HISTO-MORPHOLOGICAL EVALUATIONS OF THE CEREBELLUM IN DIHYDROCODEINE-TREATED RAT MODELS

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Abstract

The dihydrocodeine and dihydrocodeine-containing drugs have been used by opioid addicts as a substitute to heroin as it has similar metabolic pathways as codeine. In this study we assessed the histopathological and deleterious effects of DHC on the cerebellum of rats using different staining methods. Thirty-six Adult male Wistar weighing (110 ± 10 g) were randomly divided into three (3) groups (A-C, of 12 rats each). Group A served as the control (treated with 5ml/kg normal saline), group B and C rats received 15 mg/kg and 25mg/kg dihydrocodeine receptively via oropharyngeal cannula for 14days. The rats were sacrificed, and the cerebellum was processed. Each section was stained with Haematoxylin and eosin (H&E), Cresyl fast violet, and Luxol fast blue for basic and advanced histological demonstrations. Results showed a significant ($p<0.05$) decrease in body weight of group B (117.42±6.50) and group C (146.17±12.62) when compared to control group (117.42±6.50). Sections from the DHC-induced group had extensive areas of neuronal loss and showed evidence of several degeneration of Purkinje cell, fusiform-shaped nuclei of neuronal cell bodies that were centrally positioned. The cytoplasm seemed eosinophilic. Varying degree of cellular modifications including pyknosis of the nuclei were observed when compared with negative control group (group A). These were evident by a significant ($p<0.05$) decrease in molecular layer thickness, number of purkinje and granular cells of group B when compared to control group. Dihydrocodeine has a deleterious effect on the cerebellar architecture of Wistar rats

Dihydrocodeine, Cerebellum, morphometry, Cresyl fast violet, and Luxol fast blue

INTRODUCTION

The opioid, dihydrocodeine (DHC), is an analgesic and an antitussive drug. Over the years, dihydrocodeine and dihydrocodeine-containing drugs were used by opioid addicts as a substitute to heroin as it has similar metabolic pathways as codeine (Fromm *et al.* 1995; Hufschmid *et al.* 1995). These include *O*-demethylation to dihydromorphine, formation of the corresponding 6- and/ or 3-*O*-glucuronides dihydrocodeine-6-*O*-, dihydromor- phine-3-*O*- and dihydromorphine-6-*O*-glucuronide, and *N* demethylation to nordihydrocodeine (fig. 1; Helmut *et al.*, 2002).

Although both DHC and codeine possesses antitussive and analgesic properties, it must be said however, that DHC differs in its chemical structure by the saturation of the double bond between C7 and C8 (fig. 1). (Derry *et al;* 2013; Peterson *et al.,* 2015).

The request for codeine remains high and has risen by approximately 27% over the last decade (INCB, 2012). It is gaining increasing importance as step 2 of the "analgesic ladder" originally proposed by the World Health Organization (1986) for the treatment of cancer pain. It has been reported that DHC began to replace codeine when it became available as a slow-release preparation (Wotherspoon *et al.,* 1991; Ilmar *et al.,* 1997; Peter *et al.,* 2019).

The abuse of DHC is increasing globally and this has been linked to the euphoria it presents to users especially in Africa (Abudu, 2008; Ekpang & Abuo, 2015; Mohamadi *et al.,* 2018; Kiunguyu, 2018; Victor *et al.,* 2020). Although common side effects of DHC includes drowsiness, nausea, immune system disorder, endocrine disorder, nervous system disorder, eye disorder, ear and labrynth disorder, cardiac disorder, renal and urinary disorder, DHC has been reported to affect neural parts like the cerebellum, prefrontal cortex, and cerebrum (Peter *et al.,* 2019, Victor *et al.,* 2020; Theresa *et al.,*2020). Melissa (2013) linked prefrontal cortex and cerebellum (emotional and social behavior, motor coordination center) maladies to opiod consumption. Prolonged use of opioids has also been associated to physiological and behavioral malfunctions (Stimmel & Kreek, 2000). While a healthy thyroid function is necessary for normal development of the brain and life-long cognitive function, abnormal changes in cerebellar circuitry could be detrimental to the processing of emotional responses (Reiman *et al.,* 2004; Konarski *et al.,* 2005; Ebtesam *et al.,* 2017).

