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Microscopic features of the rat adrenal gland associated with chronic codeine phosphate administration

Ibsen Henric Ongidi, Fadhila Yusuf Abdulsalaam, Thomas Mombo Amuti, Wycliffe O. Kaisha, Kirsteen O. Awori, Anne Naipanoi Pulei
Department of Human Anatomy, School of Medicine, University of Nairobi, Nairobi, Kenya

Abstract: Codeine is an opioid analgesic and antitussive that has been widely abused. Some adverse effects noted with its abuse include adrenocortical insufficiency and activation of the hypothalamic-pituitary-adrenal axis. The structural basis for these dysfunctions is not clearly understood. Twenty-five adult male rats were used for the study. They were divided into intervention and control groups that were administered 40 mg/kg of codeine phosphate and normal saline respectively by gavage daily for 50 days. Subsequently, both groups were given normal saline for a further fourteen days to note recovery changes. At day 0, 50 and 64, rats were randomly selected from both groups, euthanized and adrenal glands harvested for histological processing and analysis. At day 50 of codeine administration, the adrenal glands demonstrated an increase in zona fasciculata thickness but a decrease in zona reticularis thickness. Lower values were noted in the volume density of zona reticularis and cells count of the medulla in the experimental compared to the control groups (P-value<0.05). The experimental group also showed an increase in vascularization and connective tissue in the glands. After 14 days of recovery, most of the changes observed in experimental animals were reversed and the adrenal glands in both groups had similar features. A decrease in cell count of the adrenal medulla was however observed (P-value<0.05). In conclusion administration of codeine phosphate causes discernible changes in the microscopic structure of the adrenal gland, most of which appear to be reversed after two weeks recovery period.

Key words: Codeine, Adrenal glands, Opioid, Opioid-related disorders

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Introduction

Codeine is an opioid that is used in antitussive and analgesic drug formulations as codeine phosphate. It is used as a prescription drug in many countries but remains largely accessible to the public. There are increasing reports of its abuse worldwide [1, 2]. The use of codeine remains very high especially for cough syrups and combination therapy with other analgesic.

Chronic use of codeine has been linked to endocrine disorders involving the adrenal gland including adrenocortical insufficiency [3] and opioid-induced adrenal androgen deficiency [4]. High doses codeine causes stimulation of hypothalamo-pituitary-adrenocortical (HPA) axis function as evidenced by an increase in adrenocorticotropic hormone and corticosteroid production [5]. Codeine has also been associated with decreased secretion of adrenal epinephrineand norepinephrine [6]. The effects of codeine on the histological structure of the adrenal gland, however, remains largely un-

Corresponding author:
Ibsen Henric Ongidi
Department of Human Anatomy, School of Medicine, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya
E-mail: ongidi409@gmail.com

explored.

This study describes the light microscopic features of the adrenal gland in rats administered with codeine phosphate and the changes during recovery of the gland after fourteen days. The findings of this study provide a structural basis in understanding endocrine disruptions in the adrenal gland with codeine overuse and recovery when its use is ceased.

Materials and Methods

The study followed a quasi-experimental design. Twenty-five laboratory rats of the *Rattus norvegicus* species were used in the study. The general physiology and histological structure of organs from rat models are comparable to that of human beings [7]. Adult male rats of three to six months of age were included while those with visible pathology such as obesity, gross skeletal and gonadal malformations were excluded. Female rats undergo estrous cycles that may influence the activity of endocrine glands. The Codeine phosphate used in treatment of the experimental group was manufactured by TEVA Pharmaceuticals UK limited, and bought from Malibu Pharmacy in Nairobi, Kenya. The study was conducted at the Department of Biochemistry animal house and tissue harvesting and processing of specimen done at the Department of Human Anatomy, University of Nairobi. Approval was obtained from the Biosafety, Animal Use and Ethics Committee of the Faculty of Veterinary Medicine of the University of Nairobi and the study was conducted according to the guidelines provided by the committee. The laboratory rats obtained were housed in standard laboratory rat cages measuring 109 cm length by 69 cm width by 77.5 cm height. Each cage housed 5 animals. The cages were floored with wood shavings which were replaced every two days and the cages cleaned. The animals were weighed then kept in the study area for one week prior to the start of the study for acclimatization. The animals were placed under a 12 hours light/dark diurnal cycle. They were also provided with standard rat pellets and water *ad libitum*.

