organization of the cornu Ammonis including CA1, CA2 and CA3, the neuronal cells seen are normal and not dispersed.

Discussion

Tramadol is a synthetic drug that is prescribed as a pain reliever. One of its mechanisms of action is the inhibition of the reuptake of

neurotransmitters such as epinephrine and serotonin by the neurons. The low cost and ready availability makes it an alternative to common opioids, leading to its abuse among young people [22-24]. Findings showed that youth also ingest tramadol dissolved in coke, believing it will improve their libido. There have been several reports of death through tramadol abuse [22]. Diazepam is one of the drugs classified under benzodiazepines. It is a multipurpose drug used as an antidepressant, antiepileptic, nervousness, muscle relaxant [25,26]. In addition it is used to treat alcohol and opioid addicts during their withdrawal process [27]. In surgical procedure, it is administered to calm the patient and induce amnesia [28,29]. Apart from all the above listed medical uses of diazepam, non-medical uses of diazepam include for rape and robbery, whereby the victim is administered the drug before the crime is perpetrated, in such cases, the drug is dissolved in sweet beverages such as coke to hide the taste and presence of the drugs from their victim [16]. The aim of this experiment is to evaluate the toxic effect of tramadol and diazepam suspended in coke on the brain, heart and testes of male wistar rats.

Male rats were exposed to both drugs for 24 hrs, following this; TAS and GT activities were analyzed in the serum. LDH is an important enzyme biomarker of cardiac toxicity. Decrease of the enzyme in the organ and a concomitant increase in serum LDH is a hallmark of cardiac injury caused by several drugs [30-33]. The enzyme which catalyzes the conversion of pyruvate to lactate is abundant in the heart tissue; injury to the heart caused a leakage of the enzyme into the blood. Similar to other reports, tramadol has no significant effect on heart LDH [33], however, tramadol suspended in coke reduced LDH activity in the heart, showing that coke has a negative effect on tramadol with respect to cardiac function. Coke has been reported to improve drug absorption and metabolism [15]. It can be suggested that coke enhancing the metabolism of tramadol into other products that might be responsible for this cardiotoxicity [22]. With respect to diazepam, it has been reported to cause cardiac damage [34] which is also observed in the decreased activity of LDH in the heart. However, unlike tramadol, suspension of diazepam with coke had no effect on the toxicity of diazepam.

AChE is a neurotransmitter enzyme that catalyzes the hydrolysis of acetylcholine; it is abundant in brain, playing significant role in memory. Uncoordinated metabolic

activities of the enzyme can lead to mental and neuronal disorders, both regulated by the brain [33,35,36]. Tramadol has been reported to induce neurotoxicity through the inhibition of acetylcholinesterase [37], which was affirmed in our results. The further increase in the inhibition of acetylcholinesterase activity by suspension in coke can still be linked to increase conversion of tramadol to active metabolites that further increase its toxicity. One of the mechanisms by which diazepam exerts its neuronal effect is through the inhibition of AChE [38,39]. This was consistent with our observations, showing the inhibitory effect of diazepam on AChE. However, suspension of diazepam in coke reduced the anticholinesterase activity of diazepam in the brain. Glutathione transferase is a phase II enzyme of drug metabolism and it is involved in the conjugation of drug to increase their excretion from the body [40]. Most toxic compounds often exert their toxicity by inhibiting the activity of GT. Diazepam and tramadol also exert their organotoxicity by inhibiting the activity of GT. Our results also confirm this observation. The further increase in the inhibitory activity of the drugs when suspended in coke shows the negative effect of suspending them in coke.

Glutathione (GSH) is one of the important non enzymatic antioxidant that protects against oxidative damage. Tramadol and diazepam have been reported to alter GSH homeostasis as one of their mechanisms of oxidative stress induction [41-43]. The concentration of GSH was evaluated in brain, heart and testis; it was observed that both drugs only caused decrease in GSH concentration in the heart, while their effect on testis and brain was insignificant. An interesting observation was noted in our results, the suspension of diazepam in coke increases GSH concentration in the heart, it decreased GSH concentration of the brain and testis, however, with respect to tramadol, and suspension in coke did not improve the effect on GSH concentration. Inhibition of GT has been linked to oxidative stress and further damage to various organs [40,44]. Oxidative stress is caused by an imbalance in the reactive species and antioxidant defense system. This can be quantified by evaluating the Total Antioxidant Status (TAS). The TAS, that was decreased by tramadol seems to increase with the suspension of tramadol in coke, however, diazepam has no effect on the TAS, while coke suspension of diazepam caused oxidative stress as observed in the decreased TAS in the serum. The results showed that tramadol toxicity can be linked

to oxidative stress and suspension in coke can reduce the oxidative stress.

The histopathology of brain hippocampus, heart and testis showed varying degree of tissue damage. Several report on the pathology changes after tramadol and diazepam administration has been reported in both clinical and animal studies [8,33,36]. Some of the reported abnormalities in the brain architecture include neuronal degeneration, cellular disorganization and congestion and inflammation [37,45,46]. However, with diazepam, there are conflicting report on its effect on brain morphology, some reported diazepam to maintain normal morphology [47,48], no effect [49] and deleterious effect [50]. There might be different factors responsible for these discrepancies. Tramadol and diazepam also caused alteration on the morphology of the heart. With biochemical and morphological report of the effect of the drugs on various organs, heart seems to be the organ prone to deleterious effect of tramadol and diazepam disrupting cardiac myocytes and cellular aggregation, large number of inflammatory cells, cellular congestion [37,51,52]. Acute dose of tramadol and diazepam have been reported to cause damage to testes [53-55]. Histological studies revealed abnormal seminiferous tubules that lack sperm cells with a widened lumen similar to other reports. Suspension of the drugs worsens morphological damages caused by tramadol and diazepam.

Conclusion

Suspension of diazepam and tramadol in coke caused varying degree of alteration in the various biochemical parameters evaluated with respect to the brain, heart and the testes. The effect was more pronounced in diazepam as compared to tramadol. However, histology evaluation shows that coke increased the damaging effect of both drugs on all the tissues analyzed. While several mechanism have been projected for the toxicity of diazepam and tramadol, our results shows that coke did not improve the toxicity of the two drugs, rather it aggravated their toxicities.

Author contribution

Omotayo B Ilesanmi, Omojuwa E Oluwatosin, Chinoye E Mgbagwu, and Temitope T Odewale, wrote the paper. Omotayo B Ilesanmi, Temitope T Odewale, Shereen E. Tawfeek, Naeem Qusti, Safaa Qusti, Helal F Hetta, and Gaber El-Saber Batiha revised the paper. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

Authors have declared that no competing interests exist.

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