Due to few available literatures on the extent of the histopathological consequence of misuse of opioid analgesic on the cerebellum (Peterson, 2015), this study was designed to assess the histopathological and deleterious effects of DHC on the cerebellum of rats using different staining methods (Cresyl violet and Luxol Fast Blue) and stereological principles and morphometry. This study could provide a quantitative insight to changes in the cerebellum, provide a numerical, reproducible scale of quantitative features like neurons myelin/myelinated axons and Nissl bodies and enhance sensitivity in detecting cerebellar histopathology post exposure to DHC (True, 1996).

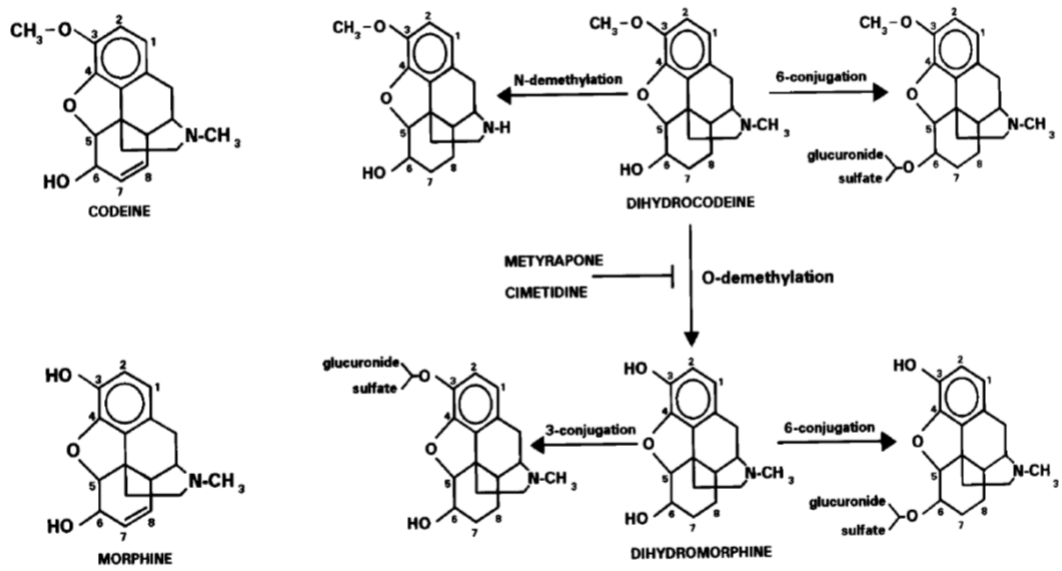


Fig. 1. Structure formulas of codeine, morphine and dihydrocodeine and the metabolic fate of dihydrocodeine. Metyrapone and cimetidine inhibit O-demethylation of dihydrocodeine by blocking the activity of cytochrome P-450.

(Helmut *et al.*, 2002)

Materials and Methods

Thirty-six Adult male Wistar weighing 110 ± 10 g/body weights were randomly divided into three (3) groups (A-C, of 12 rats each). Group A served as the control and the rats were treated with 5 ml/kg body weight of distilled water, group B received 15 mg/kg dihydrocodeine (Sigma-Tau Pharmaceuticals, Inc.) and group C were given 25mg/kg dihydrocodeine via oropharyngeal cannula. The duration of the study was for 14 days. The animals had access to rat chaw and water *ad libitum*. All procedures were carried out in accordance with the standard international guidelines on the use of animals for research (National Research Council, 2011). Approval for the study was obtained from the animal research ethical committee of Bowen University.