Treatment of animals

The intervention group were administered with 40 mg/kg of codeine phosphate solution orally by gavage every day for 50 days. Since the average duration of chronic codeine syrup abuse is 5 years [8] and the translation of rat days to human years is 10 rat days for every human year [9], an average administration period of 50 days was used to study the long term effects of the drug. The control group was administered with normal saline daily by gavage. After day 50, both groups were administered with normal saline by gavage for fourteen days. At experimental day 0, 50 and 64, rats were randomly selected from intervention and control group, euthanized, perfused with normal saline and adrenal glands harvested.

Tissue harvesting and processing

The animals were weighed then euthanized by placing them in lidded containers having cotton wool soaked in 1% halothane. Euthanasia was confirmed by the absence of heartbeat and loss of blink reflex. A midline incision was made on the chest and abdomen and the skin reflected. The sternum and the ribs were removed to allow access to the heart for perfusion. The animals were then adequately perfused through the left ventricle starting with normal saline to flush out blood then followed by 10% formal saline to fix the body organs including the adrenal glands.

The adrenal glands were harvested through the abdominal incision. To do this, the abdominal viscera including stomach, intestines and liver were reflected to expose the retroperitoneum. The kidneys together with the adrenal gland were harvested. The kidneys were used in orientation of the adrenal grand during blocking and sectioning. The harvested gland and portion of the kidney were dipped in 10% formalin in specimen bottles for at least 24 hours before routine processing and staining for light microscopy. From twenty-five rats in the study, fifty adrenal glands were harvested. Twenty-five blocks were made, which contained two adrenal glands per block oriented transversely from each animal. These blocks were sectioned to obtain ten standard sections. These sections were all stained with Hematoxylin and Eosin (H&E) according to standard protocol [10].

Histomorphometric analysis

Photomicrographs were taken using a Zeiss digital photomicroscope (Carl Zeiss AG, Oborkochen, Germany) for an examination of light microscopic features and stereological analysis. The ten serial sections obtained at uniform distance from the central part of the gland were analyzed for morphometry of the cortex and medulla in the same sections. Measures of cell counts from all four zones and volume densities of the cortical zones from five random fields on the slides were done and mean values calculated. These values across the serial sections were then summed up and the final values recorded and analyzed to ensure uniform representa-

tions of different parts of the gland. The photomicrographs were analyzed using Fiji image J software for stereological parameters. Cell counts for the three zones of the adrenal cortex and the adrenal medulla were done using grid squares superimposed on the photomicrographs at ×400 magnification [11]. Volume densities of the zones in the adrenal cortex were obtained at ×100 magnification using Cavalieri's point counting method.

Statistical analysis and presentation

Statistical analysis of quantitative results obtained was done using IBM SPSS Statistics for Windows ver. 21.0 (IBM Corp., Armonk, NY, USA). Data were expressed as the mean±standard error of mean. The Shapiro-Wilk test and histograms were used to check for normality of the obtained data. Mann-Whitney U-test was used for analysis to compare differences in median counts between the experimental

