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Despite the dangers associated with the increased use of prescription opioid drugs, limited researches have addressed the specific effects of prescription opioids on the brain. The objective of this study was to assess the effects of codeine medication on the prefrontal cortex and cerebellum of Wistar rats. The drugs, ArchilinTM with codeine syrup and Dihydrocodeine 30 mg tablets were used for this study. Thirty (30) male Wistar rats were divided into 5 groups labeled A, B, C, D, and E, n = 6. Group A served as control and was given normal saline, group B was treated with 1 mg/kg bodyweight dihydrocodeine, group C was treated with 2 mg/kg bodyweight dihydrocodeine, group D was treated with 2 ml/kg bodyweight ArchilinTM with codeine syrup and group E was treated with 4 ml/kg bodyweight ArchilinTM with codeine syrup. Drugs were administered orally and daily for 21 days. At the end of the treatment period, animals were sacrificed via intraperitoneal injection of ketamine hydrochloride, brains were perfused with phosphate-buffered saline and formal saline before harvested and postfixed in 10% neutral buffered formalin. Sections of the prefrontal cortex and cerebellum were obtained and processed for immunohistochemical studies using GFAP stain. Results from the study suggested...
that prolonged administration of codeine medication produced an inflammatory reaction in the prefrontal cortex and cerebellum of treatment groups. This neuroinflammatory reaction is an indicator of a pathologic process that could lead to neuronal degeneration, glial degeneration, and altered physiologic process in the prefrontal cortex and cerebellum.

Subjects: Mental Health; Health & Society; Allergology & Clinical Immunology

Keywords: immunohistochemistry; codeine; prefrontal cortex; cerebellum; astrocytes; Wistar rats

1. Introduction

Codeine is an opioid used to relieve mild to moderate pain and to reduce coughing. It can be taken as a tablet, but it is also found in prescription cough syrups and other stronger medicine (Smith, 2018). Young India (2015) reported that approximately 33 million people use codeine globally yearly; in 2008, the Substance Abuse and Mental Health Services Administration (SAMHSA) reported that 3 million young adults in the United States of America between the ages of 12 and 25 had used codeine-based cough syrup to get high (United State Government Accountability Office, 2018). In 2014, it was estimated that 467,000 American adolescents had used opiates such as codeine for non-medical purposes, and 168,000 of these were considered to have an addiction (Vashishtha et al., 2017). The use of prescription codeine for recreational purposes by adolescents is a source of worry. Reeves et al. (2015) reported that cough medicines containing codeine or dextromethorphan are frequently abused as recreational drugs due to their euphoric effects (the high).

The rate of prolonged use of prescription opioids such as codeine is increasing globally due to the euphoria it presents to users (Mohamadi et al., 2018). In Africa, codeine syrup addiction is a problem ravaging Kenya, Ghana, Chad, Zimbabwe, and Nigeria (Kiunguyu, 2018). In Nigeria, Akannam (2008) reported that over six million bottles of codeine syrup are sold daily in the Northwestern part of the country. Reports by Lawan and Adie (2012), Hamisu (2014), and Uthman et al. (2017) revealed that students and pupils in secondary and primary schools are active users of codeine medication due to ease of acquisition and low cost. It has been observed that codeine use amongst these sets of people has been associated with poor academic performance, property damage, physical injuries, damaged relationships, unprotected sex, and suicide (Abudu, 2008; Ekpang & Abuo, 2015). The altered social and emotional behavior exhibited by codeine abusers as reported by Melissa (2013) suggests that the prefrontal cortex which controls emotional and social behaviors may have been affected. Other characteristics presented by codeine abusers according to Melissa (2013) are impaired motor coordination which is controlled by the cerebellum. This means cerebellar functions may have been altered. The use of prescription opioids such as codeine medications either for a short or prolonged-time has been reported to alter physiological and behavioral functions (Stimmel & Kreek, 2000). However, there is limited research on the specific effect of licit (refined) opioids such as prescription codeine on the brain. Most of the researches done in the past were focused on illicit (unrefined) opioids such as heroin and the implicated cortical and subcortical limbic/paralimbic brain structures involved in emotion, reward, motivation, and impulse control (Jaymin et al., 2010). For this reason, the study aimed to assess the immunohistochemical reactions caused by codeine medication in specific brain regions such as the prefrontal cortex and cerebellum due to the reports that consumers who use codeine medication exhibit certain traits that implicate the prefrontal cortex and cerebellum.