Animal sacrifice and Sample Collection

The rats were anesthetized intraperitoneally (Ketamine- 100mg/kg) and perfused transcardially with 0.1M Phosphate Buffer (pH 7.4) and 10% formalin in 0.1 M Phosphate Buffer (pH 7.4). The cerebellum was immediately harvested and fixated in 10% buffered formalin.

Histopathological and Morphometric Evaluations

The tissues were then dehydrated, embedded in paraffin and coronal sections (5µm) were obtained with rotator microtome. Each section was stained with Haematoxylin and eosin (H&E), Cresyl fast violet, and Luxol fast blue for basic and advanced histological demonstrations.

Morphometry

This was done as described by Bianco *et al.* (2015) using Image J software (version 1.46) Molecular and granular cell layers thickness were manually measured at 50 measurements per layer on 11 random

selected fields at 100x magnification. HE-stained sections were used for granule cell neuron density. Ten randomly selected fields were acquired at 400x magnification. Nuclei count was automatic using the "Analyze particles" instruction after thresholding. Density was expressed as granule cell neurons/10000 μm2. Linear density of the Purkinje neurons was quantified on ten randomly selected fields at 200x magnification for each anatomic-functional region. Purkinje neurons were counted (neurons/millimeters).

STATISTICAL ANALYSIS

The statistical analysis applied in this study express the values in form of their mean and standard deviation. The difference of the mean between each of the groups was analyzed using one-way ANOVA in comparison with the control using Ducan method at a 0.05 significance level.

RESULTS

Body Weight

As shown in Table 1, there was a difference ($p<0.05$) increase in weight of group B (109.33±3.77) and group C (125.16±6.58) rats post-DHC treatment when compared to the control rats (111.33±25.39). After the experiment, we observed a significant ($p<0.05$) decrease in body weight of group B (117.42±6.50) when compared to control group (117.42±6.50). However, there was no significant difference in body weight of group C (146.17±12.62) when compared to control group (117.42±6.50).

Table 1: Initial and final Body weight of experimental models

Groups	Initial Body Weight (g)	P VALUE	Final Body Weight (g)	P VALUE
Group A (control)	111.33±25.39	-	144.75±27.44	-
Group B (15mg/kg)	109.33±3.77**	0.788	117.42±6.50**	0.003
Group C (25mg/kg)	125.16±6.58**	0.063	146.17±12.62**	0.873

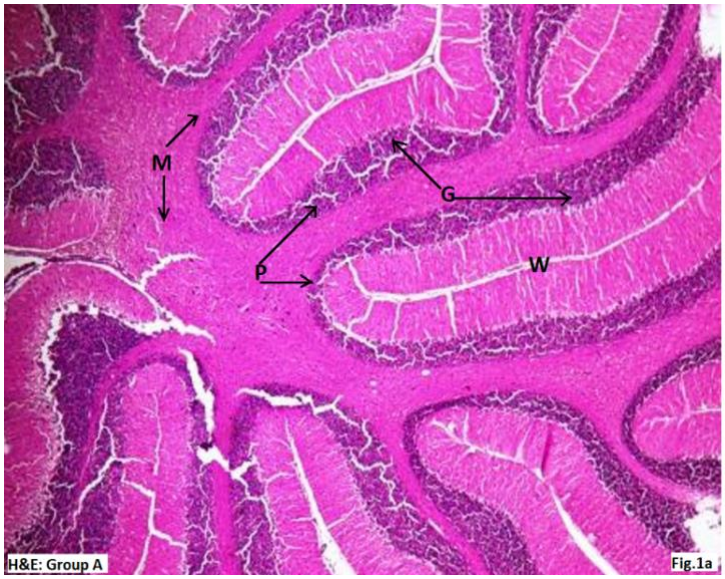
**represent significant increases or decreases at $p < 0.05$ when compared to control group (Group A). Values are means ± SD. n = 12 in each group

