Pathological effects of acetone cyanohydrin in swiss rats

Efeitos patológicos de diferentes doses de acetona cianidrina em ratos suíços

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ABSTRACT

Cassava has been widely used for animal and human nutrition. It has also been demonstrated to have antineoplastic and anthelmintic properties. Toxicity due to cassava consumption has been reported in ruminants and laboratory animals; therefore, this study aimed to investigate the toxic effects of acetone cyanohydrin, a metabolite of linamarin that is present in cassava, in Wistar rats. Six groups of five animals each were used to evaluate the toxic effects of acetone cyanohydrin administered at 25 (G1), 50 (G2), 75 (G3), 100 (G4) and 125 (G5) µmol/kg as a single oral dose. The control group received acidified water (pH 3.5). The animals were monitored after administration of acetone cyanohydrin, and clinical symptoms were recorded. Serum enzyme levels were measured to assess the kidney and liver function. During necropsy, tissue samples were collected for histopathological examination. After administration, some animals in the G2, G4, and G5 groups presented neurological symptoms such as convulsions, involuntary muscle contraction, staggering gait, motor coordination disability, prostration, and mydriasis. All of the animals in the G5 and four animals in the G4 group died seven minutes after the administration of acetone cyanohydrin. Animals in the other groups, particularly in G2, recovered from the acute phase. Biochemical analysis revealed hepatic lesions and liver dysfunction. Histopathology revealed severe lesions in both the liver and brain. In conclusion, acetone cyanohydrin has toxic effects in the liver, lung, and central nervous system in rats; however, at concentrations up to 25 µmol/kg, the animals could survive the acute phase.

Index terms: Neurotoxic; hepatotoxic; cyanide; acute intoxication.

RESUMO

A mandioca tem sido amplamente utilizada para alimentação animal e humana. Também tem sido demonstrado que possui propriedades anti-neoplásicas e anti-helmínticas. A toxicidade devido ao consumo da mandioca tem sido relatada em ruminantes e animais de laboratório. Assim, este estudo teve como objetivo investigar os efeitos tóxicos da acetona cianidrina, um metabólito da linamarina que está presente na mandioca, em ratos Wistar. Foram utilizados seis grupos de cinco animais cada para avaliar os efeitos tóxicos da acetona cianidrina administrada a 25 (G1), 50 (G2), 75 (G3), 100 (G4) e 125 (G5) umol / kg como uma dose oral única. O grupo controle recebeu água acidificada (pH 3,5). Os animais foram monitorados após a administração de acetona cianidrina e os sintomas clínicos foram registrados. Os níveis séricos das enzimas foram medidos para avaliar a função renal e hepática. Durante a necropsia, amostras teciduais foram coletadas para análise histopatológica. Após a administração, alguns animais dos grupos G2, G4 e G5 apresentaram sintomas neurológicos como convulsões, contração muscular involuntária, andar cambaleante, incordenação motora, prostração e midríase. Todos os animais do G5 e quatro animais do G4 morreram após sete minutos da administração de acetona cianidrina. Animais dos outros grupos, particularmente do G2, se recuperaram da fase aguda. A análise bioquímica revelou lesões e disfunção hepáticas. A histopatologia revelou lesões severas no fígado e cérebro. Os resultados permitem inferir que a acetona cianidrina tem efeitos tóxicos no fígado, pulmão e sistema nervoso central de ratos. No entanto, em concentrações de até 25 umol / kg os animais podem sobreviver à fase aguda.

Termos para indexação: Neurotóxico; hepatotóxico; cianeto; intoxicação aguda.

INTRODUCTION

Cassava (*Manihot esculenta)* has been cultivated since ancient times. It has a low cultivation cost and can grow even in poorly fertile soil. It is widely cultivated in tropical and subtropical regions worldwide. The foliage and roots are largely used as food for animal and human consumption (Blagbrough et al., 2010; Sornyotha; Kyu; Ratanakhanokchai, 2010).

