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Neuronal alterations in the prefrontal cortex of rats with carbon tetrachloride (CCl_4) induced hepatic damage

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In cirrhosis, some toxic substances accumulate in the brain and modify its functional integrity. In this study, we investigate the impacts of liver damage on the neuronal profile of the prefrontal cortex (PFC) in a rat model of hepatic damage induced with carbon tetrachloride (CCl_4). This study also evaluated the possible role of liver dysfunction in the etiology of neurodegenerative characteristics associated with the PFC. Ten male Wistar rats weighing 120 to 190 g body weight were used for this study. The rats were divided into 2 groups (A and B) of 5 rats each. The rats in group A (control group) were treated with phosphate buffered saline (PBS) solution only while the rats in group B (treatment group) were treated with carbon tetrachloride (CCl4). The prefrontal cortices of the rats were excised from skulls of the rats, fixed in formol calcium, while the livers were excised from the abdomen of the rats and were fixed in formol saline for cytoarchitectural study using Cresyl fast violet and hematoxylin and eosin stains respectively. The main neuropathological findings observed in this study include cortical necrosis, uneven neuronal loss with varying range of vacuolations in the prefrontal cortices of the CCl_4 treated rats when compared with the PBS treated rats. It was observed that the administration of CCl_4 induces changes in hepatocellular morphology of the treated rats and these include moderate vascular congestion and extensive cytoplasmic damage in the hepatocytes. These results could be due to loss of hepatic functions.

Key words: Liver damage, neuropathology, neurons, prefrontal cortex, rat.

INTRODUCTION

Liver cirrhosis is a pathological state that occurs when chronic hepatic damage causes diffuse scarring of the tissue, thereby preventing blood from circulating freely through the scar tissue (Crawford, 2002). The mechanisms leading to cirrhosis are alcohol abuse and chronic liver damage secondary to infection with hepatitis B or C. It is also known that tissue damage resulting from oxidative stress is one of the important factors in liver failure (Görg et al., 2010). The use of experimental models of hepatic damage has suggested some mechanisms that may be active in the development of this disease. Moreover, these models have shown the effect of these

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mechanisms on other organs, such as the brain, where they may trigger the onset of hepatic encephalopathy (HE) (Butterworth et al., 2009).In hepatic encephalopathy (HE), disorders in brain functions are the consequence of prior failure in normal liver function (Prakash and Mullen, 2010).

Liver function failure impedes the detoxification process for ammonia and other toxic substances that may reach the brain and alter its function. Hyperammonaemia is one of the factors that contribute to neurological disorders caused by both acute and chronic liver failure (Lockwood et al., 1991). Chronic liver injury is associated with profound impairments in hepatocellular regeneration. Cirrhosis develops when recurrent injury and cell loss are not adequately counterbalanced by repair mechanisms, due to inhibition of DNA synthesis, reduced energy metabolism, insulin resistance, and oxidative stress (Duguay et al., 1982).

Liver cell injury caused by various toxins such as carbon tetrachloride (CCl_4), thioacetamide, continuous alcohol consumption and microbes have been documented and reported (Salem et al., 2010). Several studies have shown that, under the condition of oxidative stress, a significant simultaneous elevation in intracellular concentration of oxidizing species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are predominant stimulators for tissue injury (Chin et al., 2009). These oxidizing species may also be associated in the etiology and pathophysiology of aging and diseases such as coronary heart disease, cancer, atherosclerosis, inflammation, cataracts and Alzheimer's disease (Nakayama et al., 1983; Fridovich, 1988; Floyd, 1990). Under a disease-free-state, there is a dynamic balance between the amount of oxidizing species produced in the body and antioxidants to quench or scavenge them and to protect the body against harmful effects. However, an overproduction of ROS and/or inadequate antioxidant defense alters this equilibrium favoring ROS upsurge that leads to oxidative stress. Several clinical disorders, implicate a deficient natural antioxidant defense as their etiological or pathological factor.