Histological Assessment

Three layers were observed on examination of the sections of the control rats (group A): an outer layer lightly stained with molecular markers, a middle Purkinje layer, and an inner granular layer. Results from group A rat showed normal Purkinje cells, and the histological cerebellar folia characteristics greatly contrast from the DHC group. They exhibited deeply stained and distributed basket cells in the molecular deposit of the cerebellum. The huge cell bodies of Purkinje cells had very pale nuclei and protruding nucleoli characterized by diverging dendrites, while

several small granular cells were compactly crammed in the inner granular layer. They were characterized by rounded dark nuclei and petite cytoplasm.

However, on examination, sections from the DHC-induced group (Group B and C) had extensive areas of neuronal loss and showed évidences of several degeneration of Purkinje cell, fusiform-shaped nuclei of neuronal cell bodies that were centrally positioned. The cytoplasm seemed eosinophilic. Varying degree of cellular modifications including pyknosis of the nuclei were observed when compared with negative control group (group A). In addition, sections from DHC group had vacuolation in their molecular layer. Aside from severed areas, focal regions of Purkinje cell loss were evident coupled with shrunken and irregularly delineated cells with extremely stained cytoplasm, and slight pyknotic nuclei.





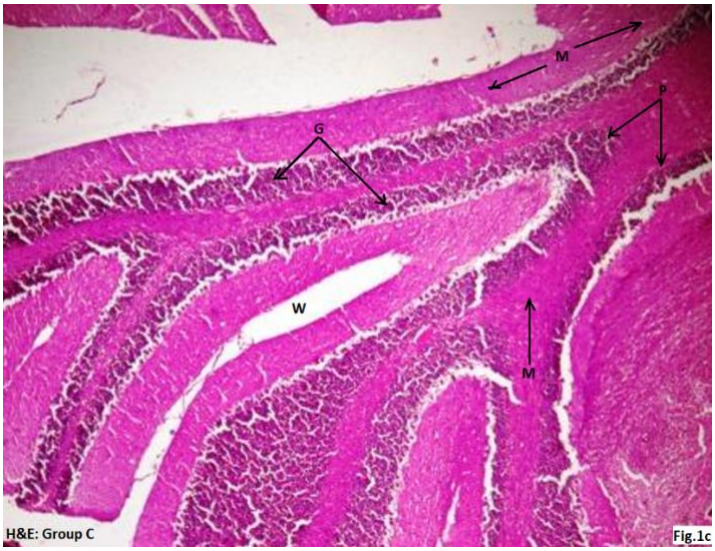
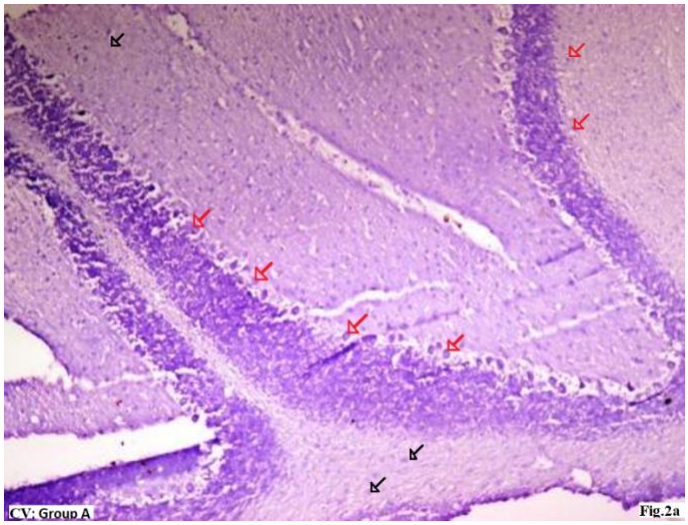
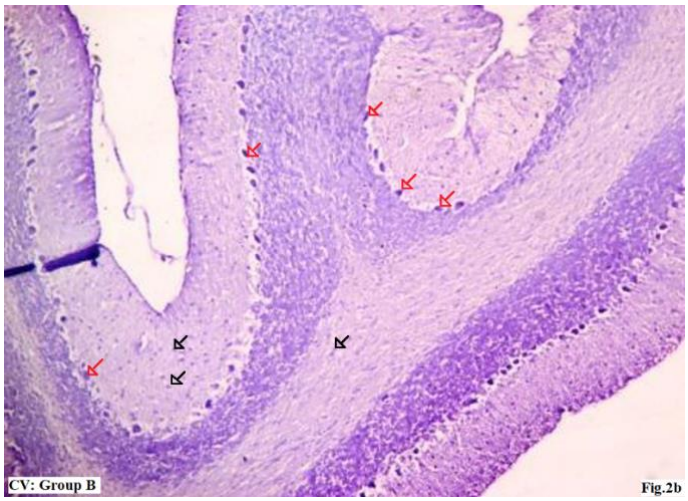