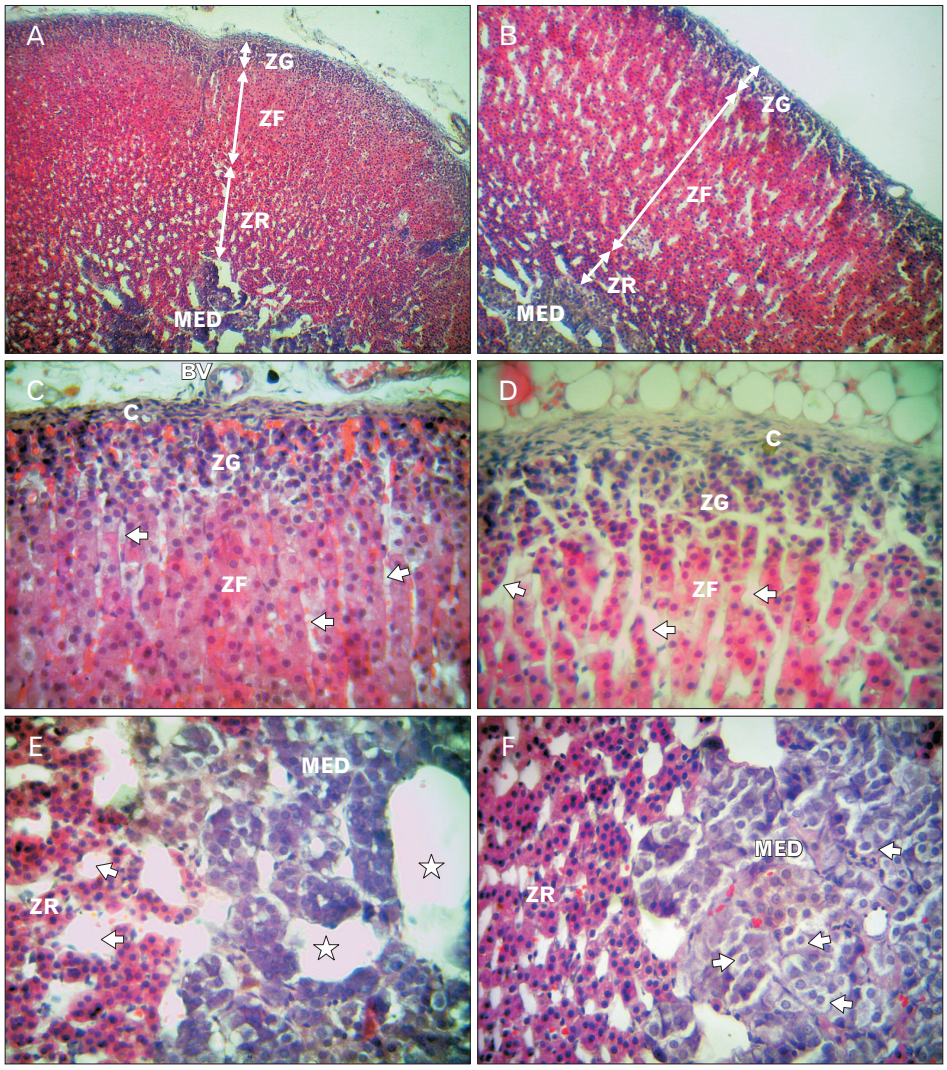

Fig. 1. Light microscopic features of the adrenal gland in the 50 days control and experimental groups. (A) Adrenal cortex and medulla in the 50 days control group. Notice the similar proportions of ZF and ZR in the cortex (H&E, ×100). (B) Adrenal cortex and medulla in the 50 days experimental group. Notice the increase in zona fasciculata thickness compared to the other cortical zones (H&E, ×100). (C) The capsule, ZG and zona fasciculata in the 50 days control group at higher magnification. Notice that the cells are densely arranged, the arrows show vascular sinusoids (H&E, ×400). (D) The capsule, zona glomerulosa and zona fasciculata in the 50 days experimental group at higher magnification. Notice the increased thickness of the capsule and the increased vascular spaces (arrowheads) compared to the control (H&E, ×400). (E) The zona reticularis and medulla in the 50 days control group at higher magnification. Notice that cells of zona reticularis are arranged in anastomosing cords (asterisk), the arrows show vascular sinusoids (H&E, ×400). (F) The zona reticularis and medulla in the 50 days experimental group at higher magnification. Notice the vesicular nuclei (arrows) in the medullary cells and smaller vascular spaces compared to the control (H&E, ×400). Asterisk, veins; BV, blood vessel; C, capsule; MED, medulla; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis.

