NEUROTOXIC AND ENZYMOLOGICAL EFFECTS OF CODEINE IN WISTAR RAT (*RATTUS NORVEGICUS*)

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ABSTRACT

Codeine is a naturally occurring opiate drug used for the treatment of pains. There have been reports of codeine abuse in some developing countries like Nigeria, however not much have been done on its effects in mammalian model. This study investigated the effect of codeine on acetylcholinesterase and other enzymological parameters in rat. Seventy-five albino rats procured for the study were divided into five groups, each containing five animals per group. Group, (A) served as the control and was administered normal saline free from the drug. The second (B), third, (C) and fourth (D) groups were given a standard therapeutic doses of 10, 15 and 20 mg/kg of codeine respectively. The drug was administered orally for a period of 20 days followed by 5 days recovery. Blood samples for enzymological parameters were obtained by puncturing the retro-orbital venous sinus of the rats. The brain tissues were later collected for the determination of AChE activity. There were significant concentration and duration dependent increase in the values of lipid peroxidation, AChE, lactate dehydrogenase, sodium adenosine triphosphatase and calcium adenosine triphosphatase in treated rats compared to the control. There were however mixed trends in the values of the studied parameters after the 5-day withdrawal of the drug. Further studies on the mechanisms of reactions of codeine in rat are thus recommended. Codeine altered the studied enzymological parameters which could not return to the control after withdrawal in rats. The use of the drug should be monitored to prevent abuse that may lead to physiological changes in animals.

Keywords: Codeine, Brain, Acetylcholinesterase, Oxidative stress, Rat

INTRODUCTION

Codeine (3-methylmorphine) is one the most commonly abused analgesic drugs used widely for the treatment of pains and cough in children and adults (Paul, 2012; Havig *et al.,* 2016). The problem associated with long-term dependence on codeine includes loss of memory, seizures, respiratory challenges, drowsiness, euphoria among others (Papich, 2015). Sudden withdrawal from the drug may lead to symptoms such as drug craving, running nose, yawning, diarrhea, muscle spasms, chills and pains (Ajayi and Akhigbe, 2021). ATPases are a group of mitochondrial enzymes that catalyze the hydrolysis of a phosphate bond in adenosine triphosphate (ATP) to form adenosine diphosphate (ADP). They harness the energy released from the breakdown of the phosphate bond and utilize it to perform other cellular

reactions. Na^+/K^+ pumps or sodium- and potassium-activated adenosine 5'-triphosphatase (Na^+, k^+-ATPase), its enzymatic version, is a crucial protein responsible for the electrochemical gradient across the cell membranes (de Lores Arnaiz and Ordieres, 2014).

Acetylcholinesterase (AChE) is a key enzyme in the nervous system that terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine. AChE is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system. AChE activity serves to terminate synaptic transmission, preventing continuous nerve firings at nerve endings. Therefore, it is essential for the normal functioning of the center and peripheral nervous system.

Ca^{2+}ATPase is a form of P-ATPase that transfers calcium after a muscle has contracted while sodium–potassium adenosine triphosphatase (Na^+/K^+-ATPase) is an enzyme found in the membrane of all animal cells (Hall and Hall, 2020). Lactate dehydrogenase (LDH) on the other hand is involved in the conversion of NAD^+ to NADH and back.

Codeine have been widely used for pain relieve but recently, there have been abuse of the drug by many youths in Nigeria (Akhigbe and Ajayi, 2020). Wrong use of codeine has been reported to cause common adverse side effects (Sehgal *et al*., 2013) and long-term effects like apathy, memory loss and diminished libido. Despite the increasing abuse of the drug, few studies have focused on effects of the drug the recovery patterns in mammalian models after exposure. There is thus a need to investigate the effect of codeine on neurotoxic and enzymological parameters and the recovery patterns in rats. The aim of the present study is to investigate whether oral administration of codeine will affect the AChE enzyme, lipid peroxidation (LPO) and other enzymological parameters in rat.