Cassava leaves and roots contain two cyanogenic glycosides, linamarin and lotaustralin, which become potentially toxic upon hydrolysis by β-glycosidase. This releases cyanhydric acid (acetone cyanohydrin), which is unstable above pH 6 and spontaneously breaks down into acetone and hydrogen cyanide (HCN), a potent inhibitor of cytochrome-c oxidase of the mitochondrial respiratory chain (Sornyotha; Kyu; Ratanakhanokchai, 2010; Shama; Wasma, 2011) a high-cyanogen variety by using plant cell

wall-degrading enzymes, xylanase and cellulase. The combination of xylanase from Bacillus firmus K-1 and xylanase and cellulase from Paenibacillus curdlanolyticus B-6 at the ratio of 1:9 showed the maximum synergism at 1.8 times for hydrolyzing cassava cortex cell walls and releasing linamarase. Combined enzyme treatment enhanced linamarin liberation from the parenchyma by 90%. In addition, when the combined enzymes were applied for detoxification during cassava starch production, a low-cyanide-product was obtained with decreased linamarin concentration (96%).

Cyanide is detoxified by sulfuration catalyzed by rhodanese, a sulfur transferase mainly found in liver cells. Since neoplastic cells have low levels of rhodanese, cyanide has been used to inhibit the growth of tumor cells (Li et al., 2010; Ramalho, Aydos; Cereda, 2010). Recently, Ramalho et al. (2014) suggested that the selective action of the cyanide is due to a deficiency in their elimination observed in tumor cells as widely discussed in the literature.

Furthermore, anthelminthic properties of cassava have been reported in ruminants fed fresh cassava (Seng; Preston 2003; Granum et al., 2007; Marie-Magdeleine et al., 2010). Since toxic effects have been observed after ingestion of fresh cassava, drying, washing by immersion in water, and cooking are recommended to remove the cyanogenic compounds, and reduce its toxicity (Nwabueze; Odunsi, 2007; Silva et al., 2011).

Determination of the correct dosage in order to prevent poisoning is the major challenge associated with the use of cyanogenic compounds. Toxicological studies show that linamarin may or may not cause tissue lesions, depending on their concentration and/ or method of administration (Ramalho; Aydos; Cereda, 2010; Soler-Martín et al., 2010) 0.5, 1.0, 2.0, 10.0, 20.0 and 30.0 microg x mL^{-1}. This study was to determine the cytotoxic effects of single oral doses of various concentrations of acetone cyanohydrin in Wistar rats.

Clinical manifestations and changes in serum levels of alanine aminotransferase (ALT), phosphatase alkaline (AP), creatinine and urea were monitored to determine the cytotoxic effects. Histopathological examination of the lungs, kidneys, liver, heart and central nervous system was performed to evaluate tissue damage.

MATERIAL AND METHODS

The toxicity trials were conducted in the pathology sector of the Dom Bosco Catholic University in Campo Grande-MS, Brazil. Newly weaned Wistar rats with weights between 80 and 160 g from the UCDB Biotery were used in this study. During the experimental period, the animals were kept in large rectangular polypropylene boxes (49 × 34 × 16 mm), which were lined with wood shavings. The animals were kept in a room at a constant temperature, with a 12 h light/12 h dark cycle and proper ventilation, and were provided natural water and food *ad libitum*. All handling procedures followed international standards for the ethical use of animals for experiments. The study was approved by the Animal Ethics Committee - AEC protocol 006/2013.

Acetone cyanohydrin was synthesized as described by Cox and Stormont (1935). To evaluate the degree of purity, free cyanide synthesized from acetone cyanohydrin was compared with free cyanide from potassium cyanide (KCN VETEC™ 96% purity) according to the method described by Brito et al. (2009). The synthesized product had 99% purity.

The rats were divided into six groups of five animals each. The acetone cyanohydrin doses were calculated according to the weight of the animals in each group (Table 1). One mole of acetone cyanohydrin is equivalent to 85 g of acetone cyanohydrin, and one mole of acetone cyanohydrin contains 26 g of cyanide (CN$^-$) (Brito et al., 2009).

