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Phenytoin-induced toxicity in the postnatal cerebellar development in rat: effect of *calotropis procera* on selective biochemical and haematological variables

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ABSTRACT

Phenytoin, an antiepileptic drug is used in managing seizures. Phenytoin-associated oxidative stress causes cellular damage by the generation of free radicals. Vitamin C, a standard antioxidant and *Calotropis procera* are believed to scavenge oxygen free radicals. The effect of *C. procera* extract on haematological and biochemical variables in an *in-vivo* model was studied. Pregnant rats were administered phenytoin (50 mg/kg body weight). Extracts of *C. procera* (300 mg/kg body weight) and vitamin C (200 mg/kg body weight) were administered one hour prior to phenytoin treatment separately, while control animals received tap water only. The animals had access to food and water *ad libitum*. Blood was collected from animals on day 50 postpartum for packed cell volume (PCV), haemoglobin (Hb) content and evaluation of levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) evaluation. Lipid peroxidase (LPO) and reduced glutathione (GSH) levels in the cerebellum were assessed as markers of oxidative stress on day 50 postpartum. Phenytoin-induced toxicity was associated with increased cerebellar LPO and decreased GSH levels. Increase in ALT and AST levels in the serum was observed. However, PCV and Hb levels were not affected. LPO, GSH, ALT and AST levels registered a tendency to shift towards normalcy on administration of *C. procera* and vitamin C to phenytoin. In conclusion, supplementation with *C. procera* leaf extract reduced the rate at which phenytoin induced toxicity in developing rat cerebellum postnatally.

Keywords: Phenytoin, *Calotropis procera*, cerebellar development, oxidative stress, antioxidants

INTRODUCTION

Phenytoin is prescribed for infants with seizure disorders (Appleton et al., 2002; Buck, 1996; McNamara, 2001). The behavioral teratogenicity induced on prenatal exposure to phenytoin is well established (Vorhees, 1994; Tachibana, 1996). Phenytoin is thought to cause chronic intrauterine hypoxia/ischaemia and embryo-foetal toxicity via reacting oxygen intermediates. Reacting oxygen species (ROS) can oxidize molecular targets such as Deoxynucleic acid (DNA), protein, lipid in a process called oxidative stress resulting in cellular dysfunction and in utero death or teratogenicity (Wells et al., 1996; Zablocka and Janusz, 2008).

Oxidative stress occurs due to an imbalance in prooxidant and antioxidant levels. Scientific evidence suggests that under oxidative stress conditions, oxygen radicals such as superoxide anion (O_2^-), hydroxyl radical (OH^-) and peroxyl radicals (ROO^-) are produced in biological systems. These oxygen radicals play important roles in degenerative processes such as ageing, cardiovascular diseases, cancer, Alzheimer's disease, stroke, Parkinson's disease and other neurodegenerative diseases, (Ames, 1983; Gey, 1990; Smith et al., 1996; Temple, 2000; Chatterjee et al., 2007). Phenytoin administration has been reported to be associated with cerebellar atrophy and persistent cerebellar ataxia with clinical manifestation presenting as confusion, slurred speech and nastagmus following acute and long-term intoxication (Imamura et al., 1992; Ney et al., 1994) and temporal cerebellar atrophy following phenytoin therapy (Guerrero et al., 1997). To prevent free radical-induced cellular damage generated by phenytoin and other neurotoxicants, the human body has developed a defense mechanism-the antioxidants system. This system includes, endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) and glutathione reductase (GSSGR), low molecular antioxidants such as glutathione (GSH) and plasma proteins, and exogenous antioxidants such as vitamins C, E and β-carotene (Zablocka and Janusz, 2008). Glutathione plays a key role in maintaining the physiological balance between pro-oxidant and antioxidants. Plasma proteins, vitamins C and E can inhibit ROS generation and lipid peroxidation by chelating free transition metals such as copper and iron (Zablocka and Janusz, 2008).