The liver is prone to numerous chemical-induced injury and/or damage due of its pivotal role in the metabolism of xenobiotic and its basic and functional anatomical structures. Most of these chemicals and drugs are not naturally toxic to the liver but cause injury secondary to the formation of a hepatotoxic metabolite through a process termed bioactivation (Vessey, 1996).

Treating cirrhosis is expensive and treatment must be increased as the disease progresses. The incidence of diseases associated with liver failure, such as HE, is high. Such diseases have a considerable impact on the quality of life of a large number of people, and in advanced stages may result in coma or death (Quiroz et al., 2010). Moreover, despite the progress that has been made in several forms of liver diseases, there is no effective treatment.

CCl_4 is an extensively studied xenobiotic that induces lipid peroxidation and toxicity (Jeon et al., 2003). CCl_4 is frequently used in animals to produce an experimental model to study the mechanisms involved in the progression of hepatic disease and the impact of various drugs on this progression (Boer et al., 2009). Metabolic activation of CCl_4 by cytochrome P450 to the free radicals, namely trichloromethyl and trichloromethyl-peroxy-radicals, is reported to enhance lipid peroxidation and protein oxidation in the liver, resulting in widespread membrane damage and liver injury (Scarpelli and Iannaccone, 1990) through the generation of free radical intermediates that can directly cause liver oxidative damage. Hepatic failure denotes a devastating clinical condition that often results in multi-organ failure and death (Sheweita et al., 2001; Konopacka et al., 2008; Kung et al., 2008).

Animal tissues are constantly coping with highly reactive species, such as superoxide anion, hydroxyl radicals, hydrogen peroxide, and other radicals generated during numerous metabolic reactions (Castillo, 1992; Hartley et al., 1999; Cabre et al., 2000; Melin et al., 2000). Oxidative stress resulting from increased free radical production after CCl_4 intoxication may play an important role in the degenerative processes in many vital tissues (Szymonik-Lesiuk et al., 2003).

Clinical and experimental studies indicate that the prefrontal cortex is critically implicated in several aspects of learning and memory (Goldman-Rakic, 1994, 1995; Fuster, 1997). The prefrontal cortex (PFC) is involved in working memory and contributes to the temporal ordering of spatial and non-spatial events as well as to the organization and planning of responses. For example, increased cellular discharge occurs in the prefrontal cortex during the delay period of delayed response tasks in the rat and the monkey (Sakurai and Sugimoto, 1986; Batuev et al., 1990; Goldman-Rakic, 1995).

According to Peterson et al. (1990), different models of chronic liver failure have shown a disruption in the functional integrity of several regions of the brain such as the cerebral cortex, hippocampus, striatum and thalamus. It has also been reported that in cases of hepatic damage, certain toxic substances may reach the brain and provoke morphological changes affecting only the astrocytes.

Nevertheless, some studies have described reports of neuronal death mediated by excitotoxicity mechanisms via activation of N-Methyl-D-aspartate (NMDA) receptors, similar to that occurring in cerebral ischemia, in models of CCl_4 hepatotoxicity (Diemer, 1997; Cauli et al., 2011). It has also been found that the metabolism of CCl_4 involves in the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum (Slater and Sawyer, 1971). With all of the aforementioned in mind, the purpose of this study is to evaluate the implication of liver-induced damage (using

CCl_4) on the neuronal profile of the prefrontal cortex in rat.

MATERIALS AND METHODS

Subject

All treatments were performed in accordance with the National Institutes of Health Guide for Care and Use of Rats. Animal treatment and care was in accordance with rules and guides of the Afe Babalola University Institutional Animal Care and Use Committee. Male Wistar rats with body weights ranging from 120 to 190 g were used for this study. The rats were housed in two home polycarbonate cages with stainless steel wire lids, at a constant temperature of 22 ± 1°C in a husbandry, under a cycle with a light phase of 12 h from 8 a.m. to 8 p.m., with free access to food and water. Two treatment groups were established: a control group treated with phosphate buffered saline (PBS, A), and a CCl_4 treated group (B). Treatments were administered 3 times a week for 8 weeks.