Table 1: Acetone cyanohydrin doses in µmol/kg body weight administered to each group of Wistar rats, and the equivalent values of C^{N-}.

Group	Weight	Concentration	Dose	CN$^-$
Control (GC)	80 ≥ 100 g	Acidified Water (pH 3.0 to 3.5)		
Group 1 (G1)	180 ≥ 205 g	25 µmol/kg	2.13 mg/kg	0.65 mg/kg
Group 2 (G2)	130 ≥ 160 g	50 µmol/kg	4.25 mg/kg	1.30 mg/kg
Group 3 (G3)	130 ≥ 160 g	75 µmol/kg	6.38 mg/kg	1.95 mg/kg
Group 4 (G4)	80 ≥ 100 g	100 µmol/kg	8.50 mg/kg	2.60 mg/kg
Group 5 (G5)	80 ≥ 100 g	125 µmol/kg	10.63 mg/kg	3.25 mg/kg

Acetone cyanohydrin was diluted to varying concentrations using acidified water at a pH of 3.0 to 3.5, which stabilizes it and prevents the volatilization of CN^- (Llorens, 2004; Soler-Martín et al., 2010).

The animals were monitored before the acetone cyanohydrin administration and during 48 hours following the administration, and symptoms were recorded.

After the animals' death, blood samples were obtained by cardiac puncture. The serum was allowed to clot and then separated for analysis. The levels of ALT and AP were determined to evaluate liver function. To evaluate the renal function, urea and serum creatinine were measured. Commercial kits using the kinetic-colorimetric method were purchased from Gold analyzes Diagnostics™ (Mendes; Lopes, 1973).

Only the animals that survived for a minimum of six hours after the oral administration of acetone cyanohydrin were included in the statistical analysis of the biochemical measurements. This was necessary since it takes six hours after a triggering event for tissue injury to occur and for the levels of serum liver enzymes (Vasconcelos; Almeida; Bachur, 2007), and renal function markers to increase, with both reaching maximum levels within approximately 48 hours, and declining after 72 hours.

The results of the biochemical measurements were statistically analyzed by the Mann-Whitney test, or by Student's unpaired t-test to compare the creatinine, urea, ALT, and AP levels between the following groups: GCxG1, GCxG2, GCxG3, GCxG4, and GCxG5. Additionally, the Kruskal-Wallis test was used to compare the creatinine, urea, ALT, and AP levels between GCxG1xG2xG3xG4xG5. Statistical analysis was performed using the statistical package InStat™ version 3.0 [DATASET.1SD] (1998), with a minimum of 5% representing significance.

The animals that died prior to 48 hours were immediately necropsied. During the necropsy, fragments of the heart, liver, kidney, and brain were collected. The samples were fixed in 10% formalin for preparation of histological slides, stained with Hematoxylin-Eosin (HE). The rats that survived for 48 hours post administration, including the control rats, were euthanized in accordance with the AEC/UCDB license (deep sedation with ketamine followed by CO_2), at the UCDB Pharmacology and Mutagenesis Laboratory. Tissues were considered altered when lesions were observed in more than 50% of the animals of each group.

RESULTS AND DISCUSSION

After the administration of acetone cyanohydrin, four animals from the G5 group (125 µmol) died. One animal had seizures and twitching seven minutes prior to death. One animal in the G5 group survived until the end of the experiment and displayed twitching, prostration, convulsions, and tetany; these symptoms disappeared after 40 minutes. All animals in G4 (100 µmol) died between zero and seven minutes after the administration of acetone cyanohydrin. These animals presented with staggering gait, motor coordination disability, prostration, and mydriasis.

One animal in the G2 group (50 µmol) died immediately after the administration of acetone cyanohydrin, while another animal presented with seizures and twitching; these symptoms appeared immediately after the administration of acetone cyanohydrin and lasted for 10 minutes. Animals of GC, G1, and G3 groups showed no apparent symptoms, and no death occurred within the 48-hour experimental period.