The antioxidant activity of some plants such as *Kolaviron, Calotropis procera* and *Vernonia amygdalina* which are thought to scavenge the oxygen free radicals have been documented (Rao and Dubey, 1990). *C. procera* also known as the giant milk weed is one of the flowering plants commonly found in tropical regions of the world. The plant is found in almost all parts of Nigeria but more abundantly in the Northern part of the country (Sofowora, 1984). *C. procera* is a perennial grayish green, woody shrub with broad ovate fleshy leaves that grow wild in the tropics and warm temperate regions (Huber, 1985; Hussein et al., 1994). It derived its common name, giant milk weed, from the thick white sap which oozes from a cut stem or from the stem when a leaf is plucked off. Hence, the family to which the plant belongs is referred to as milkweed family (Ghazanfar, 1989). Edman (1983), Mossa et al. (1991), Al-Robai et al. (1993a) and Hussein et al. (1994) reported the presence of alkaloids, flavonoids, cardiac glycosides, tannins, triterpenes steroids and uscharin in the entire plant of *C. procera*. Ethnomedical practitioners have paid considerable attention to the use of *C. procera* (NAPRALET, 1984). Anis and Iqbal (1986) showed that *C. procera* has antimalarial and anticholeral effects when used as one of the multi component decoction in traditional medicine. Srivastava et al. (1962) reported fibrinolytic and anticoagulant activities of *C. procera*, while Tariq et al. (1984) demonstrated the neuromuscular blocking activity of the plant in mouse. Padhy et al. (2007) evaluated the antioxidant and anti-inflammatory properties of the latex of *C. procera* for its hepatoprotective effect against carbon tetrachloride-induced hepatotoxicity in rats. A number of clinical studies suggest that the antioxidants in fruits and vegetables are key factors in reducing the incidence of chronic diseases including heart disease and some cancers (Salah et al., 1995; Gerber et al., 2002; Kris-Etherton et al., 2002; Serafini et al., 2002). This research will test the hypothesis that leaf extracts of *C. procera*, can protect or reduce phenytoin-induced oxidative stress in developing rat cerebellum postnatally.

MATERIALS AND METHODS

Animals

Twenty sexually matured female rats weighing about 160 g of Wistar strain were obtained from the central animal house of the Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Oyo State. The animals were randomly divided into five groups of four animals per group. They were mated and pregnancy confirmed by the presence of vaginal plug. The animals were fed with standard diet of rat pellets and water provided *ad libitum*. All procedures on animal handling conformed to the acceptable guidelines on the ethical use of animals in research.

Drug preparation and administration

Phenytoin dissolved in tap water was administered orally in pre and postnatal life at the dose of 50 mg/kg. Sodium salt of Phenytoin (capsule) manufactured by Man care Pharmaceuticals PVT Ltd India purchased from the pharmacy, University College Hospital (UCH), Ibadan was used for the experiment.

Two hundred (200) mg/kg body weight vitamin C was administered one hour orally prior to phenytoin administration, to the experimental animals in pre and post natal life.

Extraction of *C. procera* leaves

The leaves of *C. procera* were harvested within the main campus of the University of Ibadan and authenticated by Dr. O.A. Ugbogu of the Forestry Research Institute of Nigeria (FRIN), Ibadan with a Forestry Harberium Identification number (FHI) 108221. They were air-dried at room temperature for two and a half months, blended and made into powdered form of about 1.2 kg. Cold methanolic extraction for about 72 hours was done. The methanolic solution was concentrated with rotatory evaporator at a temperature below 50 ^{o}C for 7 hours. The concentrated extract (187.0 g) was stored in the refrigerator until use. The concentrated extract was reconstituted with water before administration.

Phytochemical studies of the leaves of *C. procera* was done in the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan. The following compounds were screened for: alkaloids, flavonoids, cardenolides, saponins and tannins.

Three hundred (300) mg/kg body weight methanolic extract of *C. procera* was administered one hour orally prior to phenytoin administration, to the experimental animals in pre and post natal life. The dose of *C. procera* extracts used in the research was based on a safety evaluation studies carried out by Mossa et al. (1991) that the use of the extract in a single high doses (up to 3 g/kg body weight) does not produce any visible toxic symptoms or mortality.