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and control animals. Differences were considered significant at $P \leq 0.05$ (95% confidence interval). Data were presented in tables and photomicrographs.

Results

The adrenal glands obtained from the rats at the end of the 50-day experiment were larger compared to the baseline. It was noted at ×100 magnification that the proportion of zona fasciculata (ZF) was similar to that of zona reticularis (ZR) in the control group (Fig. 1A). The proportion of ZF in the cortex was much larger than that of ZR in the experimental group (Fig. 1B). The ZR thickness was also greatly reduced in the experimental group compared to the control group. The overall size of the adrenal cortex was however reduced in the experimental group compared to the control (Fig. 1A, B).

At ×400 magnification, it was discernible that the adrenal gland in the control group had a thin connective capsule (Fig. 1C). The experimental group had thick gland capsule (Fig. 1D). The adrenal glands in the experimental group had thicker capsules with larger blood vessels compared to the control (Fig. 1C, D). Zona glomerulosa (ZG) cells in the control group were compact with few vascular spaces (Fig. 1C). The ZG in the experimental group was loosely arranged with larger spaces between the cells (Fig. 1D). The ZF in the control group had a compact arrangement of cells while those in the experimental group had large spaces between the parallel cords of cells (Fig. 1C, D). The ZR in the control group had cells arranged in anastomosing cords with large capillary spaces (Fig. 1E). The ZR in the experimental group had cells that were arranged in clumps with small vascular spaces in-between (Fig. 1F). The adrenal medulla in both the control and experimental groups were relatively uniformly staining (Fig. 1E, F). Cells of the medulla in the control group had a compact arrangement with large venous spaces separating the clumps of cells (Fig. 1E). The cells in the experimental group were compact and had a notable perinuclear space (Fig. 1F). The vascularity of the medulla comparable between the experimental and control groups.

Analysis of the cell counts of different regions of the adrenal gland demonstrated that cell counts of the cortical zones were not significantly different between the two animal groups after fifty days of codeine administration (*P*-value>0.05). The ZG revealed a reduction in cell count in the experimental group compared to the control (*P*-value=0.222) while the ZF had comparable cell count between the two groups (*P*-value=0.69) (Table 1). Both the ZG and ZF showed increased thickness in the experimental compared to the control groups (Table 2) but the differences were not significant (*P*-value=0.421 and 0.095 respectively). The ZR demonstrated a reduced cell count (*P*-value=0.151) and a significantly reduced thickness (*P*-value=0.008) in the experimental paralleled to the control group (Tables 1 and 2). The adrenal medulla revealed a significantly reduced cell count in the experimental compared to the control group after fifty days of codeine administration (*P*-value=0.016) (Table 1).

Examination of the slides of the adrenal gland from animals which underwent a 14-day recovery period at the end of the 50-day experiment revealed that the experimental and control groups had similar features and size. At ×100 magnification, it was noted that the thickness of the three adrenal cortex zones was similar in the control and experimental groups (Fig. 2A, B). The ZR in the experimental group regained its normal thickness and the ZF constituted a similar proportion of the cortex as ZR (Fig. 2B). Examination of the slides at X400 magnification showed that the gland capsule was of similar thickness between the experimental and control groups (Fig. 2C, D). There was a compact arrangement of

Table 1. Comparison of cell counts in the adrenocortical zones and adrenal medulla between experimental and control groups at day 50 (n=5)

Adrenal gland zone	50 days control group	50 days experimental group	*P*-value
Zona glomerulosa cell count (per μm^3)	17.00	15.50	0.222
Zona fasciculata cell count (per μm^2)	9.00	9.00	0.690
Zona reticularis cell count (per μm^2)	14.50	12.00	0.151
Medulla cell count (per μm^2)	10.50	7.50	0.016*

*Significant *P*-value.