The severity of symptoms caused by cyanide have been shown to be dose-dependent, with higher concentrations causing faster cell death (Gupta, 2009). This explains why the animals that received high doses of acetone cyanohydrin (G5 125 µmol/kg and G4 100 µmol/kg) died after administration, while those receiving lower concentrations (up to 25 µmol/kg) survived the acute phase. Although individual characteristics may explain the fact of one animal in G5 had survived and animals in G2 had died.

Neurological symptoms such as incoordination, tetany, hyperexcitation, convulsions, and prostration preceded the acetone cyanohydrin-induced deaths observed in this study In severe cases of acetone cyanohydrin poisoning, periods of unconsciousness progressing to coma, convulsions and death have previously been reported (Hassan et al. 2015). It has been demonstrated that the cyanide initially stimulates and then depresses the CNS (Gupta 2009; Soler-Martín et al., 2010). Similar to what was reported by Hariharakrishnan, Satpute and Bhattacharya (2010), we observed that the effects of cyanide on motor activity were most evident in the acute poisoning phase. Convulsions with tetany and involuntary muscle contractions are most likely related to disturbance of calcium homeostasis (Mathangi; Namasivayam, 2004; Anwikar et al., 2011). In the CNS, manifestations of cyanide toxicity were more pronounced owing to the low capacity of this tissue to obtain energy through the anaerobic pathway, high energy demands, and small reserve of energy in the tissue.

The levels of AP were significantly different in the G1 (25 µmol), G2 (50 µmol), and G3 (75 µmol) groups compared to the levels of AP in GC group after 48 hours. We only subjected the biochemical measurements of G1, G2, G3, and GC groups to statistical analysis, because

G4 and G5 group animals died immediately after the administration of acetone cyanohydrin (Table 2). No statistical differences were observed in creatinine, urea, and ALT levels between groups. Although we did not observe significant differences in ALT levels between the test and control groups, the values obtained for ALT increased with increasing doses of acetone cyanohydrin.

We detected that the intensity of lesions was proportional to the concentration of acetone cyanohydrin. Animals belonging to the G5 (125 µmol) group had markedly severe morphological tissue changes compared to the other groups, while in the G1 group (25 µmol) only discrete changes were observed.

Hydropic or vacuolar degeneration of kidney tubular cells was observed in G5 and GC group animals (Figure 1a). In addition, a moderate quantity of intratubular protein was found in all groups (Figure 1b). The normal levels of serum

creatinine and urea observed in this study were consistent with the minimal renal changes observed. This observation indicates that the inactivation of cyanide by rhodanese was quite efficient, and as a result, there was insufficient formation of free radicals to promote renal cell membrane peroxidation (Aletor, 1993).

Histopathology of the lungs of animals of G3, G4 and G5 groups showed edema and inflammation. When compared to the GC group (Figure 2a), thickening of the alveolar septa was a finding observed in all treatment groups (Figures 2b, 2c, 2d). The inflammatory response observed in the parenchyma of the lung is associated with vascular changes in the local microcirculation that increase the capillary permeability, resulting in edema, hemorrhage, and congestion.

The main histopathological changes in the liver were hydropic degeneration of the hepatocytes and necrosis (Figure 3b), when compared to morphology of hepatocytes of the GC group (Figure 3a). Inflammatory

Table 2: Mean values and their standard deviations of the results of measurements of creatinine, urea, alanine amino transferase and alkaline phosphatase after the administration of acetone cyanohydrin.