Grouping of animals

Group I: Control group received water
Group II: Received 50 mg/kg phenytoin
Group III: Received 200 mg/kg Vitamin C + phenytoin
Group IV: Received 300 mg/kg *Calotropis procera*
Group V: Received *Calotropis procera* + phenytoin

At the end of the experiment, blood samples were collected under anaesthesia from the animals on day 50 postpartum (ten per group), five animals for haematological studies, pack cell volume (PCV) and haemoglobin (Hb) content, and five animals (serum) hepatocellular enzyme assays, alanine transaminase (ALT) and aspartate transaminase (AST). The animals (five per group) were then killed, the cerebellum dissected out and preserved in phosphate buffer at 4 ^{o}C and a pH of 7.4 for lipid peroxidation (LPO) and reduced glutathione (GSH) levels.

Estimation of packed cell volume (PCV) and haemoglobin (Hb)

Having collected the blood samples from day 50 postpartum animals into the EDTA bottles, heparinized capillary tubes were used to collecte blood from the EDTA bottles. One end of the capillary tube was sealed with plastacin. The capillary tubes were then centrifuged using a microhaematocrit centrifuge at 5000 g, after which the PCV were determined using a haematocrit reader. PCV was expressed in percentage. Haemoglobin concentration was estimated from the PCV by dividing the value of the PCV by a factor of 3. It was expressed in mg/dl.

Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities

The quantitative *in vitro* determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were carried out with the aid of Randox kits. (Randox Laboratories Ltd., United Kingdom). ALT and AST activities were determined following the principles described by Reitman and Frankel (1957).

Assessment of lipid peroxidation (LPO)

Lipid-peroxidation was determined by measuring the thiobarbituric acid reactive (TBAR) products present in the cerebellar tissue using the procedure of Vashney and Kale (1970) and expressed as micromolar of malondialdehyde (MDA)/g tissue.

Determination of reduced glutathione (GSH) levels

The levels of reduced glutathione (GSH) in the cerebellar tissues were determined by the method described by Beutler et al. (1963).

Statistical analysis

The data obtained was further analyzed with unpaired student *t*-test using computer software, Microsoft Excel. The Mean, Standard Deviation, SD, and the level of significance at 95% Confidence Interval calculated.

RESULTS

Phytochemistry

Phytochemical studies carried out on the leaves of *C. procera* showed that it contained mainly flavonoids, alkaloids and some cardenolides.

Haematological changes in the blood samples of day 50 postpartum animals

No significant difference was observed in the packed cell volume and haemoglobin content in the blood samples on day 50 postpartum in the control and treated animals, $P>0.05$ (Table 1).

Biochemical changes in the cerebellum of day 50 animals postpartum

An increased rate of lipid peroxidation (LPO) (4.00±2.19 µmol/g cerebellar tissue vs 1.8±1.49 µmol/g cerebellar tissue) and a decreased glutathione (GSH) levels (1.6±0.5 µg/cerebellar tissue vs 2.0±0.7 µg/cerebellar tissue) at $P>0.05$ was observed in phenytoin-treated animals when compared with the control group. Administration of vitamin C and extracts of *C. procera* to phenytoin-treated animals decreased the rate of lipid peroxidation and increased the glutathione levels in the cerebellum when compared with phenytoin-treated animals (Table 2).

Changes in the hepatocellular enzymes, ALT and AST on day 50 postpartum

A non significant elevation in ALT, $P>0.05$ and a significant elevation in AST, $P<0.05$ (203.4±34.5 IU/l vs 162.0±15.5 IU/l) was seen in the phenytoin-treated animals compared with the control group. Administration of vitamins C and extracts of *C. procera* decreased the total activities of ALT and AST at $P>0.05$ when compared with phenytoin-treated animals. Vitamin C administered to phenytoin-treated animals significantly decreased the level of ALT when compared with phenytoin-treated group, $P<0.05$ (60.0±3.8 IU/l vs 73.6±10.4 IU/l) (Table 3).

Table 1: Packed cell volume (PCV) and Haemoglobin level (Hb) in the control and treated groups on day 50 postpartum.

Parameter/Gp	PCV (%)	Hb (mg/dl)
Control	39.6 ± 1.8	13.2 ± 0.6
Pheny	38.8 ± 3.1	13.0 ± 1.1
Pheny+vit C	40.4 ± 2.1	13.5 ± 0.6
Cp	39.0 ± 1.9	13.0 ± 0.5
Pheny+*cp*	40.0 ± 2.4	13.4 ± 0.8

Values are given as mean±SD, (n=5). Pheny=phenytoin; vit C=vitamin C; *Cp= Calotropis procera* extract. P>0.05 in the treated groups versus the control group.