Inducing liver damage with CCl_4

Hepatic damage was induced by intraperitoneal administration of a blend of 0.2 ml CCl_4 and olive oil in a ratio of 1:1 (v/v), 3 times a week for 8 weeks, decreasing the volume of the initial blend of olive oil and CCl_4; first week 1:6, second week 1:5, third week 1:4 and fourth week 1:3, continuing until the eighth week. The choice of CCl_4 dosage was according to the dose employed in the study of Mu˜noz et al. (1998) and Fregozo et al. (2012).

Twenty-four hours after the administration of the last dose, all rats were sacrificed under ether anesthesia. Craniotomy was performed and intact brains were dissected and removed for histopathological studies. The excised brain regions were isolated and washed with normal saline followed by 50 ml (4%) of paraformaldehyde in PBS, postfixed in 10% formalin for 7 days. For cresyl violet staining, a 1:5 series of sections was mounted onto coated slides, dried, and stained in 2.5% cresyl violet acetate (Sigma, St. Louis, MO). Liver tissue was fixed overnight in 10% formol saline and embedded in paraffin. Tissue sections 5 µ thick were stained using the Leica Autostainer XL for Hematoxylin and Eosin.

The activities of marker enzymes; alkaline phosphatase (ALP), alanine aminotransferase (ALT) and asparate aminotransferase (AST) were determined in serum using standard assay kits. For the concentration of oxidative stress; glutathione peroxidase (GP_X) and malondialdehyde (MDA), each of the liver and PFC tissue from the control and CCl_4 treated rats were weighed and homogenized, respectively with a Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in bi-distilled water (1.0 g tissue/10.0 ml bi-distilled water). Samples were centrifuged 15 min at 3000 rpm. Following centrifugation, the respective supernatant was separated and pipetted into separate tubes.

Determination of GP_X in the PFC and liver tissues respectively

The glutathione peroxidase (GP_X) concentration was measured using the GP_X cellular activity assay kit CGP-1 (Sigma, Aldrich). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GP_X, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH). The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GP_X activity.

Determination of MDA in the PFC and liver tissues respectively

The concentration of malondialdehyde (MDA) was determined by thiobarbituric acid reactive substances (TBARs) assay. 200 µl of PFC and liver homogenate, respectively was added and briefly mixed with 1.0 ml of trichloroacetic acid at 50%, 0.9 ml of TRIS-HCl (pH 7.4) and 1.0 ml of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100°C for 20 min. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm. The signal was read against an MDA standard curve, and the results were expressed as nmol/mg protein as described by Padurariu et al. (2010).

Statistical analysis

The data obtained were expressed as means (±standard error of mean (SEM)). The inter-group variation was measured by one way analysis of variance (ANOVA; 95% confidence interval) followed by Fischer's least significant difference (LSD) test. Statistical significance was considered at $p<0.05$.

RESULTS

Treatment with CCl_4 induced neuronal cell death in the cytoarchitectural profile of the PFC as shown in arrow heads in Figure 2A and B compared with the control rats (Figure 1A and B). The pattern of cell death seen in the prefrontal cortex of the CCl_4 treated rats include neuro-degenerative features of neuronal necrosis with enlarged cell bodies and distorted membranes, degeneration of cytoplasm and nuclear materials was also observed. The neuronal damage was pronounced with lost neuronal connections. The Nissl's bodies are eccentrically placed signifying chromatolysis.

Hematoxylin and eosin staining were used to examine the effects of CCl_4 on the livers of the treated and control rats, respectively. It was observed that the administration of CCl_4 induced changes in hepatocellular morphology of the treated rats and these include moderate vascular congestion and extensive cytoplasmic damage in the hepatocytes (Figure 4A and B). The hepatocellular morphology of the rats in the control group was well preserved (Figure 3A and B).