Figure 1a-c: Cross-section of cerebellum of group A (5ml/kg water), group B (15mg/kg DHC) and group C (25mg/kg DHC). M: Molecular layer; P: Purkinje layer; G: granular layer and W: white matter. H&E (x40).





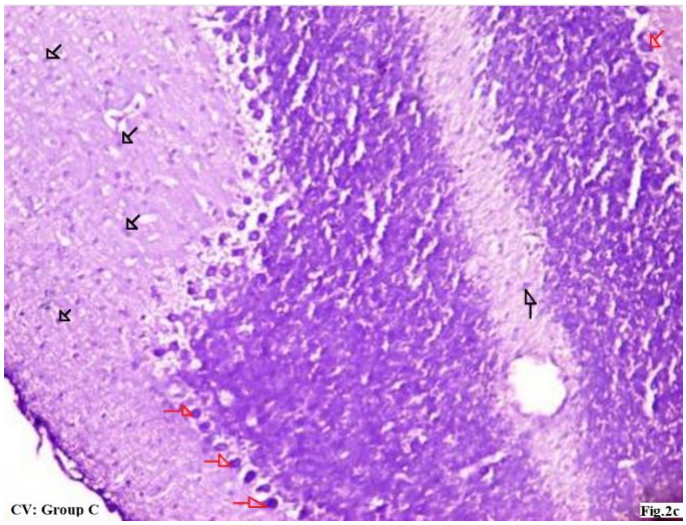
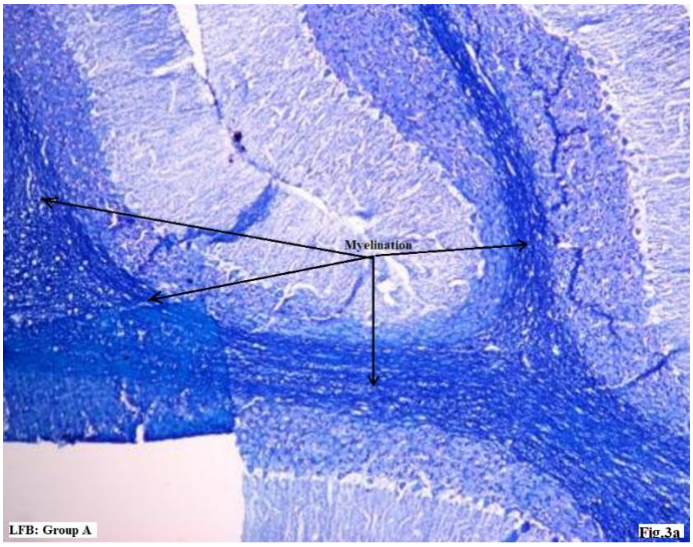
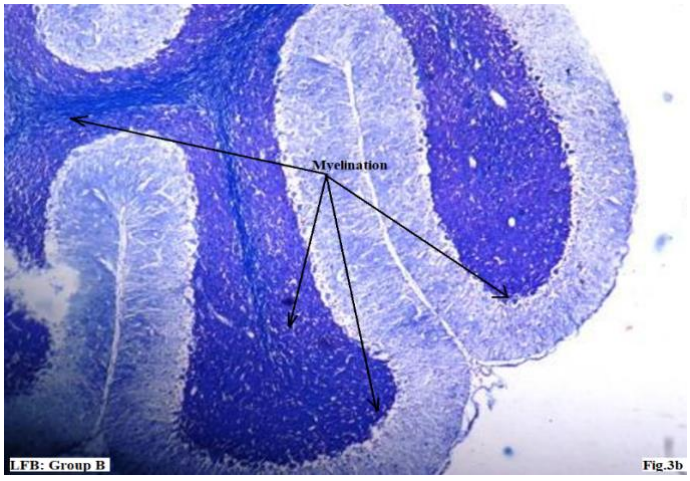


