ORIGINAL COMMUNICATION

Anatomy Journal of Africa. 2021. Vol 10 (1):1881-1887

SUBACUTE ORAL ADMINISTRATION OF CODEINE CAUSES UP-WARD REGULATION OF PRO-APOPTOTIC GENES IN FORE BRAIN TISSUE OF RABBITS

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ABSTRACT

Addictive drugs trigger strong and persistent neuroadaptive changes in the brain through a series of gene regulatory mechanisms leading to addiction. The objective of the study was to assess the effect of 28day oral administration of codeine on the expression of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) genes in the frontal lobe of the brain of adult rabbits. Dihydrocodeine tablets were obtained from the National Drug Law Enforcement Agency (N.D.L.E.A) and subjected to cold water extraction method to separate the active ingredient from other formulation excipients. Twenty-four adult rabbits that were priory acclimatized for ten days, were divided into four groups of six each. The first three groups were tagged as experimental while the fourth as control group. The three experimental groups were orally administered with graded doses of codeine of 3mg/kg (low dose),5mg/kg (medium dose) and 7mg/kg (high dose) respectively for 28 days, while the fourth group (the control group) were given distilled water only .On the 29th day, biological sampling of two rabbits from each group was done, and the experimental animals were humanely sacrificed . Frontal brain tissues were obtained from each rabbit and subjected to gene expression analysis using standard methods. Gene of interest were Bax and Bcl-2. Molecular analysis for pro apoptotic gene (bax) in the frontal lobe showed a highly significant increase at low-dose and high-dose rabbits relative to control. For the anti-apoptotic gene Bcl-2 however, there was a highly significant decrease in the frontal lobe of the brain of medium and high dose groups respectively, it was concluded that codeine causes upward regulation of pro apoptotic gene (bax) and downward regulation of anti-apoptotic gene (Bcl-2) in the rabbit forebrain.

Key words: Codeine, Pro-apoptotic gene, Anti-apoptotic gene, Fore brain.

INTRODUCTION

Addictive drugs, such as cocaine, opioids (including heroin) and amphetamines, trigger strong and persistent neuroadaptive changes in the brain through a series of gene regulatory mechanisms leading to addiction. Recent studies have reported involvement of miRNAs in drug addiction (Hollander et al. 2010; Dreyer 2010; Schaefer et al.2010), mainly associated with cocaine-related drug addiction, although there is no direct report of miRNAs involved in opioid-related addiction so far. Recent studies have demonstrated that miRNAs are highly expressed in the CNS including the brain and spinal cord where biological action of opioids, as well as nociception takes place (Dave and Khalili 2010; Sanchez-Simon et al. 2010; He et al. 2010; Zheng et al. 2010). A growing body of exciting evidence suggests that miRNAs are important regulators of opioid-associated biological processes such as drug addiction (Zheng et al. 2010a; He et al. 2010), pain perception (Kusuda et al. 2011), neuron development (Gao, 2010), viral infection (Wang et al. 2011b; Dave and Khalili 2010), and opioid receptor regulation (Wu et al.2009).

Submitted 11th June 2019. Published 1st November 2020. To cite: Abubakar AA, Sani GH, Uthman GS. 2021. Subacute oral administration of codeine causes up-ward regulation of pro-apoptotic genes in fore brain tissue of rabbits. Anatomy Journal of Africa. 2021. Vol 10 (1): 1881-1887.

Apoptosis or programmed cell death is a normal process to remove candidate cells for death (Takata et al. 2014). Accordingly, this is a physiological process for progression and homeostasis of human systems such as immune and nervous systems (Paganelli et al. 2008). Previous investigations revealed that several endogenous and exogenous agents can induce apoptosis (Khalil et al., 2012). Alkaloids (morphine, heroine and codeine) are the important exogenous agents which induce apoptosis (Aydin et al. 2010). There are more than 20 alkaloids and more than 70 components in opium, thus, its effect on the cells could be different from its derivatives.