To confirm that the morphologic alterations shown in Figure 4A and B represent significant hepatocellular damage, the serum activities of hepatocytes cytosolic enzymes (alanine aminotransferase and aspartate aminotransferase) were measured in the same rats examined in Figures 3A, B, 4A and B. Administration of CCl_4 produced significant ($p<0.05$) increase in the activities of these enzymes (ALT and AST) in the treated rats when compared with the control rats (Figures 5 and 6).

The concentration of lipid peroxidation index, MDA in the livers and prefrontal cortices of the CCl_4-treated rats were significantly ($p<0.05$) higher when compared with the control rats (Figure 7). Also, compared with the control rats, GP_X concentration in the livers and prefrontal cortices were significantly ($p<0.05$) higher in the CCl_4-

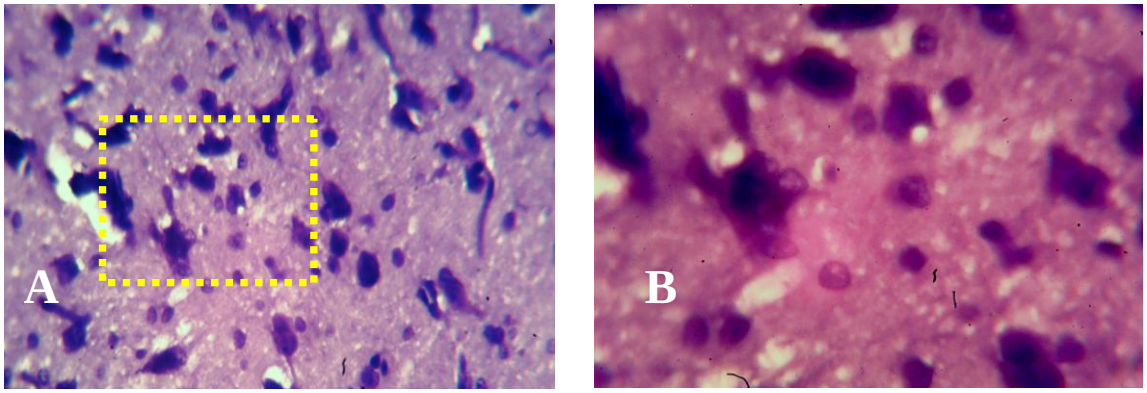

Figure 1. Cresyl violet-stained cells in the PFC showing normal neuronal profile of the neurons in the prefrontal cortex of control rats. The adjacent photomicrograph (B) is that of the yellow boarder region on (A) at higher magnification (A = 400x; B = 1000x).