Group	Creatinine	Urea	ALT	AP
	—— mg dL-1——		—— U L-1 ——	
Control (-)	0.6±0.1a	37.8±3.6a	121.4±29.0a	100.1±18.7a
1 (25 µmol)	0.6±0.1a	35.8±3.9a	143.9±22.0a	137.4±28.6b
2 (50 µmol)	0.5±0.1a	45.1±8.8a	152.0±46.3a	171.2±70.8b
3 (75 µmol)	0.5±0.1a	37.9±6.8a	161.9±32.3a	158.1±28.2b

Different letters on lines represent significant statistical difference among the groups. ALT: Alanine Amino Transferase. AP: Alkaline phosphatase.
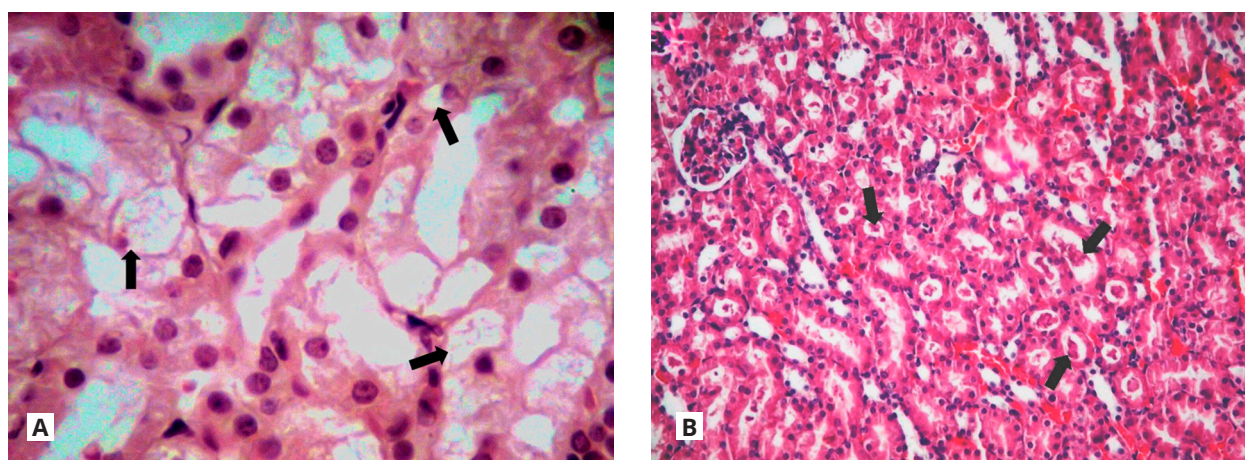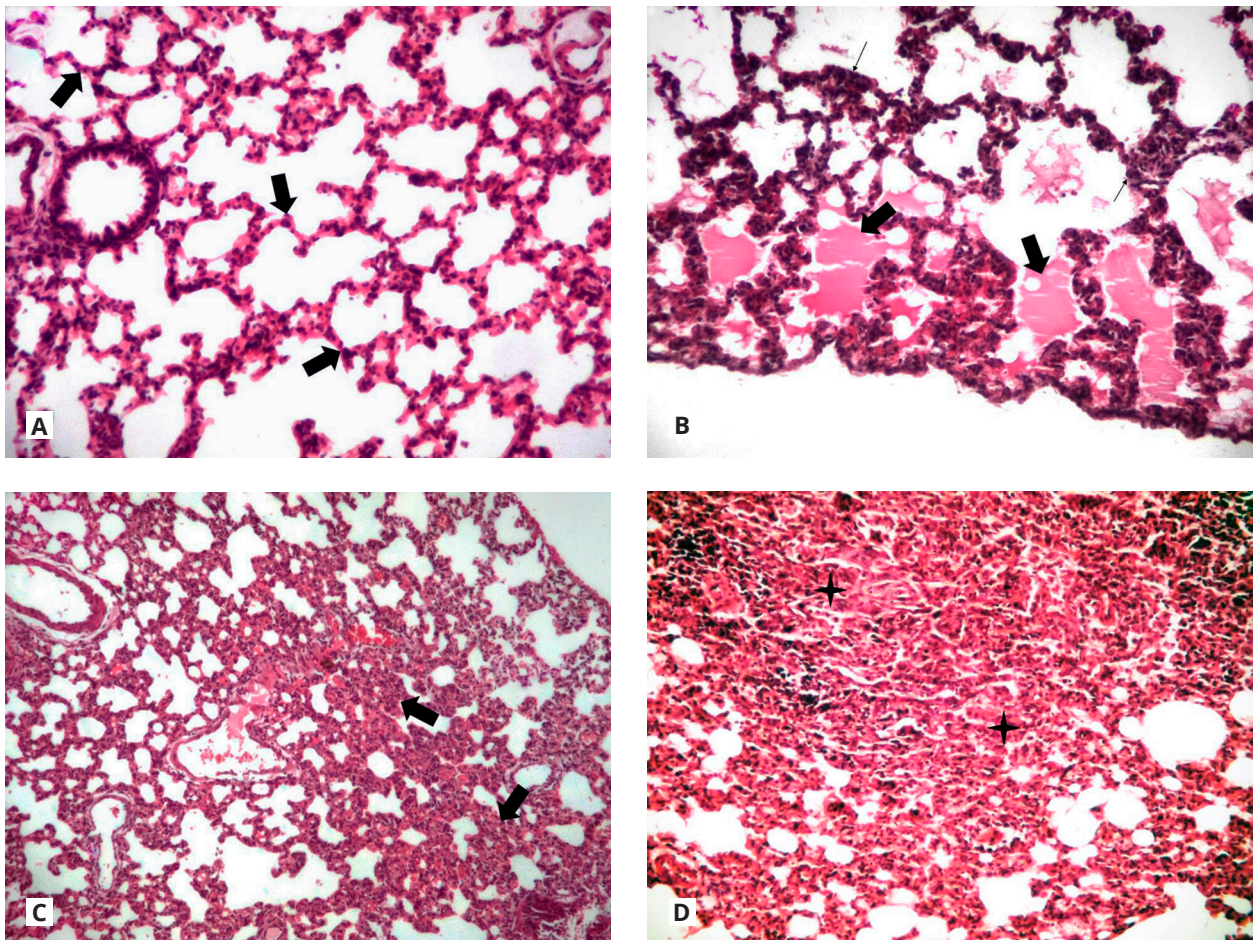