MATERIALS AND METHODS

Experimental Animals and Drug: A total of 75 six weeks old Wistar rats (*Rattus norvegicus*) weighing between 50 – 85 g, were obtained from the Department of Biochemistry, University of Nigeria, Nsukka, Animal house. They were maintained at a room temperature (27 ± 4°C) under natural lighting condition. The rats were fed *ad libitum* with pelleted standard diet (crude protein 10.66 + 0.76%, metabolizable energy 2543.20 kcal/kg) (Vital Feed Broiler Finisher, Grand Cereals Limited, Jos, Nigeria) and clean water. They were handled in accordance with international guidelines for Care and Use of Laboratory Animals as promulgated by the National Research Council (NRC, 2010). The experimental drug, codeine syrup was purchased from the manufacturer Archy Pharmaceutical Limited, Lagos, Nigeria. All other chemicals and the reagents used in the study were obtained from Sigma-Aldrich Chemical Sciences Company, St. Louis, Missouri, United States.

Experimental Design: The seventy-five albino rats (67.7 ± 17.5 g) were used for the study. The experiment was laid down in a complete randomized design (CRD) of five treatments replicated three times with each replicate having five rats. Group (A) served as the control and was given normal saline free from the drug. The second (B), third (C) and fourth (D) groups were given 10, 15 and 20 mg/kg doses of codeine respectively. The selection of the doses and duration were based on previous published reports of codeine in rats (Owoade *et al.,* 2019). The drug was administered once every 5 days for 15 days after which the drug was withdrawn for 5 days for recovery. Blood samples were collected from the rats by puncturing the retro-orbital venous sinus for enzymological parameters. Serum was obtained from the blood samples by centrifugation at 2000 rpm for 15 minutes and was collected in vials and used for the determination of LDH. The rats were later sacrificed and the brain collected for determination of AChE activity. Sampling was done on day 1, 5, 15, 20 and after 5-days recovery period. The experimental rats were kept for a 5-day recovery period where the drugs weren't administered.

Evaluation of Enzymatic Parameters: LPO was estimated by the methods of Draper and Hadley (1990). Approximately 3 ml of glacial

acetic acid was added into a blank test tube and a sample test tube. A total 3 ml of 1% thiobarbituric acid was also added to each of the test tubes, 0.6 ml of distilled water was added to the blank tube, 0.6 ml of sample was also added into the sample test tube. The solutions were thoroughly mixed and incubated in a boiling water bath for 15 minutes then was allowed to cool. After cooling, the solutions were centrifuged for 5 minutes at 2000 rpm and supernatant collected from each test tube. The supernatant of the blank was used to zero the spectrophotometer before the absorbance was read at 532 nm.

Acetylcholinesterase was assayed following Michel (1949) method. Approximately 3 ml of distilled water was placed in a glass container of capacity 10 ml, 0.2 ml of tissue homogenate was added along with 3 ml of phosphate solution and 0.12 ml of acetylcholine iodide solution. The mixture was incubated in a water bath at 37℃ for 30 minutes. The change in absorbance was monitored using the spectrophotometer at 412 nm. The enzyme activity was expressed as µmol/min/mg tissue.

Sodium-Potassium Adenosine Triphosphatase (Na^+,K^+-ATPase) was determined by measuring the amount of inorganic phosphate liberated following the hydrolysis of ATP by ATPase (Sarkar, 2002).

Estimation of calcium adenosine triphosphatase (Ca^{2+}ATPase) was done according to the method of Hjertén and Pan (1983). The amount of Ca^{2+}ATPase was determined by using prepared reagent that was prepared specifically for Ca^{2+}ATPase at pH 7.0.

The LDH in serum was separated into five different isoenzymes based upon their electrophoretic mobility. This method measures total LDH activity in serum. Elevations can occur in megaloblastic anaemia, carcinoma, shock muscular disorders, nephritic syndrome, cirrhosis, myocardial, leukaemia, haemolytic anaemia and non-viral hepatitis. The initial rate of NADH formation was directly proportional to the catalytic LDH activity and was determined by measuring the increase in absorbance at 340 nm (Mekkawy *et al.*, 2017).

