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ORIGINAL ARTICLE

Inhibition of hepatic energy metabolizing enzymes in murine model exposed to diisononyl phthalate

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ABSTRACT

Background and objectives: Diisononyl phthalate (DINP) is a class of phthalates and phthalates are known to be metabolism disrupting chemicals (MDCs). Numerous MDCs, to which humans are exposed, have an effect on every aspect of energy transduction. They affect the liver by impairing insulin secretion in pancreatic cells and altering the liver's insulin-dependent glucose metabolism.
Methods: For this study, eighteen male albino rats weighing 200±20g were randomly assigned to three groups (of six rats each) and followed for a 14-days period. The groups were: group A or control which was given Tween-80 orally, group B or $DINP_1$ group which was given 20 mg/kg b.wt. DINP, and Group C or $DINP_2$ group which received 200 mg/kg b.wt. DINP. The rats were then sacrificed, their livers were removed, and the glycolytic and oxidative phosphorylation enzyme activities were evaluated.
Results: Activities of the glycolytic, tricarboxylic acid cycle and electron transport chain enzymes under investigation were significantly down-regulated with severity observed in decreased activities of hepatic oxidative phosphorylation enzymes when compared with control (P<0.05). Hepatic tissue sections of 20 and 200mg/kg DiNP group revealed distorted cytoarchitecture of hepatocytes ranging from histocellular disarrangement to vaocular changes suggestive of loss of liver integrity or fibrosis.
Conclusions: Finally, DINP exposure impairs hepatic energy transduction enzymes as evident in down-regulation of the various enzymes of energy metabolism under investigation and this may invariably be a good tool for diagnosis for hepatic energy impairment as seen in some disease conditions.

Keywords diisononyl phthalate, liver, oxidative phosphorylation, phthalates

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INTRODUCTION

The liver is an important metabolic organ that controls the body's energy metabolism. It serves as a central metabolic node for a number of tissues, such as skeletal muscle and adipose tissue.[1] After food is broken down in the gastrointestinal tract, the nutrients such

as glucose, fatty acids, and amino acids are absorbed by the walls of small intestine into the bloodstream; and then, they are carried to the liver by the portal venous system.[1] Metabolic activities of the liver are regulated by the insulin and other metabolic hormones.[1]

Glycolysis converts glucose to pyruvate in the cytoplasm. The mitochondrial tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation completely oxidize pyruvate to produce ATP. In the fed state, *de novo* lipogenesis produces fatty acids from glycolytic waste products.[2] Hepatocytes convert long-chain fatty acids into triacylglycerol, phospholipids, and cholesterol esters. The complex lipids are then either kept in lipid droplets and membrane structures or released into the bloodstream as very low-density lipoprotein (VLDL) particles.[2] The liver secretes glucose while fasting through gluconeogenesis and glycogenolysis. Hepatic gluconeogenesis is the primary endogenous glucose generator during prolonged fasting.[2] Fasting also encourages the release of non-esterified fatty acids from adipose tissue, which are then converted into ketone bodies in the liver via mitochondrial oxidation and ketogenesis. Extrahepatic tissues can use ketone bodies as a source of metabolic energy.[2] Neuronal and hormonal systems have a strong grip on the regulation of liver metabolic activities.[2]

Phthalates are 1,2-dibenzene dicarboxylic acid esters that are primarily used as plasticizers to enhance the transparency, toughness, flexibility, and lifespan of plastics. They are extensively used in the production of a variety of cosmetics, paints, adhesives, and plastics.[3] Because phthalates are weakly bonded to their parent substances, they can easily leak, causing environmental contamination and a variety of public exposures. When using a product that contains phthalates, a person may be exposed to them through their breath, food, and skin as well as through direct contact with contaminated environments.[1] Exposure to phthalates and other endocrine disrupting chemicals (EDCs) generated by plastic, also referred as metabolism-disrupting chemicals (MDCs), has raised concerns about human health.[1]

The extensively used plasticizer diisononyl phthalate (DiNP) is the diisononyl ester of benzene-1,2-dicarboxylic acid. Di(2-ethylhexyl) phthalate (DeHP), one of the previously used phthalates, has been linked to toxicity issues.[4] Thus, substitutes such DiNP have been examined. Disruption of energy metabolism have been strongly linked to DiNP in prior investigations on experimental animals involving the heart and testes.[5,6] Therefore, this study was designed to investigate the potential energy metabolic disruptions in the liver by DINP using glycolytic, TCA and electron transport chain (ETC) enzymes as markers for evaluation.