Table 2. Comparison of volume densities of the adrenocortical zones between control and experimental groups at day 50 (n=5)

Adrenal gland zone	50 days control group	50 days experimental group	*P*-value
Zona glomerulosa volume density	0.063	0.070	0.421
Zona fasciculata volume density	0.532	0.679	0.095
Zona reticularis volume density	0.404	0.237	0.008*

*Significant *P*-value.

cells of the ZG in both the control and experimental groups. The ZF in both experimental and control groups had a compact arrangement of cell cords (Fig. 2C, D). The ZF in the experimental group, demonstrated few vascular spaces. The ZR appeared to be more compact in the experimental than the control group (Fig. 2E, F). In the adrenal medulla, the cells were slightly more compact in the experimental group compared to the control group.

Analysis of cell counts of the adrenocortical zones showed that they were similar between the control and experimental groups after the 14-day recovery period. The differences observed in the cell counts were not significant for any of the three zones (*P*-value>0.05). There was a however significant increase in the cell count of the medulla in the experimental compared to the control group (*P*-value=0.008) (Table 3). The differences in the volume densities of the three adreno-

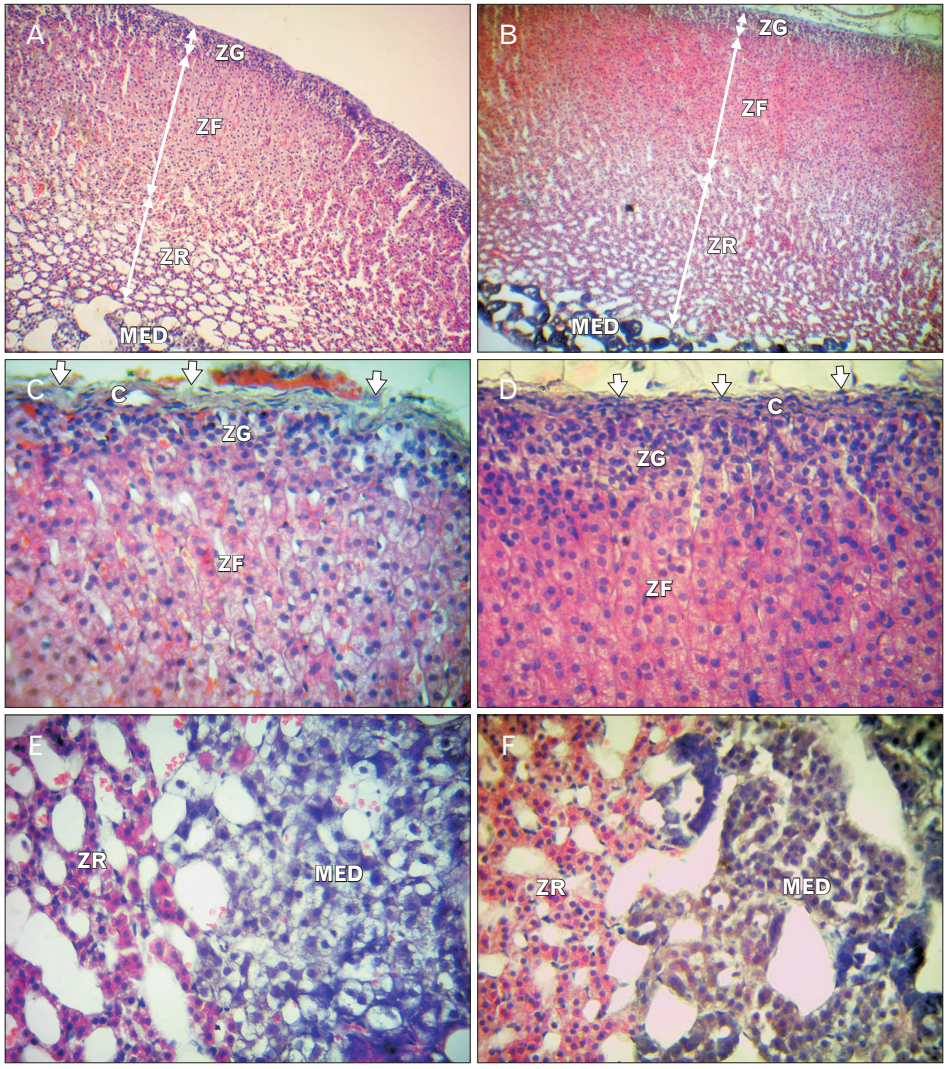

Fig. 2. Light microscopic features of the adrenal gland in the 14-day recovery control and experimental groups. (A) The drenal cortex and medulla in the 14-day recovery control group (H&E, ×100). (B) The adrenal cortex and medulla in the 14-day recovery experimental group. Notice relative thickness of the three cortical zones is similar to the control group (H&E, ×100). (C) The capsule, zona glomerulosa and zona fasciculata in the 14-day recovery control group at higher magnification. Arrows show the capsule (H&E, ×400). (D) The capsule, zona glomerulosa and zona fasciculata in the 14-day recovery experimental group at higher magnification. The thickness of the capsule is similar to the control. Arrows show the capsule (H&E, ×400). (E) The zona reticularis and medulla in the 14-day recovery control group at higher magnification (H&E, ×400). (F) The zona reticularis and medulla in the 14-day recovery experimental group at higher magnification. The features are comparable to the control group (H&E, ×400). C, capsule; MED, medulla; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis.