Statistical Analysis: Data was analyzed using Statistical Packages for Social Sciences (SPSS) version 23.0 (IBM Corporation, Armonk, USA). Generalized linear model was used to estimate the effect of codeine concentration and duration of exposure on neurological and enzymatic activities of *Rattus norvegicus*. Least significant difference was used as post-hoc test.

RESULTS

Administration of the drug induced significant increase in LPO (Figure 1). The effect increased as time of administration increased from day-5 to day-20 ($F = 144.202$, $p<0.0001$, $\eta_p^2 = 0.935$). LPO reduced in rats administered codeine after the 5-days withdrawal (Figure 1).

Acetylcholinesterase activity in rat significantly increased after administration of codeine (Figure 2). The effect was significant from day-5 and continued till day-20, and even after 5-day withdrawal period. AChE increased in a manner that depended on both concentration and duration of administration of codeine (Drug: $F = 208.842$, $p<0.0001$, $\eta_p^2 = 0.940$; Duration: $F = 102.406$, $p<0.0001$, $\eta_p^2 = 0.911$).

The effect of codeine on serum Na^+ATPase, Ca^{2+}ATPase and lactose dehydrogenase were presented in Table 1. Serum Ca^{2+} ATPase concentration were modified by administration of codeine to the rats. The effects on Ca^{2+} ATPase depended on concentration of the drug ($F = 73.480$, $p<0.0001$, $\eta_p^2 = 0.846$) and the duration of administration ($F = 47.949$, $p<0.0001$, $\eta_p^2 = 0.827$). The concentration of Ca^{2+} ATPase increased in all rats administered codeine; the effect was most pronounced at the highest concentration (20 mg/L) and on prolonged administration. Similarly, Na^+ ATPase increased in a manner dependent on drug concentration ($F = 323.198$, $p<0.0001$, $\eta_p^2 = 0.960$) and duration of administration ($F = 256.731$, $p<0.0001$, $\eta_p^2 = 0.963$). The increase Na^+ ATPase was most pronounced at the highest codeine concentration and on day-20. Activities of the enzyme LDH were up-regulated by codeine.