Figure 2a-c: Cross-section of cerebellum of group A (5ml/kg water), group B (15mg/kg DHC) and group C (25mg/kg DHC). Cresyl violet depicted cellular degenerations in terms of lightly stained condensed nissl substance, loss of nucleus and reduced number of purkinge cells (Red Arrow). Additionally, cells in the molecular and granular layer were reduced in number with small lighly stained cell bodies presenting chromatolysis. (black arrow) (x40).





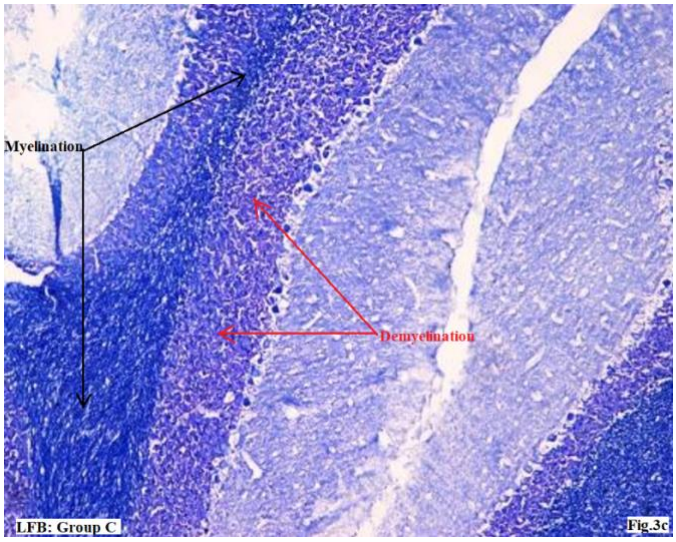


Figure 3a-c: Cross-section of cerebellum of group A (5ml/kg water), group B (15mg/kg DHC) and group C (25mg/kg DHC). Luxol Fast Blue depicted myelinations (Black Arrow) in the treatment groups except for some few demyelinations in group C (Red Arrow) (x40).

Morphometry

There was a significant ($p<0.05$) decrease molecular layer thickness, number of purkinje and granular cells of group B (221.45±62.3; 42.08±1.78 and 54.11±10.18 respectively) and group C (242.18±43.31; 33.92±0.90 and 57.92±0.90 respectively) when compared to control group (262.34±43.5; 49.92±1.08 and 86.01±2.03 respectively) (Table 2).