Table 2: Reduced glutathione (GSH) levels and lipid peroxidation (LPO) in the cerebellum of the control and treated groups on day 50 postpartum

Parameters/Gp	GSH	LPO
Control	2.0 ± 0.7	1.80 ± 1.49
Pheny	1.6 ± 0.5	4.00 ± 2.19
Pheny+vit C	1.8 ± 0.4	2.40 ± 0.90
Cp	2.0 ± 0.0	2.43 ± 0.21
Pheny+*Cp*	1.8 ± 0.8	2.44 ± 0.12

Values are given as mean±SD, (n=5). Reduced GSH is expressed in µg/g tissue. Rate of LPO is expressed in µmmol/g tissue. Gp= group; Pheny=phenytoin; vit C=vitamin C; *Cp= Calotropis procera* extract. P>0.05 in the treated groups versus the control group.

Table 3: Alanin aminotransferase (ALT) and Aspartate aminotransferase (AST) levels in the control and treated groups on day 50 postpartum

Parameters/Gp	Total activity of ALT	Total activity of AST
Control	65.2 ± 7.1	162.0 ± 15.5
Pheny	73.6 ± 10.4	203.4 ± 34.5[a]
Pheny+vit C	60.0 ± 3.8 [d]	168.2 ± 13.1
Cp	68.8 ± 2.4	169.4 ± 14.1
Pheny+*cp*	67.2 ± 6.2	168.6 ± 11.0

Values are given as mean±SD, (n=5). Total activity of ALT is expressed in IU/l. activity of ALT is expressed in IU/l. Gp= group; Pheny=phenytoin; vit C=vitamin C; *Cp= Calotropis procera* extract. [a]P<0.05 versus control, [d]P<0.05 vs phenytoin group.

DISCUSSION

Phenytoin has been found to induce neurotoxicity by generating free radicals resulting in oxidative stress which leads to cellular damage and dysfunction (Liu et al., 1997). The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods and / or supplements. These antioxidants act as “free radical scavengers” by preventing and repairing

damages caused by ROS, and therefore can enhance the immune defenses and lower the risk of cancer and degenerative diseases (Valko et al., 2006).

The antiepileptic drug concentration profiled in breast milk follows the plasma concentration curve (Crawford, 2005). The total amount of drug transferred to infants via breast milk is usually much smaller than the amount transferred via the placenta during pregnancy. However, as drug elimination mechanisms are not fully developed in early infancy, repeated administration of antiepileptic drug via breast milk may lead to accumulation in the infant (Crawford, 2005). The results of the haematological parameters, PCV and Hb in this present study showed no statistically significant difference from the values of the control and other treated groups. These results could mean that anaemia was not induced and tend to confirm the works of Weber et al. (1977) that the haematological parameters (red cell foliate, red blood cell and haemoglobin count) did not show any alterations and no patient developed any sign of anaemia on long-term phenytoin treatment, several months and years after administration.

A number of observations suggest that detoxification of a xenobiotic free radical intermediate with antioxidants may provide embryoprotection (Wells et al., 1997). Glutathione, a low molecular weight endogenous antioxidant may be involved in the detoxification of a teratogenic reactive intermediate of phenytoin and/or in cytoprotection against oxidative stress. Glutathione plays a key role in maintaining the physiological balance between pro-oxidants and antioxidants. It has been reported to be first line of defense against oxidative stress (Xi and Chen, 2000). Glutathione depletors or inhibitors of glutathione synthesis potentiate phenytoin teratogenicity in mice (Wong and Wells, 1989).

In the present study, the decrease in the glutathione levels in the brain of phenytoin treated animals could be positively correlated to the increased rate of lipid peroxidation observed in the brain. Increased generation of ROS and LPO followed by apoptosis has been shown recently in cultures of cortical neurons from foetal brains with Down's syndrome (Busciglio and Yanker, 1995). The known moderate cerebellar hypoplasia in patients with Down's syndrome might reflect the effect of early degeneration of cerebellar progenitor cells by increased LPO and apoptosis *in vivo* (Olanow, 1993). Increased malondialdehyde, one of the end products of LPO can be interpreted as the result of cellular membrane damage initially caused by increased formation of radicals from LPO. Johnson et al. (1986) reported that increased intraneuronal calcium and lipid peroxidation might be mechanisms by which xenobiotics produce nerve injury.