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Table 3. Comparison of cell counts in the adrenocortical zones and adrenal medulla between experimental and control groups after 14-day recovery period (n=5)

Adrenal gland zone	14-day recovery control	14-day recovery experimental	*P*-value
Zona glomerulosa cell count (per μm^2)	13.00	13.50	1.000
Zona fasciculata cell count (per μm^2)	7.50	7.60	0.690
Zona reticularis cell count (per μm^2)	10.00	9.50	1.000
Medulla cell count (per μm^2)	5.00	8.00	0.008*

*Significant *P*-value.

Table 4. Comparison of volume densities of the adrenocortical zones between control and experimental groups after 14-day recovery period (n=5)

Adrenal gland zone	14-day recovery control	14-day recovery experimental	*P*-value
Zona glomerulosa volume density	0.077	0.072	0.548
Zona fasciculata volume density	0.585	0.539	0.056
Zona reticularis volume density	0.337	0.396	0.056

cortical zones between the control and experimental groups were not significant (*P*-value>0.05) (Table 4).

Discussion

The present study demonstrates an increase in the thickness of ZF and a decrease in thickness of ZR in the adrenal glands of animals which received codeine phosphate for fifty days. This is consistent with findings by Abdelaleem et al. [12] who reported hypertrophy of cells and an increase in the thickness of the ZF layer in the adrenal cortex of tramadol administered rats. The study also showed an increase in capsule thickness, adrenal gland vascularization and vacuolation of the cytoplasm which were correspondingly observed in the present study. Salbacak et al. [13] likewise described a thickening in the ZF layer in the adrenal gland of rats administered with morphine. The similarity of the findings of the present study to previous studies on other opioids suggests that codeine has similar effects on the adrenal cortex as more potent opioid drugs when administered chronically.

It is possible that codeine, like other opioids, causes a physiological increase in the secretion of steroid molecules by the ZF cells [12, 14, 15]. This is evidenced in light microscopic examination by an increase in vacuolation of these cells. Abdelaleem et al. [12] observed that tramadol administered rats demonstrated pathological changes such as degenerated mitochondria, increased caspase 8 expression and oxidative damage due to the generation of free radicals. The ultrastructural mechanisms of changes caused by codeine on the adrenal gland are however not fully explicated.