The prefrontal cortex (PFC) is the cerebral cortex which covers the front part of the frontal lobe (Elisabeth et al., 2016). This brain region has been implicated in planning complex cognitive behavior, personality expression, decision-making, and moderating social behavior (Yang & Raine, 2009). The basic activity of this brain region is considered to be the orchestration of thoughts and actions following internal goals (Miller et al., 2002). The cerebellum is a region of
the brain that plays an important role in motor control and cognitive functions such as attention, language, and in regulating fear and pleasure responses. However, its movement-related functions are the most solidly established (Wolf et al., 2009). The cerebellum receives input from sensory systems of the spinal cord and other parts of the brain and integrates these inputs to fine-tune motor activity. Cerebellar damage produces disorders in fine movement, equilibrium, posture, and motor learning (Gray's Anatomy, 2008). The immunohistochemical study of codeine medication on the prefrontal cortex and cerebellum was demonstrated using a polyclonal Glial Fibrillary Acidic Protein (GFAP) antibody. The present study was limited to the use of prescription codeine medication (syrup and tablet) and how it affects neuroinflammatory response in the prefrontal cortex and cerebellum of Wistar rats.

2. Materials and method

2.1. Experimental animals

Thirty adult male Wistar rats of average weight (200 g) were obtained, authenticated, and housed in the animal house of the Faculty of Basic Medical Sciences, University of Calabar. The animals were kept in standard cages constructed for rats measuring 40 cm x 35 cm with adequate space and ventilation to encourage free movement. Animals were kept for 2 weeks before commencement of treatment to allow them to acclimatize and fed with normal commercial pellet grower mash (Vital Feed Grand Cereal Ltd, Jos, Nigeria) and clean water ad libitum. Ethical approval (No: 008AN30418) was obtained from the Faculty of Basic Medical Sciences Ethics Committee of the University of Calabar, and animals were handled according to international guidelines as laid down by the National Institute of Health (NIH) of the United States of America for the regulation of laboratory animals (Institutional Animal Care and Use Committee (IUAUC), 2015).

2.2. Drug purchase and preparation

The codeine medication used for the study was Archilin™ with codeine syrup (Archy Pharmaceuticals Ltd, Lagos Nigeria) and Dihydrocodeine 30 mg tablets (Dihydrocodeine BP, Bristol Laboratories Ltd, Berkhamsted, Herts, UK). Archilin™ with codeine contains 10.95 mg of codeine phosphate and each tablet of dihydrocodeine 30 mg contains dihydrocodeine tartrate 30 mg and lactose. The medications were obtained from Anijah Pharmacy in Calabar, Cross River State Nigeria. The dihydrocodeine solution was prepared by dissolving 30 mg of dihydrocodeine in 10 ml of distilled water to yield a working stock solution of 3 mg/ml of dihydrocodeine. The dihydrocodeine suspension and Archilin™ with codeine syrup were administered to the animals based on their body weight daily and orally with the aid of oropharyngeal tubes.

2.3. Experimental procedure

The LD50 of the drugs was determined following the method as described by Chinedu (2013) before animals were assigned the following doses; Group A served as control and animals in this group were given normal saline. Group B was treated with 1 mg/kg body weight of dihydrocodeine, Group C was treated with 2 mg/kg bodyweight of dihydrocodeine, Group D was treated with 2 ml/kg bodyweight of Archilin™ Codeine syrup and Group E was treated with 4 ml/kg bodyweight of Archilin™ Codeine syrup. Drugs were administered orally by the aid of oropharyngeal tubes for a period of 21 days. At the end of the administration, rats were anesthetized via intraperitoneal injection of 0.5 ml ketamine hydrochloride. Full organ perfusion was carried out by first drawing blood from the right ventricle with a 5 ml syringe followed by the introduction of phosphate-buffered saline (PBS) and formal saline in the left ventricle. A change in color of the heart, liver, and lungs was used as an indicator for full organ perfusion. After organ perfusion, the perfused brains were harvested, rinsed in normal saline, and fixed in 10% neutral buffered formalin. Sections of the prefrontal cortex (PFC) and cerebellum were obtained and processed for the immunohistochemical analysis using the Avidin-Biotin Complex (ABC) method of Brathauer (1999). The polyclonal Glial Fibrillary Acidic Protein (GFAP) antibody was used to demonstrate the integrity of astrocytes. Sections were viewed under an Olympus BX53 microscope (Olympus, Tokyo, Japan).
3. Results