Among the known regulators of apoptosis, the Bcl-2 protein stands out for its ability to suppress cell death induced by a wide variety of insults and stimuli, including some of the mediators suspected to be inducers of neuronal cell death with reperfusion injury. For example, gene transfer-mediated elevations in Bcl-2 protein levels in immortal neural cell lines have been shown to markedly inhibit cell death induced by L-glutamate, Ca2+-ionospheres, hypoglycemia, free radicals, and glutathione depletion (Zhong et al. 1993; Behl et al. 1994). In addition ,a role for Bcl-2 in the control of neuronal susceptibility to ischemia-induced death has recently been suggested by studies of transgenic mice that over express bcl-2 in the brain, in which infarct sizes caused by focal ischemia were reduced by-50% compared to non-transgenic litter mate controls (Martinou et al. 1994). Bcl-2 is an integral membrane protein associated primarily with the outer mitochondrial membrane, the nuclear envelope, and endoplasmic reticulum (Krajewski et al. 1993) that appears to block a distal event in what may represent a final common pathway for apoptotic cell death (reviewed by Reed,1994). Though the biochemical mechanism by which the Bcl-2protein prevents cell death remains enigmatic, evidence has been obtained suggesting an effect on regulation of intracellular Caz+ fluxes across membranes (Baffy et al., 1993; Lam et al., 1994)or control of an antioxidant pathway (Hockenbery et al., 1993; Kane et al.,1993), among other possibilities (Reed,1994).

Recently, a family of genes encoding homologs of Bcl-2 has been discovered. Some of these proteins, such as Bcl-X-L, function as blockers of cell death similar to Bcl-2 (Boise et al., 1993), whereas others, such as Bax, render cells more sensitive to apoptotic stimuli (Oltvai et al., 1993). Gene-targeting experiments in mice have recently demonstrated that loss of bcl-X expression is associated with massive death of immature post mitotic neurons in developing embryos, suggesting that this antiapoptotic member of the bcl-2 gene family is important for mammalian neuronal survival at least during development (Motoyama et al., 1995). It has been shown that the Bcl-2 protein can physically interact with several of its homologous proteins, in the form of heterotypic dimers (Oltvai et al., 1993; Sato et al., 1994). The aim of this study therefore was to find out how short-term administration of codeine affects levels of Bax and Bcl2 in rabbit forebrain.

MATERIALS AND METHODS

Two hundred (30mg) Dihydrocodeine tablets were obtained from the National Drug Law Enforcement Agency (N.D.L.E.A) and subjected to cold water extraction method to separate the active ingredient from other formulation excipients. Purity was tested using high profile liquid chromatography (HPLC). Dihydrocodeine tablets are crystalline, odourless, white to off white in colour and are readily soluble in both water and ethanol. Water was used as dissolving agent in this study.

Twenty-four healthy adult rabbits that were priory acclimatized for ten days, were divided into four groups of six each. The first three groups were tagged as experimental while the fourth as control group. The three experimental groups (low dose, medium dose and high dose

groups) were orally administered with graded doses of codeine of 3mg/kg ,5mg/kg and 7mg/kg respectively for 28 days, while the fourth group (the control group) were given distilled water only. 5ml disposable syringes were used to administer codeine solution to the experimental animals.

Written permission was obtained from the National drug law enforcement agency (NDLEA) and the Animal Ethics Committee of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Maiduguri, Borno State, Nigeria, prior to the commencement of the study.

Total RNA was isolated from rabbit fore brain using Trizol reagent (ambion) as per manufacturer instructions. Briefly,100mg sample of forebrain tissue was removed from RNA storing agent and 1000µl Trizol agent was added directly to the sample in the centrifuge tube and was lysed using tissue homogeniser.

After incubation at room temperature for 5 minutes, 0.2ml of chloroform was added and shaken vigorously and kept at room temperature for 10 minutes. Following centrifugation at 12000g for 5 minutes at 4 degrees Celsius, the aqueous layer at the top was pipetted into a new micro centrifuge tube and RNA was precipitated by adding 0.5ml of 100% isopropanol, then was incubated for 10 minutes at room temperature and centrifuged at 12000g for 10 minutes at 4 degrees Celsius. Supernatant was decanted. RNA pellet was washed with 70% ethanol, the tube was vortexed briefly, and then centrifuged at7500g for 15 minutes at 4 degrees Celsius.

Supernatant was be decanted and pellet air dried for two hours and suspended in 2µl of nuclease free water (NFW). The purity of the RNA was quantitated by using bio spectrometer and only RNA samples with an A260/A280ratio were used for reverse transcription.

cDNA was synthesized from the isolated RNA using iScript cDNA synthesis kit (Bio red). A reaction mixture containing 4ul of 5x iScript reaction,1µl of script reverse transcriptase,8µl of RNA template and 7µl of NFW was incubated for 5 minutes at 25 degrees Celsius for 20 minutes at46 degrees Celsius and for 1 minute at 95 degrees Celsius. cDNA was stored for further use.

Table 1: Grouping of animals, codeine administration and biological sampling.