MATERIALS AND METHODS

Chemicals and reagents

CYPRESS® Diagnostics (Langdorp, Belgium) supplied the lactate dehydrogenase test kit while Relonchem Ltd. (Gorsey Lane, Widnes, Cheshire, UK) provided the DINP. All other chemicals were of the highest analytical grade available, including phospho-

enolpyruvate, mannitol, sorbitol, sucrose, glucose-6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide (NADH), fructose-1,6-bisphosphate, succinate, oxaloacetate, and rotenone was products of Sigma-Aldrich (USA), and BDH Chemicals Ltd. (England, UK).

Animal studies and experimental design

Eighteen male albino rats weighing 200±20g and aging 10 weeks were procured from College of Medicine Animal House of the University of Ibadan (Nigeria). The animals were housed in compartmentalized cages for acclimatization and treatment at the animal facility of Ajayi Crowther University (Nigeria). The rats were given a week to adjust and were allowed unlimited access to pelletized food and water. The Departmental Committee on Animal Ethics approved the use of the animals (FNS/ERC/2021/006). The animals were divided into three groups randomly (each group with six rats) and each group received a different treatment for 14 days, as follows: The Group A (or control) received Tween-80 orally, Group B (or $DINP_1$) rats are given DINP of 20 mg/kg b.wt., and Group C ($DINP_2$) rats are given DINP of 200 mg/kg b.wt. Oral route of administration was used and earlier studies led to the choice of 20 and 200 mg/kg/day DiNP.[4,6,7]

Sample collection

The official guidelines for the care and management of laboratory animals were followed when handling and caring for the rats.[8] After the final administration, rats were sacrificed, and liver excised. The removed liver, using a potter-elevehjem homogenizer, was homogenized in 0.1M phosphate buffer (pH 7.4) after being washed in ice-cold 1.15 percent KCl, blotted, and weighed. Centrifugation of the homogenate was done at $10{,}000_g$ for 15 minutes at 4 ^oC to obtain the supernatant for further biochemical analyses.

Isolation of mitochondrial fraction

The liver of male wistar rats was processed, and the mitochondria were isolated, as previously described by Fernández-Vizarra (2010).[9]

Determination of activities of glycolytic enzymes

Determination of hepatic hexokinase activity

To determine the activity of hexokinase (HK), the technique previously described by Colowick (1973) was used.[10] The assay's basic principle is based on the fact NAD^+ will decrease when glucose-6-phosphate dehydrogenase is coupled (G-6-P-D) with it. Following an increase in absorbance, the activity was determined spectrophotometrically at 340nm.

Determination of hepatic phosphofructokinase activity

To determine the phosphofructokinase (PFK) activity, the change in absorbance at 340nm was measured after adding 10 to 20 uL of homogenate to reach a final volume of 1 ml of test mix (pH= 8.0), which comprised 2mM ATP and 4mM fructose-6-phosphate.[11]

Determination of hepatic aldolase activity

The method described previously by Jagannathan et al. (1956) which entailed generating a hydrazone (Boyer's adaption of the hydrazine assay) by reacting 3-phosphoglyceraldehyde with hydrazine, was utilized to determine the aldolase (ALD) activity.[12] The generated hydrazone is measured spectrophotometrically at 240nm.

Determination of the activity of hepatic lactate dehydrogenase

The lactate dehydrogenase (LDH) kit was used to determine LDH activity according to the manufacturer's instructions (LABKIT). The rate of pyruvate concentration reduction by NADH, as measured spectrophotometrically at 340 nm, is determined by the amount of catalytic lactate dehydrogenase present in the sample.

Determination of hepatic NADase activity

NADase activity was evaluated by the Tatsuno et al. method (2007).[13] The maximum absorbance is found at 340nm in the addition product produced when cyanide interacts with NAD+'s quaternary nitrogen form.

Assay of mitochondrial metabolizing enzymes

Determination of hepatic citrate synthase activity

Using a previously described spectrophotometric enzyme test, the citrate synthase (CS) activity was measured.[14] By detecting the reaction product thionitrobenzoic acid (TNB) of DTNB and CoASH at 412nm, citrate synthase activity was determined. The rate of absorbance increase is inversely correlated to enzyme activity.