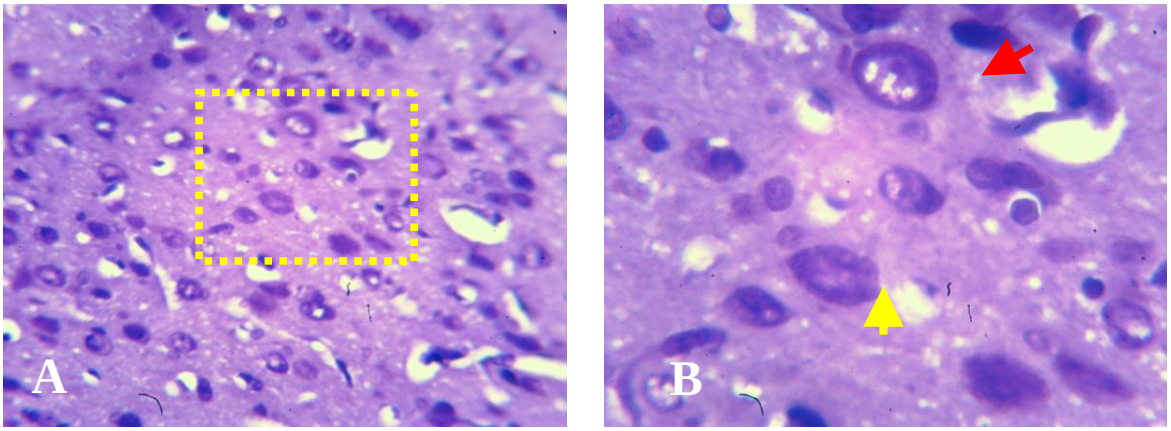

Figure 2. Cresyl violet-stained cells in the PFC showing histological evidence of neuronal loss in the prefrontal cortex of CCl_4 treated rats. The adjacent photomicrograph (B) is that of the yellow boarder region on (A) at higher magnification. The red arrow head depicts cell bodies with fragmented cytoplasm and nucleus. The yellow arrow head depicts neuron with degenerated axons' free ending and vacuolar spaces this may probably occur as a result of peroxidation of lipids and fast progressing degeneration of cell body and axons (A = 400x; B = 1000x).

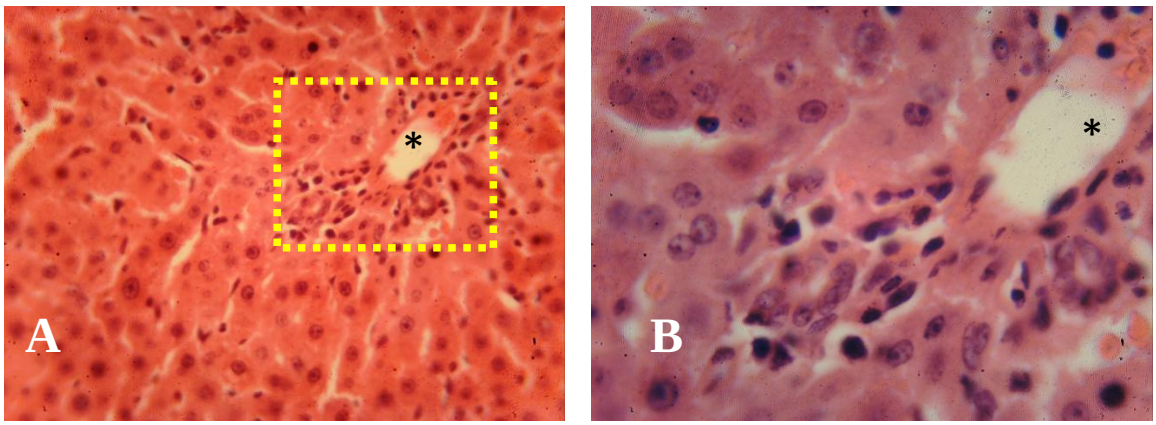

Figure 3. Hematoxylin and eosin stained section of the liver of the representative rat in the control group with preserved and well defined hepatic profile (magnification, A = 400x; B = 1000x).