Figure 1a-e are sections of the prefrontal cortex of control and treated rats, respectively. Animals which received 1 mg/kg bodyweight of dihydrocodeine (Figure 1(b)), 2 mg/kg body weight of dihydrocodeine (Figure 1(c)), 2 ml/kg body weight of Archilin™ Codeine syrup (Figure 1(d)) and 4 ml/kg body weight of Archilin™ Codeine syrup (Figure 1(e)) showed strong staining/immunohistochemical reactivity of glial fibrillary acidic protein (GFAP) reactive astrocytes when compared to the control (Figure 1(a)). Figure 2(a-e) shows sections of the cerebellum of control and treated rats, respectively. The sections of the cerebellum of rats treated with 1 mg/kg bodyweight of dihydrocodeine (Figure 2(b)), 2 ml/kg body weight of Archilin™ Codeine syrup (Figure 2(d)), and 4 ml/kg body weight of Archilin™ Codeine syrup (Figure 2(e)) revealed strong staining/immunohistochemical reactivity of glial fibrillary acidic protein (GFAP) reactive astrocytes while sections of the cerebellum treated with 2 mg/kg bodyweight of dihydrocodeine (Figure 2(c)) revealed mild staining/immunohistochemical reactivity of glial fibrillary acidic protein (GFAP) astrocytes when compared to the control (Figure 2(a)).

The following slides show the effects of codeine-induced changes on the prefrontal cortex of Wistar rats in control and treated groups, respectively.

The following slides show the effects of codeine-induced changes on the cerebellum of Wistar rats in control and treated groups, respectively.
4. Discussion
The study revealed that the rats given dihydrocodeine and Archilin™ Codeine syrup showed strong and mild staining/immunohistochemical reactions of glial fibrillary acidic protein (GFAP) and this is a biomarker for GFAP astrogliosis in the prefrontal cortex and cerebellum (Table 1). GFAP astrogliosis occurs when there is trauma to the brain; in the event of this, astrocyte hypertrophy proliferates, and there is an upregulation in the synthesis of intermediate proteins such as GFAP (Pekny & Nilsson, 2005). Glial fibrillary acidic protein (GFAP) is an intermediate protein that is expressed by numerous cell types of the CNS including astrocytes (Jacque et al., 1978) and is believed to help maintain astrocyte mechanical strength (Cullen et al., 2007). GFAP is important in
the repair of the central nervous system (CNS) injury because of the role it plays in the formation of glial scars in a multitude of locations throughout the CNS. This glial scarring is believed to be caused by the up-regulation of GFAP (Paetau et al., 1985; Smith & Eng, 1987; Tuccari et al., 1986).

During brain damage, most of the astroglial cells rapidly become reactive astrocytes and this critical conversion is important for the development of reactive astrogliosis. The reactive GFAP astrogliosis itself is a defensive brain reaction that aims to isolate the damaged brain areas from the undamaged areas of the CNS tissues. The mechanism of reactive astrogliosis involves the reconstruction of the

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blood-brain barrier which was damaged and also facilitates the remodeling of brain neuronal circuits around areas surrounding the lesion (Auld & Robitaille, 2003). By doing this, the reactive GFAP expressing astrocytes help to cushion the damage that occurred in that region. Also, astrocytes that are located immediately around the damaged region become hypertrophied and proliferate to replace the previously existing cytoarchitecture leading to the formation of a glial scar.

The glial scar is formed as a result of secretions that the reactive astrocytes release in the lesioned area. Reactive astrocytes produce chondroitin, keratin, and mucopolysaccharides in response to brain damage. The chondroitin and keratin are meant to inhibit axonal regeneration and prevent nerve processes from entering the damaged area while the mucopolysaccharide cements the damaged area by producing an astrocytic scar (Seifert et al., 2006). The astrocytic