Group	Number of Animals	Treatment for 28 days	Number of Animals sampled After 28 day	Gene targeted
Low dose	6	Codeine at 3mg/kg Body weight	2	Bcl-2 and Bax
Medium dose	6	Codeine at 5mg/kg Body weight	2	Bcl-2 and Bax
High dose	6	Codeine at 7mg/kg	2	Bcl-2 and Bax

Previously published standard primers were used for gene expression studies. The cDNA was subjected to amplification with the respective primers at various annealing temperatures to optimize the PCR conditions. Real time PCR analysis was carried out after checking each primer for specifity.

Polymerase chain reaction (PCR) was performed to confirm the synthesis of cDNA using primer pairs used for Beta actin gene which was also used as internal control in q PCR experiments. PCR reaction was done to ascertain the specifity of all the primer pairs used for the different genes under study by amplicon size. The reaction mixture containing 6.25µl of PCR master

mix(2X),3µl of cDNA template,0.5ul of 10µM sense and antisense primers and 2.25 nuclease free water to make final reaction volume of 12.5µl. Gradient PCR was run to assess the annealing temperature range of the primer pairs. The cycling conditions was carried out as follows: initial denaturation at 95ºC for 3 minutes, followed by 40 cycles of denaturation at 95 ºC for3 seconds. annealing at different temperatures namely; 56ºC, 57ºC,58º,60ºC,61ºC,62ºC, and 63ºC for 1 minute, extension at 72ºC for 30 seconds at 72ºC for 7 minutes.

Table 2: Description of primers used.

Gene	primer used	annealing temp, (ºC)	Product size	Aee No.NCIII	Reference
Bax sense	5'-CCAGGACGGATCCACCAGAAGC-3'	58	136bp	AF_235993.1	Li et al.2009
Antisense	5'-TGCCACACGGAAGACCTTCTCG-3'	58	76bp	AF_235993.1	Li et al. 2009
Bcl-2 sense	5'-GGAATGACTTCTCTCGTCTACCGT-3'	56	76bp	AF_51283.1	Li et al. 2009
Antisense	5'-ATCCCTGAAGAGTTCCTCACCAC-3'	56	76bp	AF_ 5122831	Li et al.2009
B-actin sense	5'-TCCTAGCACCATGAAGATCAAG-3'	59	132bp	NC_005111	Kant et al. 2015
Antisense	5'-GACTCATCGTACTCCTGCTTG-3'	59	132bp	NC_005111	Kant et al. 2015

RESULTS

As shown in both table 3 and figure 1, Molecular analysis for pro apoptotic gene bax in the frontal lobe showed a highly significant increase in cycle threshold (ct) values at low-dose and high-dose rabbits relative to control. For the anti-apoptotic gene Bcl-2 however, there was a highly significant decrease in cycle threshold values in the frontal lobe of the brain of medium and high dose groups respectively.

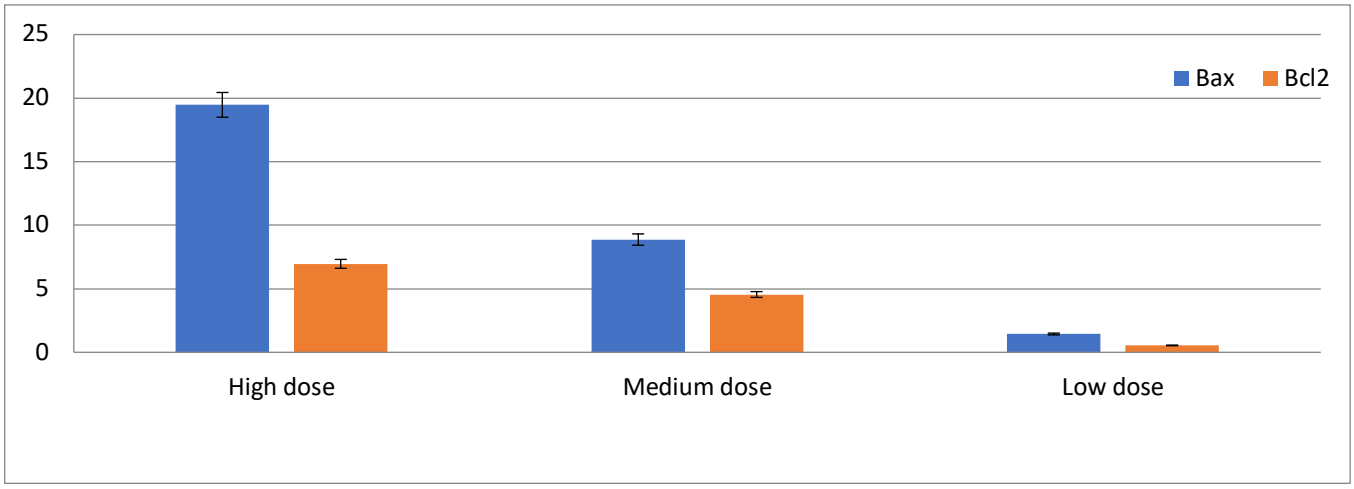

Figure 1: Bar chart showing concentration of pro apoptotic gene (Bax) and antiapoptotic gene (bcl-2) in high dose(7mg) medium dose(5mg) and low dose(3mg) rabbits respectively