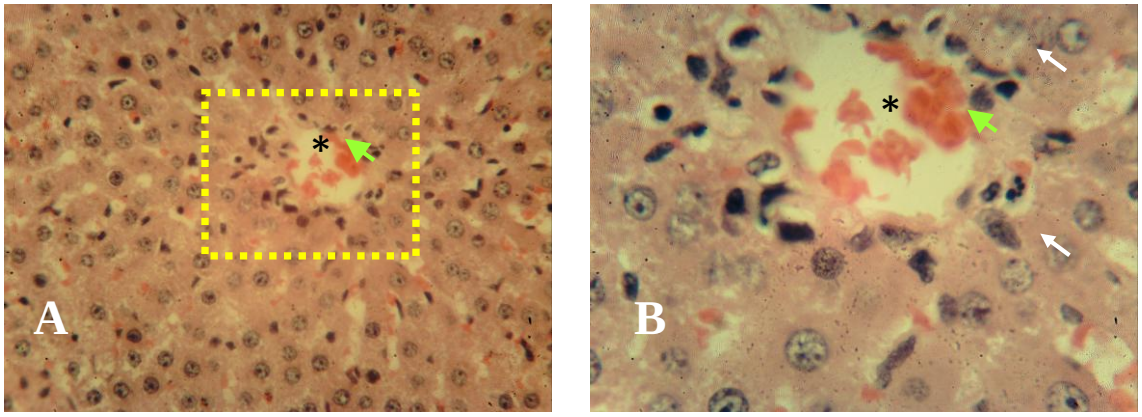

Figure 4. CCl_4 causes hepatocellular damage visible by hematoxylin and eosin staining. Hematoxylin and eosin staining was used to examine the effects of CCl_4 on the liver of treated rats. A representative image of a hepatic lobule near central vein is shown (asteriks denote central vein). The image in this figure is the representative of all the rats in the treated group. Evident in this slide is moderate vascular congestion (green arrow head). This congestion may be responsible for the extensive cytoplasmic disappearance in the hepatocytes of the CCl_4 treated rats (white arrow) (magnification, A = 400x; B = 1000x).

Figure 5. Serum AST activities after treatment with PBS and CCl_4 respectively in the livers of the control and treated rats.

treated rats (Figure 7).

DISCUSSION

It has been reported that the safety assessment in experimental animals of both medicinal and non-medicinal biologically active chemicals (be it plant based or synthetic) has been very successful in predicting toxicity in humans (Zhao et al., 1995). It has also been documented that the major advantages of preclinical safety assessment studies are the known responses of experimental species, the controlled conditions under which they can be maintained and the establishment of appropriate metrics, such as tissue volume rates, which can be applied to extrapolation of findings in laboratory animals to assessment of possible side effects in human (Zhao et al., 1995).

An organism's good state of health depends on the proper function of and interactions among its different

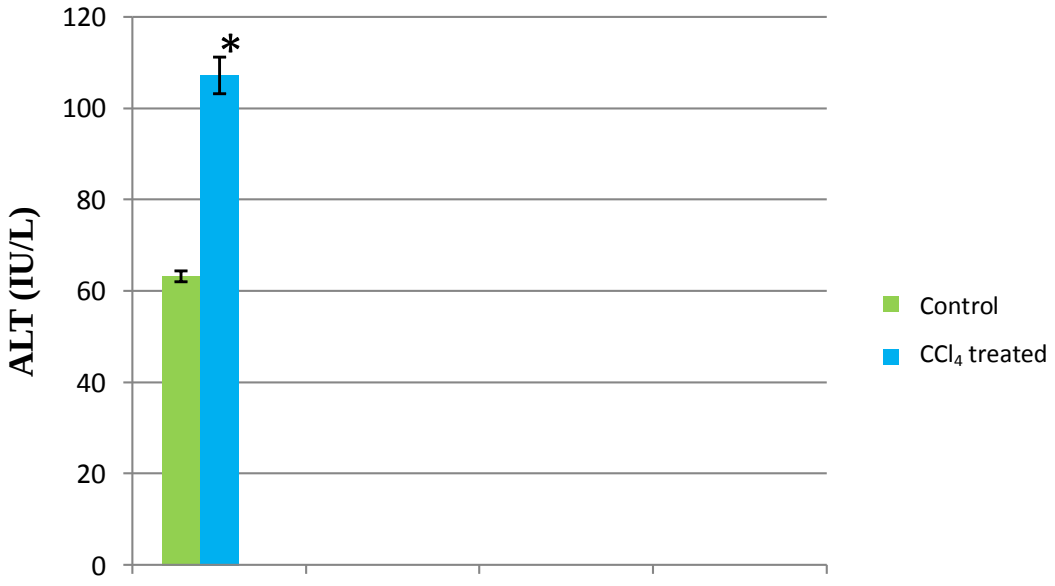

Figure 6. Serum ALT activities after treatment with PBS and CCl_4 respectively in the livers of the control and treated rats.