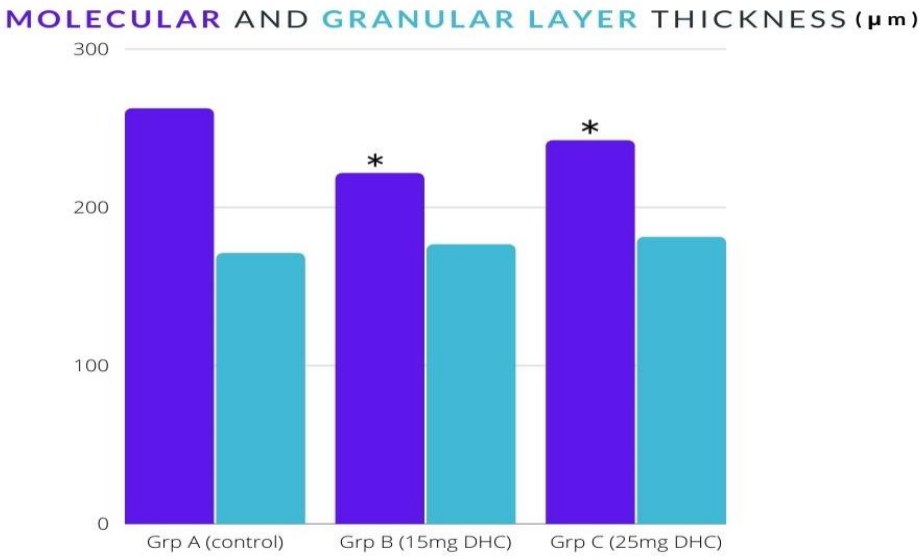


Figure 4: Result Showing the Molecular layer thickness**(**µm**)** and Granular layer thickness **(**µm**).** *represent significant increases or decreases in molecular and granular layers at $p < 0.05$ when compared to control (Group A). Values are means ± SD. n = 12 in each group.

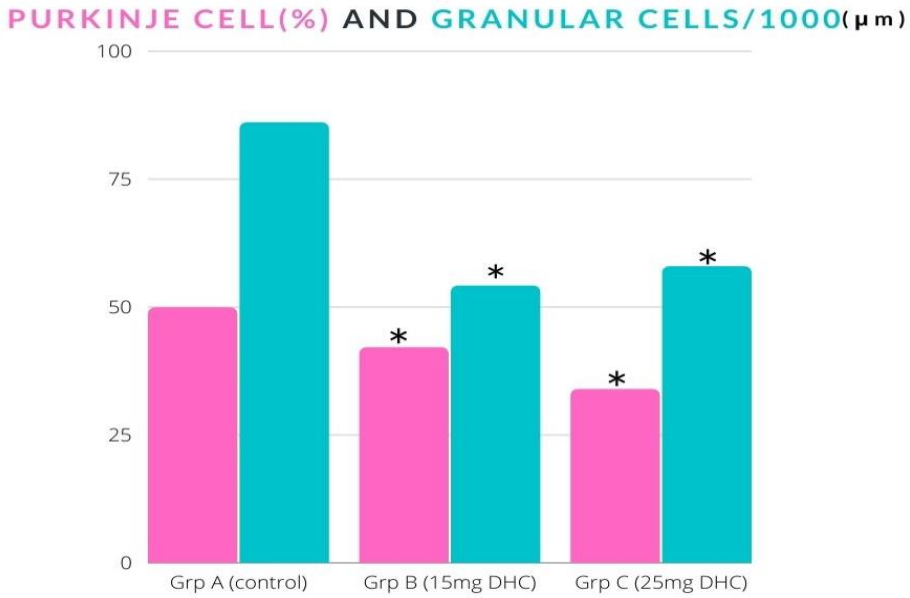


Figure 5: Result showing the number of Purkinje and granular cells. *represent significant increases or decreases in molecular and granular layers at $p < 0.05$ when compared to control (Group A). Values are means ± SD. n = 12 in each group.

DISCUSSION

Previously, the cerebellum has been classified to be involved only in motor function (Holmes, 1939) and motor learning (Bernard and Seidler, 2013). However, there is a growing literature suggesting that the cerebellum also contributes to non-motor behavior (Chen *et al.*, 2014; Schmahmann, 2018). Rightly so, what else can be said about the largest structure in the human brain in terms of sheer number of neurons (Buckner, 2011).