Figure 1: Photomicrograph of the kidney tissue of Wistar rats. (a) Tubular epithelium degeneration was observed in an animal from G2. Observe the vacuolated cytoplasm and lateral displacement of some nuclei (arrows) (HE, 40×). (b) Accumulation of intratubular protein in the proximal contoured tubules (arrows) was observed in an animal from G5 (HE, 4x).

infiltration in portal areas was also observed in animals that received higher concentrations of acetone cyanohydrin (G3 and G5 group) (Figure 3c). In fact, inflammatory cells are responsible for removing the debris of cellular catabolism, which, if not removed, act as irritant stimuli. In addition, pro-inflammatory cytokines were reported to have a significant role in the degeneration of hepatocytes, during the onset of liver diseases (Nagata; Suzuki; Sakaguchi, 2007). The slight dose-dependent increase in ALT levels is consistent with the extensive damage in the liver observed in the histological analysis of G4 and G5 groups.

CNS examination of the animals in the G5 group revealed neuronal degeneration (chromatolysis) (Figure 4a),

congestion, edema of the cerebrum, and demyelination of the cerebellum, as evidenced by the presence of large vacuoles in the white matter (Figure 4b).

The respiratory, locomotor, and neurological symptoms observed in this study, as well as the histopathological changes in the liver, lung, and CNS is most likely associated with cellular and biochemical factors induced by high concentrations of cyanide. These factors include: (i) the inability of hemoglobin to carry O_2 owing to the high affinity of cyanide to the ferric ion of heme, leading to tissue hypoxia (Hamel, 2011) and (ii) metabolic acidosis caused by high lactate concentrations that results in the inactivation of cytochrome oxidase