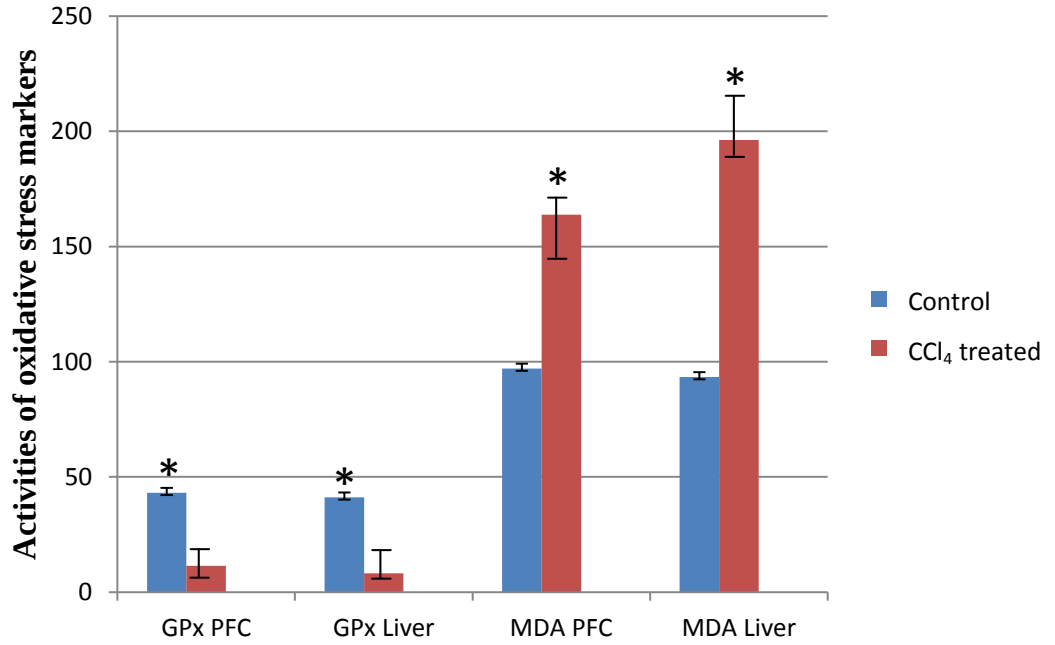

Figure 7. GPx and MDA concentration after treatment with PBS and CCl_4 respectively in the PFC and liver of the control and treated rats.

organs. In particular, the liver and the brain closely interact given that the liver provides the brain with nutrients which the brain itself cannot produce, and eliminates toxic substances from the blood. These include those substances released by the brain itself or by another organ, and which are necessarily released outside of that organ. As a consequence, liver dysfunction may bring about significant disorders in the functions of the brain.

Results from this study showed that the livers of rats with CCl_4-induced hepatic damage displayed a number of traits typical of hepatic damage. Due to this close interaction between the liver and brain, the metabolism, synthesis and delivery of the nutrients necessary for maintaining cerebral function probably decreased

following CCl_4-induced hepatic damage. This may reduce the number of nerve connections that naturally occurred in the brain, which is likely to be reflected as a distortion in the neuronal profile of the PFC in the CCl_4 treated rats. Comparing the treatment groups in Figure 2A and B with the control (Figure 1A and B), neuronal degeneration has occurred.

It has been shown that hepatic damage decreases the expression of both GLT-1 protein and its mRNA. It also increases the extracellular concentration of glucose, which may over activate NMDA receptors, increase calcium influx into the postsynaptic neurons, and cause neuronal damage (Knecht et al., 1997; Michalak and Butterworth, 1997).

Although CCl_4 is primarily metabolized in liver, its determinant effects on the brain are well documented (Boer et al., 2009). The lipid solubility of CCl_4 allows it to cross cell membranes and deposits it in different tissues such as liver, brain and testes (Szymonik-Lesiuk et al., 2003). Free radicals and reactive oxygen species (ROS) in biology gains more attention and there is increasing awareness of the ubiquitous role of oxidative stress in neuropathology (Bray, 1999; Sayre et al., 2008). Free radicals have been implicated in multiple CNS disorders (Kuloglu et al., 2002). This is understandable since this tissue is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses (Carbonell and Rama, 2007).