Determination of hepatic isocitrate dehydrogenase activity

Romkina and Kiriukhin (2017) previously reported that the decrease in NAD^+ at 340nm was used to evaluate isocitrate dehydrogenase (IDH) activity.[15]

Determination of hepatic succinate dehydrogenase activity

Succinate dehydrogenase (SDH) catalyzes the reaction that changes succinate into fumarate. The activity of SDH was assessed using the procedure described by Dervartanian and Veeger (1964).[16]

Determination of hepatic malate dehydrogenase activity

According to López-Calcagno et al. (2009), malate dehydrogenase (MDH) activity was determined by measuring the decrease in absorbance at 340nm brought on by NADH oxidation when L-malate and oxaloacetate are interconverted by MDH using nicotinamide adenine dinucleotide (NAD) as a catalyst.[17]

Determination of hepatic Complex I-IV activity

As described previously, the activities of Complex I (NADH ubiquinone oxidoreductase), Complex II (succinate ubiquinone oxidoreductase), Complex III (cytochrome c oxidoreductase), and Complex IV (Cytochrome C Oxidase) were determined spectrophotometrically in isolated hepatic mitochondria.[18]

Total protein determination

According to Gornall et al. (1986), the maximum absorbance of the purple-colored chelate produced between Cu^{2+} and the protein's peptide bonds at 540nm was used to calculate the total protein concentration of the mitochondria isolation.[19]

Histopathological examination

The liver was cut into 4 μm frozen sections and stored at 20°C. Following that, the sections were fixed in 4% paraformaldehyde at room temperature for 30 minutes, stained with 0.3% oil red O solution for 30 minutes, counter-stained with hematoxylin and eosin (H&E) for 30 seconds, and sealed with glycerin.[20] An optical microscope (X200) was utilized to examine and evaluate each kidney slide. Normal appearance (—), mild (+), moderate (++), and severe (+++) were the scores assigned for the extent of the changes that were observed.

Statistical analysis

The results are presented as mean±SEM. Analysis of variance (ANOVA) and Tukey's test were used to analyze data statistically. All analyses were performed with GraphPad Prism® version 8. *P* values less than 0.05 were considered statistically significant.

RESULTS

Effect of DINP on hepatic glycolytic enzyme activities

Figure 1 indicates a significant ($P<0.05$) decrease in hepatic glycolytic enzymes (HK, PFK, ALD, LDH and NADase) activities after DiNP exposure compared to control. DiNP significantly reduced the activity of hepatic HK and PFK at doses of 20 and 200 mg/kg

body weight compared to the control, with percentage decreases of 60, 40, 63, and 27%, respectively. In comparison to the control group, the activities of ALD, LDH, and NADase were all significantly reduced at 20 mg/kg and 200 mg/kg, respectively.