Figure 2: Photomicrograph of the lung tissue of Wistar rats. (a) A normal lung from an animal in the control group, demonstrating normal lung histology. The thickness (arrows) and delicate texture of the interalveolar septum (HE, 4x). (b) Pulmonary edema, noted by the filling of the alveoli by serous plasma exudate (large arrows) and thickness of interalveolar septa (thin arrows) were observed in an animal from G5 (HE, 20x). (c) Slight inflammatory reaction (arrows) consisting of mononuclear cells in an animal from G5 (HE, 10x). (d) Intense inflammatory reaction of mixed composition (star) was observed in an animal from G4. Note the filling of the alveolar spaces by inflammatory cells (HE, 10x).
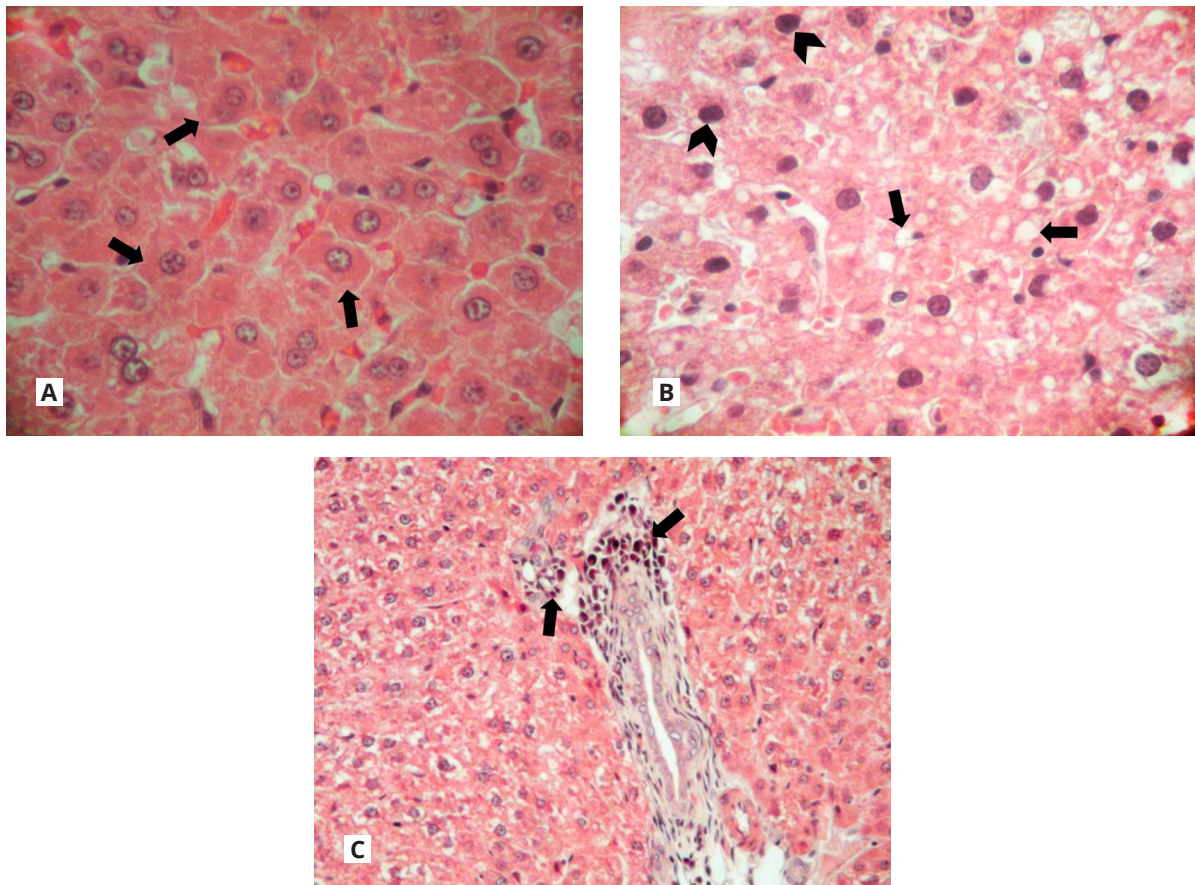
Figure 3: Photomicrograph of the liver tissue of Wistar rats. (a) Liver from an animal in the control group, with no morphological changes observed. Note the preservation of hepatocytes (HE, 40×). (b) Hydropic degeneration of the hepatocytes, necrosis observed by vacuolization of the cytoplasm of hepatocytes (arrows), and some pyknosis of the nuclei (head arrow) were observed in an animal from G5 (HE, 40x). (c) A slight inflammatory reaction of mixed composition (arrows) was observed in an animal from G3 (HE, 10x).
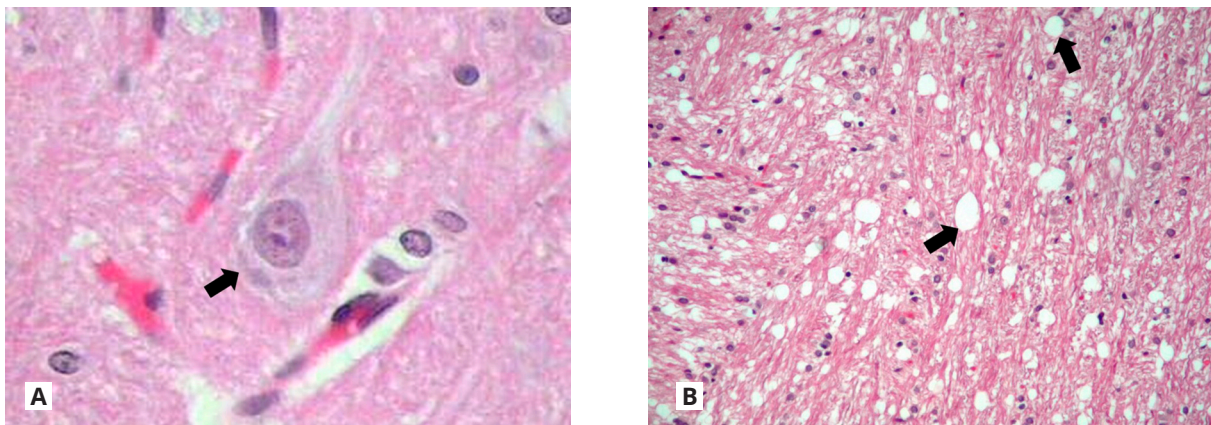
Figure 4: Photomicrograph of the central nervous system tissue of Wistar rats. (a) Neuronal degeneration (arrow) was observed in an animal from G5 (HE, 40x). (b) Neuropil spongiosis (star) in an animal of G5. Observe numerous vacuoles in different sizes (arrows) (HE, 40x).