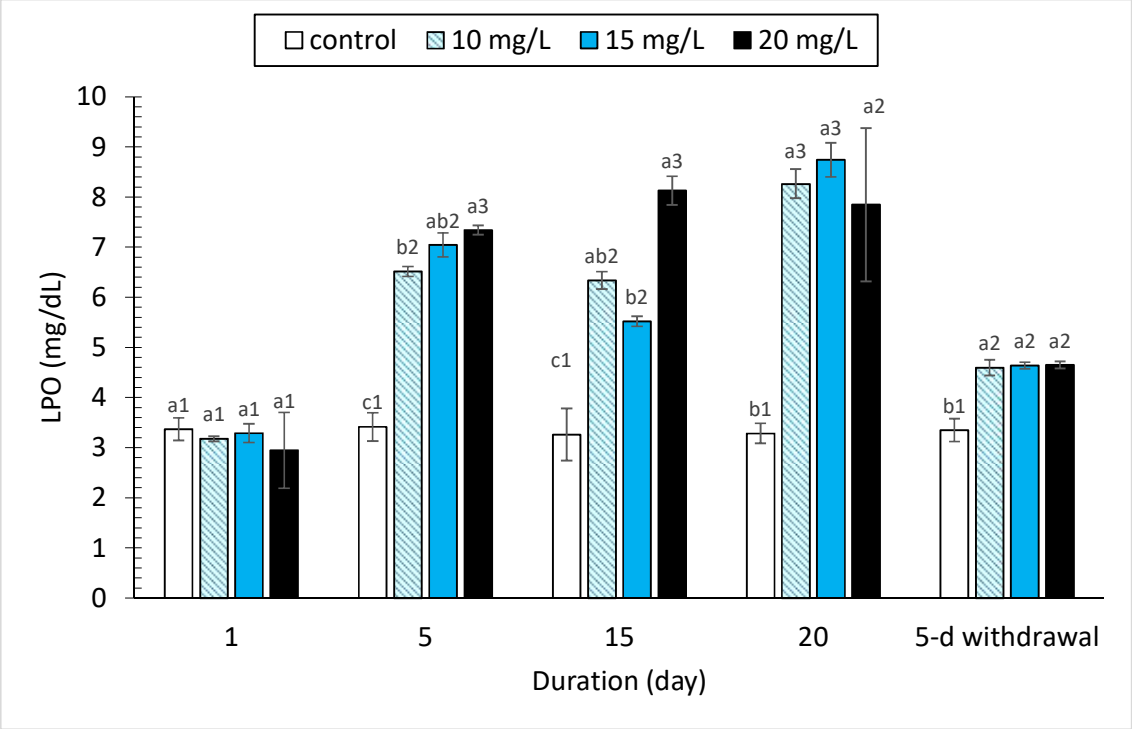

Figure 1: Lipid peroxidation (LPO) in *Rattus norvegicus* administered codeine. ***Keys:*** *Bars with different alphabet labels for a given day (5, 10, 15, 20, and 5-day withdrawal) was significantly different, while bars with different numeric label across the days for a given group were significantly different (p<0.05), values plotted as mean ± SD*

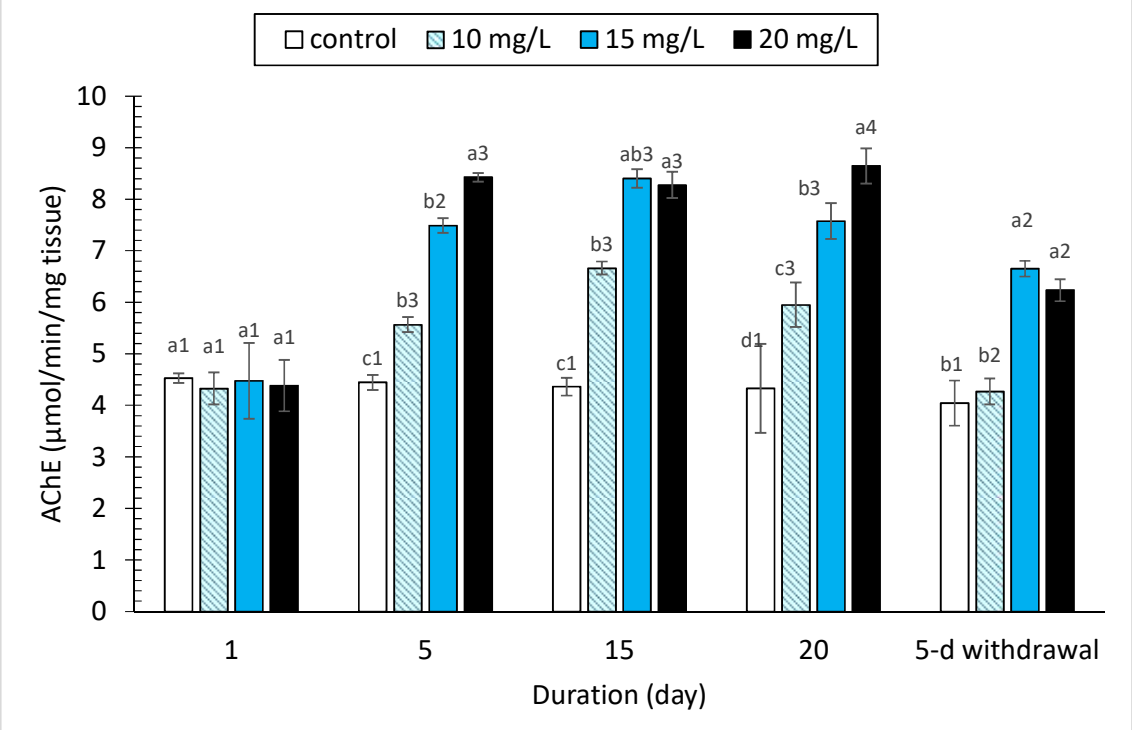

Figure 2: Acetylcholinesterase (AChE) activity in *Rattus norvegicus* administered codeine. ***Keys:*** *Bars with different alphabet labels for a given day (5, 10, 15, 20, and 5-day withdrawal) was significantly different, while bars with different numeric label across the days for a given group were significantly different (p<0.05), values plotted as mean ± SD*

LDH activities increased significantly in rats administered codeine from day-5 (Drug: F = 154.884, $p<0.0001$, $\eta_p^2 = 0.921$; Duration: F = 109.543, $p<0.0001$, $\eta_p^2 = 0.916$). There was significant interaction of drug concentration and duration of administration on all three parameters (Table 1). The values of the studied parameters were reduced after the 5-day withdrawal from the drug.