Ferriero (2001) indicated that the increased susceptibility to oxidative injury can be explained by several mechanisms that facilitate the production of ROS from the increased presence of hydrogen peroxide that tends to accumulate because of immature defense mechanisms. It is indicated that the inadequate scavenging abilities inherent to developing systems are not sufficient to maintain glutathione store in neonatal neural tissue that also contains more available iron than is present in mature neural tissue. Glutathione depletion observed in the phenytoin-treated animals may be a contributory factor in the cascade of events leading to neurotoxicity in the developing cerebellum of the rats.

Vitamin C and extracts of *C. procera* extracts improved the glutathione levels and reduced the rate at which lipid peroxidation occurred in the brain. The increased LPO and reduced GSH registered a tendency to shift

towards normalcy on co-administration of *C. procera* extracts. This confirms the position of *C. procera* as one important member of the flavonoid's family in ameliorating the effect of oxidative stress in biological tissues. The mechanism of the antioxidant activity demonstrated by *C. procera* and its fractions were not specifically studied in this research. However, Roy et al. (2005) reported that *C. procera* extracts increased hepatic levels of endogenous antioxidants such as superoxide dismutase, catalase and glutathione in alloxan-induced diabetes and that the antioxidant and antidiabetic properties of the extract was comparable to standard antidiabetic drug, glibenclamide. In this study, the methanolic extract of the leaves of *C. procera* was found to possess strong antioxidant activity which is comparable to the standard antioxidant supplement, vitamin C. The free radical scavenging property of *C. procera* may be one of the mechanisms by which the drug is effective as a traditional medicine.

About four to five percent of phenytoin is excreted unchanged in the urine and the half-life is 6-24 hours while the remainder is metabolized by hepatic microsomal enzymes (Rang and Dale, 1987). ALT and AST enzymes are excellent markers of hepatocellular injury reflecting, the hepatocellular integrity of the liver. Hepatocellular injury, and not necessarily cell death, is the trigger for the release of these enzymes into circulation (Katkov et al., 1991). The release of these cellular enzymes is one of the most sensitive parameters for the recognition of minute disturbances of cellular integrity. The elevation of these enzymes in the phenytoin treated animals showed a leakage of the enzymes from its intracellular compartment into the blood which may be due to relatively low oxygenation. These findings support the concept of phenytoin chronic intrauterine hypoxia/ischaemia and embryo-foetal toxicity (Wells et al., 1996). Vitamins C and extracts of *C. procera* decreased the ALT and AST levels probably by inhibit the generation of reacting oxygen species (ROS) and oxidative stress induced by phenytoin thereby preventing the release of free radical intermediates, which have been reported by Zablocka and Janusz (2008) to cause tissue necrosis and cellular damage.

In conclusion, a shift in the levels of reactive oxygen species towards pro-oxidants in developing rat brain can induce an oxidative stress on the cerebellum. To this end, the present study has shown that phenytoin administered to rats in pre and postnatal life induced oxidative stress in the cerebellum. The administration of vitamins C and methanolic leaf extracts of *Calotropis procera* appeared to reverse these changes when compared with the phenytoin-treated animals and as such tend to reduce the rate at which phenytoin induced toxicity in the developing rat cerebellum postnatally. Since the exposure to oxidants such as antiepileptic drugs (phenytoin) in the first trimester has been seen to be associated with an increased risk of major congenital anomalies (as most vital organs in the body develop within this period) in offspring, vitamins C and plants extract containing high amount of flavonoids, alkaloids and tannins supplementation in diets of pregnant epileptics receiving long term phenytoin therapy should be prescribed by physicians within this period and throughout pregnancy. Also, the antioxidants properties of these plants extract may prevent oxidative damage in neurodegenerative diseases and as such play a critical role in wellness and health maintenance. Characterization of the different fractions of *C. procera* should be further investigated to determine the maximum antioxidant activity. Also, as a result of the high medicinal and pharmacological values, availability and affordability of *C. procera*,

and the results obtained from this study, further pharmacological and toxicological studies are required to establish the therapeutic uses of the plant and particularly with its active principles, so as to advise ethnomedical practitioners on the dosage and usage of the plant.

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