To the best of our knowledge, the concurrence of liver toxicity and brain damage after exposure to CCl_4 had not been examined. While the effects of CCl_4 on the liver have been extensively studied, the possible damage to several of the brain regions and the brain as a whole has received less attention. The current study is the first to show evidence of neurotoxicity relating to the cytoarchitectural profile of the PFC of rat model of CCl_4 hepatotoxicity. These were considerable venous congestion in the liver (Figure 4A and B). This congestion as suggested by Hong et al. (1991) and Wijetunga et al. (2003) is a typical early marker of hepatotoxicity. It could also be an evidence of posthepatic vascular constriction (Chen, 2007; Wang et al., 1990), and/or direct drug-induced hepatotoxic damage to the liver which may ultimately compromise the metabolic functions of the liver.

When hepatocytes are damaged, cytosolic contents, such as AST and ALT, are released into the blood and can be measured. Serum AST activity was 102.7±4.11 IU/L in the control rats and significantly elevated to 507.19±47.9 IU/L in CCl_4-treated rats (Figure 5). At this time point, ALT activity was 63.24±1.19 IU/L in the control rats and significantly increased to 107.23±4.01 IU/L (Figure 7).

There was clear evidence that CCl_4-induced hepatic damage was associated with free radical injury and oxidative stress as evidenced in the concentration of the markers of oxidative stress. This is characterized in the concentration of GP_X and MDA. In the PFC, GP_X concentration was 43.12±2.13 nmol/mg protein in the control rats and significantly reduced to 11.43±0.87 nmol/mg protein in the CCl_4 treated rats. In the liver, GP_X activities was 41.21±2.11 nmol/mg protein in the control rats and significantly reduced to 8.21±0.01 nmol/mg protein in the CCl_4 treated rats.

Furthermore, the concentration of MDA was observed to be 97.09±5.11 nmol/mg protein in the PFC of the rats in the control group and significantly elevated to 163.77±2.26 nmol/mg protein in the PFC of the CCl_4 treated rats. In the liver, MDA concentration was 93.41±0.01 nmol/mg protein in the control rats and significantly increased to 196.22±0.12 nmol/mg protein in the CCl_4 treated rats (Figure 6).

Similarly, serum AST and ALT were well established and sensitive markers for liver damage and were elevated after CCl_4 treatment. Although increases in AST or ALT could represent damage to other tissues, the elevations in both, along with the hepatic histopathological findings, support the claim that CCl_4 does cause liver damage (Charles et al., 2012; Adewole et al., 2007; Junnila et al., 2000). The adverse effect of CCl_4 on the livers of the treated rats also include severe global cytoplasmic disappearance (Figure 4A and B), suggestive of cellular dgeneration (Rautou et al., 2008).

It has been suggested that compromised liver functions resulting from inborn error of metabolism or frank liver damage can contribute to compromised neurological symptoms, including altered mental status, peripheral neuropathy, and coma (Felipo and Butterworth, 2002).

The current findings showed that CCl_4 produced significant elevated activities of AST and ALT in parallel with the compromised histopathology of the liver (Figures 3 to 6). The concentration of the markers of oxidative stress were statistically consistent in both the liver and PFC, therefore CCl_4 appeared to cause neurotoxic effect via oxidative stress. Similar result was observed in liver and brains of animals with alcohol-induced liver damage (de la Monte et al., 2009).

Metabolic stresses impair the cellular functions of the CNS, including myelin maintenance. The extent to which liver-damage-related neurodegeneration occur, would likely correlate with a compromise in the functional integrity of the gastrointestinal and hepatic detoxification systems that overwhelm the liver-brain axis. In essence, CCl_4-induced hepatotoxicity with increased oxidative stress and a mark alteration in the activities of AST and ALT, would likely mediate their adverse effects on the brain regions (most precisely the prefrontal cortex) and functions via a liver-brain axis of neurodegeneration.

In conclusion, this study identify that CCl_4-induced hepatic damage caused neurotoxic damage via oxidative stress and also compromises the integrity of the neuronal profile in the PFC of the treated rats. This finding suggests that comorbid conditions affecting liver functions may negatively contribute to the neurotoxic

consequences of CCl_4 exposure.

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