DISCUSSION

Investigation on the administration of codeine on AChE, ATPase and other enzymes activities in the brain of rats provides valuable information on the safe level, toxicity and damage that may result from codeine use. It has been shown that oxidative stress is associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Uttara *et al.*, 2009; Chen *et al.*, 2012). In addition, post mortem examination of patients with these diseases show that the region of the brain affected by neurodegeneration displayed increased ROS indices (Allan Butterfield *et al.,* 2002).

In the present study, there were duration and concentration dependent significant increase in ATPase (Na^+ and Ca^{2+}), LPO, LDH and AChE in rat administered various doses of codeine compared with the control. After 5 days of withdrawal of the drug, there were mixed trends in the values of the studied parameters and these calls for further studies on mechanisms of reactions of the drug. An increase in Ca^+ accumulation triggers an activation of the mitochondrial metabolic machinery and thus, increases the ATP synthesis in the mitochondria. Ca^{2+}ATPase dysfunction has been associated with heart failure (Díaz *et al.,* 2004), diabetes (Belke and Dillmann*,* 2004), atherosclerosis (Adachi *et al.,* 2004), restenosis (Lipskaia *et al.,* 2005) as well as aging skeletal muscles (Sharov *et al.,* 2006). Indirect inhibition of Na^+/ Ca^{2+} exchange causes a buildup of Ca^{2+} intracellularly because Ca^{2+} is not allowed to exist the cell if it cannot accept Na^+ into the cell. This increased intracellular Ca^{2+} then increases cardiac contractility, this stimulate the vagus nerve, causing a decrease in heart rate and can lead to heart failure. In the present study, there was an increase in the synthesis of Na^+-ATPase and this may lead to an increase in basal metabolic rate which then increases oxygen consumption, respiratory rate, body temperature and calorigenesis (Lei *et al.,* 2003). Furthermore, Archibong *et al.* (2021) reported inhibition of the Na^+-ATPase activity in rat administered various doses of codeine.

Acetylcholinesterase is the degenerative cholinergic enzyme of acetylcholine (ACh) found at the post synaptic neuromuscular junctions (Milatovic *et al.,* 2006; Ezinwa *et al.,* 2020). From the study, it is shown that there is an increase in the level of AChE in rats treated with codeine. When this high level of AChE in the brain and muscles do not break down acetylcholine into acetyl and choline, ACh will remain bound to its receptors and this will cause unwanted extended muscle contraction. This extended muscle contraction can result to paralysis, cramps, increased salivation, lacrimation, muscular weakness, muscular fasciculation, diarrhea and blurry vision (Kothari*,* 2004; Lacomis *et al.,* 2005; Silvestri *et al.,* 2016). When there is little ACh in the body due to rapid degradation, it causes learning difficulties, memory impairment, dry eyes, dry mouth, constipation/gastroparesis, low muscle tone, depressed mood and chronic inflammation (Grodner *et al.*, 2021). Thus this study demonstrated that AChE activity is modulated by codeine exposure by increasing an imbalance in the cholinergic system. Contrary to our results, Ajayi and Akhigbe (2021) reported significant decrease in AChE in rabbit administered different doses of codeine.

Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes, leading to the cell death (Melefa and Nwani*,* 2021). An increase in LPO as seen in the study cause a disturbance in the assembly of membrane, causing changes in fluidity and permeability, alterations of ions transport and inhibition of metabolic process. Increased LPO thus can lead to increased toxicity which can cause serious damage to the tissues.