Figure 2. (a): Section of the cerebellum of the control group given normal saline showing astrocytes (AST) with weak staining/immunohistochemical reactivity of GFAP, molecular layer (ML), Purkinje cell layer (PKL), granular layer (GL), (GFAP), (Mag.x400). (b): Section of the cerebellum of the group treated with 1 mg/kg of dihydrocodeine showing strong staining/immunohistochemical reactivity of GFAP reactive astrocytes (RA), molecular layer (ML), Purkinje cell layer (PKL), granular layer (GL), (GFAP), (Mag.x400). (c) Section of the cerebellum of the group treated with 2 mg/kg of dihydrocodeine showing mild staining/immunohistochemical reactivity of GFAP astrocytes (AST), molecular layer (ML), Purkinje cell layer (PKL), granular layer (GL), (GFAP), (Mag.x400). (d): Section of the cerebellum of the group treated with 2 ml/kg of Archilin™ codeine syrup, showing strong staining/immunohistochemical reactivity of GFAP reactive astrocytes (RA), molecular layer (ML), Purkinje cell layer (PKL), granular layer (GL) (GFAP), (Mag.x400). (e): Section of the cerebellum of the group treated with 4 ml/kg of Archilin™ codeine syrup, showing strong staining/immunohistochemical reactivity of GFAP reactive astrocytes (RA), molecular layer (ML), Purkinje cell layer (PKL), granular layer (GL), (GFAP), (Mag.x400).
scar helps to allow the lesioned area to heal properly. The strong staining/immunohistochemical reactivity of GFAP reactive astrocytes as seen in this present study suggests neuronal and glial pathology which could ultimately result in neuronal and glial degeneration. Some authors have reported a similar trend of astrocyte pathology as a biomarker for brain damage (Archibong et al., 2017; Barres, 2008; Ekanem et al., 2008; Fawcett & Richard.A, 1999; Kumar et al., 2007; Peter et al., 1998; Seifert et al., 2006; Sofroniew, 2005; Tani et al., 1996; Udoh et al., 2020).

Other studies done on prescription opioids reported a significant reduction in the volume of the brain, white tract abnormalities, and alterations in functional connectivity (Jaymin et al. 2010). Famitafreshi
et al. (2015) reported that prolonged use of morphine had adverse effects on neurogenesis thus affecting memory, emotional reactivity, and anxiety levels. Ruping Fan et al. (2018) reported that changes in white matter, deformation of axonal tracts, reduction in the size of axonal fascicles, loss of myelin basic protein, and accumulation of amyloid precursor protein beta (β-APP) are associated with prolonged use of oxycodone. It is believed that oxycodone causes neuronal degeneration by activation of the integrated stress response which led to an induction of apoptotic signaling in neurons.

Our study is unique as it targets expression of GFAP reactive astrocytes as a marker for inflammatory reactions in the brain which may lead to neuronal and glial degeneration. Ultimately, our findings are in
Table 1. Scores for immunohistochemical reactivity of GFAP reactive astrocytes in PFC and cerebellum

| Groups               | A | B | C | D | E |
|----------------------|---|---|---|---|---|
| Prefrontal cortex    | 1 | 3 | 3 | 3 | 3 |
| Cerebellum           | 1 | 3 | 2 | 3 | 3 |
| X % of IHC+ labeled cell | Y Intensity of reaction | Final score |
| 0 = 0%               | 0 = No staining/reaction | (X + Y) = 0 |
| 1 = <30%             | 1 = Weak staining/reaction | (X + Y) = 2 |
| 2 = 30-60%           | 2 = Mild staining/reaction | (X x Y) = 4 |
| 3 = >60%             | 3 = Strong staining/reaction | (X x Y) = 9 |

Klein et al. (2001) and Klein et al. (1999) scoring system.

line with the studies done by Jaymin et al. (2010), Famitareshi et al. (2015), and (Ruping Fan et al., 2018) which have all reported evidence of neuronal degeneration from prolonged use of prescription opioids.

5. Conclusion

From this study, it can be seen that prolonged use of codeine medication triggers the expression of GFAP reactive astrocytes in the prefrontal cortex and cerebellum of the brain. Increased GFAP expressing reactive astrocytes has been documented to be a biomarker for brain injury/damage. Therefore, damage to the prefrontal cortex and cerebellum from continued use of codeine medication could manifest in the form of impaired cognitive control, altered physiology, altered behavioral functions, and motor skill impairment. These manifestations are peculiar to users of codeine medication (Albein-Urios et al., 2012; Areal et al., 2017; Charlet et al., 2014; Melissa, 2013; Otis & Mueller, 2017; Stimmel & Kreek, 2000; Viola et al., 2013). Licit or refined codeine present in prescription syrups or tablets poses the same risk to users in the same vein as illicit codeine if abused. For these reasons, codeine medications should be tightly regulated and taken strictly according to prescription and not for recreational purposes.

6. Recommendation

Molecular studies to ascertain how codeine affects the expression of glial fibrillary acidic protein in astrocytes can be explored.

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Conflicts of interest

There is no conflict of interest.

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