The decreased ZR thickness and volume density as shown in rats administered with codeine for 50 days complements findings from previous reports by clinical studies on patients taking high doses of opioids. Daniell [16] has previously described a decline in production of adrenal androgens in the use of sustained action prescription opioids i.e. opiate induced adrenal androgen deficiency. He discovered that production of Dehydroepiandosterone (DHEA) and Dehydroepiandosterone sulfate (DHEAS) was lower in patients who took opioids compared to those who did not. Similarly, experimental studies demonstrate a reduction in serum DHEAS levels in opioid administered rats [12].

The reduced ZR thickness and volume density in codeine administered rats shows that this drug may also cause such deficiencies. DHEA deficiency can cause fatigue, depression, weakness and sexual dysfunction and hence caution should be observed in opioid therapy to avoid these adrenal androgen deficiencies. Daniell [16] also demonstrated that Adrenocoticotropic Hormone (ACTH) levels in serum of patients using opioids remained normal, suggesting that suppression of the adrenal cortex by opioids may result from direct effects on the adrenal gland.

Findings of reduced cell counts, sparse arrangement of cells and increased size of the adrenal medulla in the experimental group in this study are consistent with observations seen with other opioids. Salbacak et al. [13] demonstrated an increase in thickness of the adrenal medulla with morphine administration in rats. Morphine and other opioids are thought to induce immunosuppression by either stimulating production of adrenal corticosteroids or activation of the sympathetic nervous system and catecholamine release from the adrenal medulla [17]. On the contrary, *in vitro* studies have shown that opioids inhibit the secretory activity of the medulla [18]. This is effected by inhibition of potassium ion evoked acetylcholine release from nerve terminals of the adrenal medulla by action at mu opioid receptors [19]. A possible explanation to our findings of reduced cell counts in the adrenal medulla may therefore be inhibition of neuronal input to the medullary chromaffin cells by codeine.

The adrenal glands from animals in the 14-day recovery period had a marked improvement on the structure, with most features being restored to the reference observed in the control group. This was similar to findings by Abdelaleem et al. [12] in a two-week recovery period from chronic tramadol administration in rats. The study reported that the structure was most improved after two weeks of recovery. Houshyar et al. [20] also found that rats recovered normal adrenal size sixteen days after withdrawal of morphine administration. The rats in the study, however, exhibited diminished and briefer responses of ACTH and corticosterone to restraint stress [20]. The restoration of the adrenal cortex can be explained by migration of stem cells from progenitor cell zone of the adult adrenal gland, an observation which has been made by studies on adrenal gland regeneration [21, 22].

In conclusion, the results of the study reveal that administration of codeine phosphate causes discernible changes in the light microscopic structures of the adrenal gland. These include increase in ZF thickness, decrease in ZF cell count, decrease in ZR thickness, reduction of medullary cell count and increase in vascularization and stromal connective tissue. Most of these changes appear to be reversed after two weeks recovery period without administration of codeine. The structural changes determined from this study may explain endocrine derangements of the HPA axis that are observed in high dose or long-term opioid therapy and in opioid abuse. Further studies could be done to elaborate ultrastructural and immunohistochemical features in adrenal glands of codeine administered rats in order to better understand mechanisms of the morphologic changes.

ORCID

Ibsen Henric Ongidi:
https://orcid.org/0000-0003-3255-5388
Fadhila Yusuf Abdulsalaam:
https://orcid.org/0000-0002-3890-4537
Thomas Mombo Amuti:
https://orcid.org/0000-0003-0532-3653
Wycliffe O. Kaisha:
https://orcid.org/0000-0003-4398-9771
Kirsteen O. Awori:
https://orcid.org/0000-0002-6941-4944
Anne Naipanoi Pulei:
https://orcid.org/0000-0003-2437-8596

Author Contributions

Conceptualization: IHO, WOK, KOA, ANP. Data acquisition: IHO, FYA, TMA. Data analysis or interpretation: IHO, FYA, TMA. Drafting of the manuscript: IHO, FYA, TMA. Critical revision of the manuscript: IHO, WOK, KOA, ANP. Approval of the final version of the manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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