Table 1: Calcium ion (Ca^{2+}ATPase) and sodium ion (Na^{+}ATPase) concentration, and lactate dehydrogenase activity in *Rattus norvegicus* administered codeine

Parameter	Conc. (mg/L)	Duration (day)				
		5	10	15	20	5-d withdrawal
Ca^{2+}ATPase	Control	8.47 ± 0.50^{a1}	8.50 ± 0.30^{b1}	8.63 ± 0.59^{c1}	8.63 ± 0.59^{c1}	8.67 ± 0.47^{b1}
	10	8.40 ± 0.20^{a1}	10.10 ± 0.53^{a2}	10.57 ± 0.31^{b2}	10.57 ± 0.31^{b2}	10.23 ± 0.57^{a2}
	15	8.50 ± 0.40^{a1}	10.47 ± 0.15^{a2}	11.33 ± 0.31^{ab2}	11.65 ± 0.22^{a2}	10.13 ± 0.45^{a2}
	20	8.83 ± 0.76^{a1}	10.80 ± 0.20^{a2}	11.53 ± 0.31^{a2}	11.87 ± 0.31^{a3}	10.27 ± 0.35^{a2}
Na^{+} ATPase	Control	6.35 ± 0.20^{a1}	6.61 ± 0.29^{c1}	6.51 ± 0.30^{c1}	6.27 ± 0.29^{c1}	5.90 ± 0.38^{a1}
	10	6.25 ± 0.22^{a1}	8.74 ± 0.29^{b2}	9.25 ± 0.14^{b2}	10.28 ±0.26^{b2}	8.26 ± 0.14^{a2}
	15	6.21 ± 0.22^{a1}	9.15 ± 0.18^{b2}	10.38 ± 0.16^{a3}	10.65 ± 0.30^{a3}	8.28 ± 0.25^{a3}
	20	6.32 ± 0.68^{a1}	9.88 ± 0.23^{a2}	10.69 ± 0.29^{a3}	11.06 ± 0.13^{a3}	8.22 ± 0.26^{a3}
LDH (U/L)	Control	160.67 ±2.08^{a1}	160.00 ±2.00^{c1}	162.67 ±7.57^{c1}	163.0±3.61^{d1}	163.00 ± 4.36^{c1}
	10	161.00 ±2.64^{a1}	192.00 ±6.25^{b2}	194.00 ±2.00^{b2}	188.0 ± 4.00^{c2}	173.33 ± 3.06^{a2}
	15	160.67 ±3.06^{a1}	194.33 ±5.13^{b2}	197.33±4.04^{ab2}	202.33±4.51^{b2}	183.33 ± 3.21^{a2}
	20	161.67 ±3.51^{a1}	206.67 ±3.06^{c2}	203.00 ±5.57^{b2}	213.33±7.02^{c4}	182.33 ± 2.52^{a3}

Keys: *Values presented as mean ± SD. Values with different alphabet superscript along a column for each parameter were significantly different, while values with different numeric superscript across a row were significantly different ($p<0.05$)*

The results of this study are similar to the findings of Archibong *et al.* (2021) who reported elevation of LPO in rat administered various doses of codeine. Furthermore, Ajayi and Akhigbe (2021) also reported significant increase in LPO in rabbits administered codeine.

Lactate dehydrogenase plays an important role in synthesis of the body's energy as it is found in almost all the body's tissues, including those in the blood, heart, kidney, brain and lungs. When the LDH in blood or fluid level is high as seen in the study, it means certain tissues in the body have been damaged by drugs, disease, xenobiotics or other chemicals. When these tissues are damaged, they release LDH into the blood stream or other body fluids (Farhana and Lappin, 2023). In other words, LDH can be used as an important biomarker for tissue damage in organisms. The mixed trends in the values of the studied parameters after the 5-day withdrawal period calls for further insights on the pharmaco-dynamics or mechanisms of reactions of the drug.

Conclusion: The present study showed that codeine elicited increase in the oxidative stress parameters as indicated by the significant increase in LPO. Also, the increase in AChE, ATPase enzymes (Ca^{2+}ATPase and Na^{+}ATPase) and LDH levels suggests that codeine is toxic to rat. The integrated use of these biomarkers would be useful to regulatory agencies in determining the extent to which codeine affects animals as well as man.

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