Table 3: Ct Values of Bax, Bcl-2 and Beta actin (housekeeping) genes in high, medium and low dose rabbits.

Sample gene Bax	*High dose 7mg/kg*	*Medium dose 5mg/kg*	*Low dose 3mg/kg*	*control*
Ct value	25.28	25.17	25.13	24.77
Ct value	24.9	24.11	25.47	23.91
Sample gene Bcl-2	High dose	Medium dose	Low dose	control
Ct value	24.06	26.55	27.48	26.41
Ct value	25.09	24.06	24.79	24.8
House keeping Gene Beta actin	*High dose*	*Medium dose*	*Low dose*	*control*
Ct value	21.05	21.62	23.26	23.01
Ct value	21.64	20.58	24.2	23.47

*Ct: cycle threshold

DISCUSSION

The rate of abuse of drugs and other chemotherapeutic agents seem to be increasing daily. A lot of patients are placed on long-term administration of some clinically useful drugs such as Codeine which may have molecular adverse effects. High levels of the cell death promoting protein Bax and concomitant low levels of the apoptosis-blocking protein Bcl-2 were found in some populations of neurons that are particularly sensitive to cell death induced by transient global ischemia, such as the CA1 sector of the hippocampus and the Purkinje cells of the cerebellum. Moreover, within 0.5 to 3 hr after an ischemic episode, immunostaining for Bax was markedly increased within neurons with morphological features of degeneration in many regions of the brain. Use of a two-color staining method for simultaneous analysis of Bax protein and in situ detection of DNA-strand breaks revealed high levels of Bax immunoreactivity in many neurones undergoing apoptosis. Post ischemic elevations in Bax protein levels in the hippocampus, cortex, and cerebellum were also demonstrated by immune blotting. At early times after transient ischemia, regulation of Bcl-2 and Bcl-X protein levels varied among neuronal subpopulations, but from 3 hr on, those neurons with morphological evidence of degeneration uniformly contained reduced levels of Bcl-2 and particularly Bcl-X immunoreactivity. The findings suggest that differential expression of some members of the bcl-2 gene family may play an important role in determining the relative sensitivity of neuronal subpopulations to ischemia and that post ischemic alterations in the expression of bax, bcl-2, and b &X may contribute to the delayed neuronal cell death that occurs during the reperfusion phase after a transient ischemic episode.

In the current study, the effects of subacute administration of low (3mg/kg), medium(5mg/kg) and high(7mg/kg) doses of codeine in adult rabbits were studied. Result showed that pro apoptotic (bax) gene expression was markedly induced and dose dependent suggesting that bax gene was up regulated and played an important role in the induction of apoptosis in rabbit forebrain after subacute codeine exposure.

This result is consistent with the findings of Xiao and Zhang (2008), who demonstrated that prenatal cocaine exposure induces apoptosis in the fetal brain and suggested that up-regulating Bax/Bcl-2 gene expression may be involved in opiod-induced apoptosis. Nivikova et al.2005 has detected cocaine exposure-induced changes in expression of some apoptosis-related genes in the fetal mouse cerebral wall by microarray analysis and demonstrated that maternal cocaine exposure could influence transcriptional expression levels of multiple apoptosis related genes in fetal cerebral wall.

In conclusion, this study has demonstrated that subacute administration of codeine causes upward regulation of pro apoptotic genes (Bax) in the rabbit fore brain. As reported previously, Recent studies also suggest that multiple mechanisms may be involved in opiod-induced apoptosis in the brain. One of the apoptotic pathways is regulated by specific genes. Of these genes, Bax is the key gene in upregulation of opiod induced apoptosis in the brain. However, Bcl-2 expression could be an important factor that promotes survival of opiod-injured neurons. These findings demonstrate that genes can be orchestrated in codeine-induced apoptosis in rabbit forebrain and provide a rationale for the further development of pharmacological and molecular therapies targeting programmed cell death after codeine use.

Conflict of interest: None

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