a3 (Beasley; Glass, 1998). Inhibition of the respiratory chain (Sornyotha; Kyu; Ratanakhanokchai, 2010; Shama; Wasma, 2011) compromises ATP production and proper functioning of the calcium pump, leading to an imbalance in Ca^{++} concentrations in the intra- and extracellular spaces. An increase in intracytoplasmic calcium leads to free radical generation, lipid peroxidation, mitochondrial dysfunction, and consequently irreversible cell injury (Kamalu, 1995). Furthermore, the accumulation of calcium leads to the release of intracellular enzymes, with detrimental effects to the cells.

Additionally, liver damage as observed in the present study, causes hyperammonemia and consequently encephalopathy, leading to loss of consciousness (Yamamoto, 1989). Moreover, the intracellular accumulation of Ca^{++} decreases the amount of gamma-aminobutyric acid (GABA), and elevates glutamate levels in the CNS. GABA and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, in the mammalian CNS; both play an important role in the regulation of neuronal excitability (Hariharakrishnan; Satpute; Bhattacharya, 2010). Persson, Cassel and Sellström (1985) observed cyanide-induced decrease in GABA levels in the brains of rats.

CONCLUSIONS

The nervous system symptoms and tissue damage observed in Wistar rats after oral administration of acetone cyanohydrin is dose-dependent. At concentrations lower than 25 μmol/kg body weight, the animals generally survive the acute phase with minor morphologic tissue changes.

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