The cerebellum has an extremely high input-to-output axon ratio (Nitsche *et al.*, 2008; Buckner, 2011; Oldrati and Schutter, 2018). Inhibition and shifting ability have also been linked to it (Neau *et al.*, 2000; Ravizza and Ivry, 2019). Its posterior lobules have been implicated in this debate (Stoodley and Schmahmann, 2010; Buckner *et al.*, 2013; Maldonado *et al.*, 2019). However, it must be said that the extent of its involvement has not fully been elucidate even in human subjects. And one thing is sure, disruption of its circuitry by any organizational damage could deter the processing of emotional responses and results in momentous personality changes.

Programmed cell death in the cerebellum has been known to result from cytotoxic effect of various neurotoxins. Exposure to opioid hastened this likelihood. For instance, chronic morphine administration in rats has been linked to significant changes in the principal proteins involved in the apoptosis signalling (Peter *et al.*, 2019).

Employing morphometry in the evaluation of degenerative disorders is very promising and could afford an exciting involvement to cases with more subtle histological changes.

In this study, we evaluated the histopathological effect of DHC on the cerebellum using morphometry that proved

a useful ancillary tool in measuring cerebellar cellular populations.

There was an indication that DHC could cause cortico-cerebellar degeneration (loss of Purkinje neurons accompanied by a consequential reduction of granule cell neurons (Summers *et al.,* 1995).
The control groups of rats had normal Purkinje cells and histological cerebellar folia in addition to exhibiting deeply stained and distributed basket cells in the molecular deposit of the cerebellum with huge cell bodies of Purkinje cells with pale nuclei and protruding nucleoli characterized by diverging dendrites. Granular cells were compactly crammed in the inner granular layer and characterized by rounded dark nuclei and petite cytoplasm.

However, same cannot be said about DHC-induced group that had extensive areas of neuronal loss and showed evidence of several degeneration of Purkinje cell, fusiform-shaped and pyknotic nuclei of neuronal cell bodies with cytoplasm that seemed eosinophilic. These histopathological changes are in accordance with several other study (Summers *et al.,* 1995; Cartabuke *et al.*, 2006; Vandevelde *et al.,* 2012;).

The cerebellar pathology observed in this study might have been as a result of dysregulation of plasma membrane calcium ATPase (PMCA2). PMCA2 is a calcium pump expressed primarily in neurons and Purkinje cells (Stahl *et al.,* 1992; Stauffer *et al.,* 1997; Tachibana *et al.,* 2004; Michael *et al.,* 2007). This is in line with reports by Kozel *et al.* (1998) of an increase in the density of Purkinje neurons and a decrease in the density of granule cells, thickness of the molecular layer.
Cerebellar degeneration is commonly linked with reactive gliosis (Summers *et al.,* 1995; Vandevelde *et al.,* 2012; Bianco *et al.,* 2015; Peter *et al.,* 2019; Victor *et al.,* 2020; Theresa *et al.,*2020). It has been stated that that Purkinje cell death is "patterned" and topographically complex (Sarna and Hawkes, 2003).
Since the cerebellum shares physiological connections with all major functional divisions of the CNS.3, cerebellar pathology observed in this study might make clear in several ways. According to the theory by Allen and Courchesne (1998), it might lead to significant diminishing in a neurobehavioral function. While it seems strange that damage to the cerebellum does not affect activities known to activate it, we align with the proposed theory of cerebellar function. We are not suggesting that damage to the cerebellum is at the forefront. However, the likely contribution of cerebellar abnormalities to disorders characterized by non-motor symptoms can no longer be dismissed especially in DHC treated models. This theory might have given an insight and possible explanation for the behavioral and psychological changes associated with codeine abuse.
We conclude from the results obtained therein that Dihydrocodeine has a deleterious effect on the cerebellar architecture of Wistar rats.

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