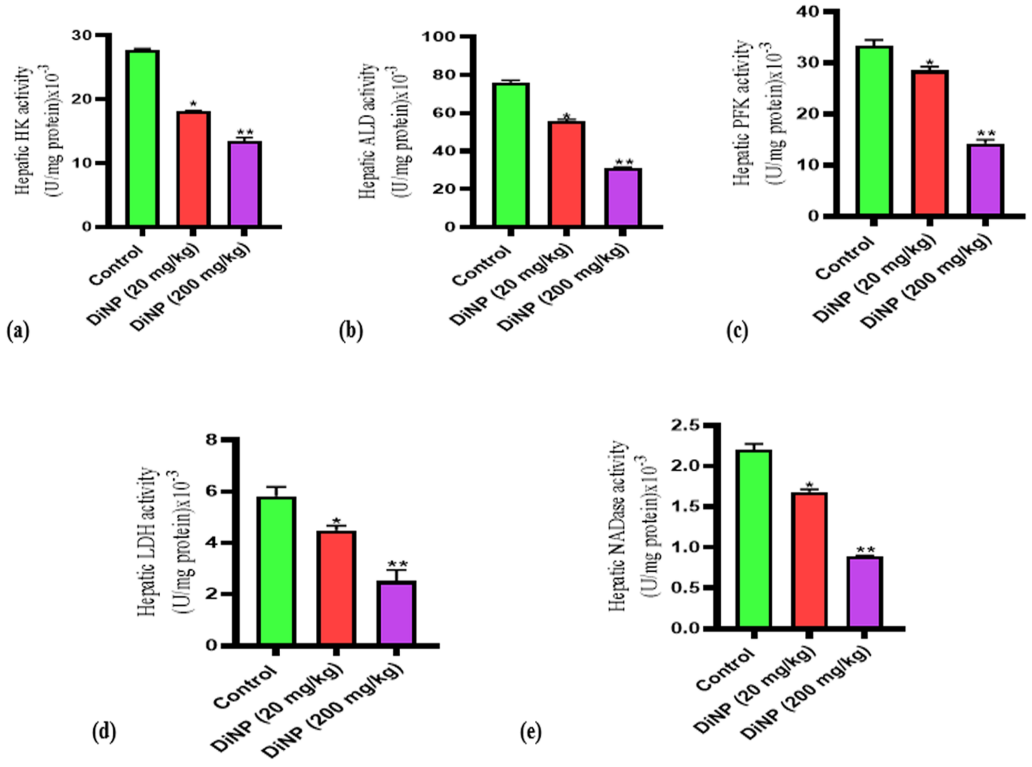

Figure 1 **Effects of DiNP on the activities of hepatic glycolytic enzymes in rats. The results are presented as mean±SEM of six rats in each group. * indicates a significant difference from the control (P<0.05). ** indicates a significant difference from the DINP (20mg/kg) group (P<0.05).**

Effect of DiNP on hepatic TCA enzyme activities

Figure 2 depicts the effect of DINP on the activities of hepatic tricarboxylic acid cycle enzymes (CS, IDH, MDH and SDH). Hepatic CS, IDH, MDH, and SDH activities were significantly (P<0.05) lower in the animals exposed to the two doses (20 and 200 mg/kg) under investigation. The CS activity is reduced by 67% and 55%, IDH by 89% and 81%, MDH by 70% and 38%, and SDH by 66% and 38%.

Effect of DiNP on hepatic electron transport chain enzyme activities

Figure 3 shows that the activities of the hepatic electron transport chain enzymes under investigation were significantly reduced (P<0.05) in rats exposed to DiNP compared to controls. In comparison to the control, 20 and 200 mg/kg DiNP reduced hepatic complex I (63% and 30%), complex II (57% and 39%), complex III (66% and 33%), and complex IV (65%

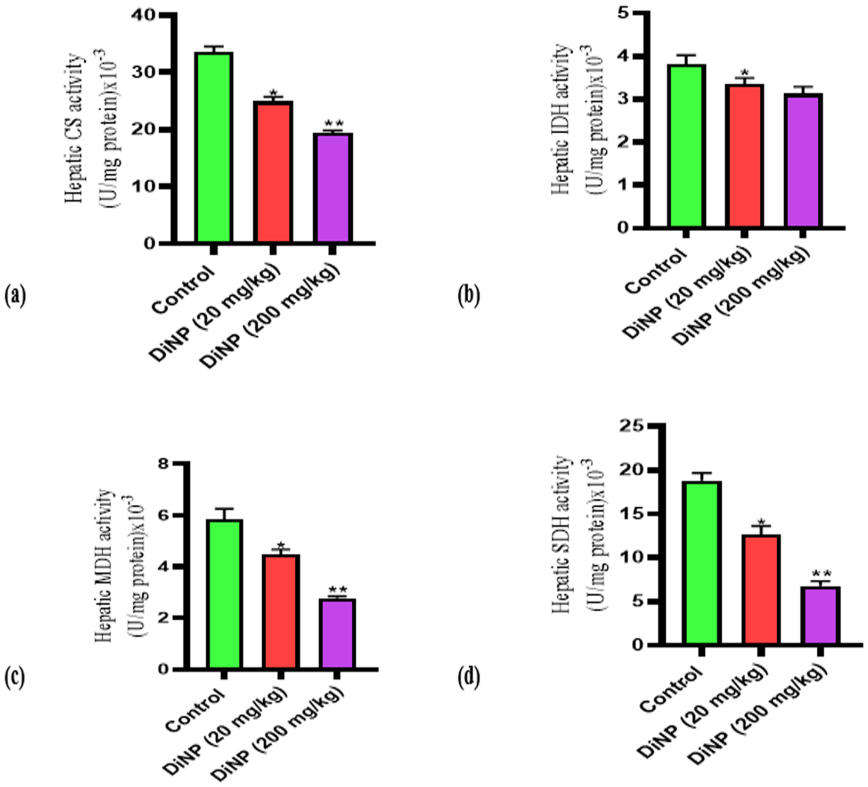

Figure 2 Effects of DiNP on the activities of hepatic TCA enzymes in rats. The results are presented as mean±SEM of six rats in each group. * indicates a significant difference from the control (P<0.05). ** indicates a significant difference from the DINP (20mg/kg) group (P<0.05).

and 55%) activities.

As seen in Figure 4 and Table 1, hepatocytes in the livers of control rats are normal; they fill sinusoidal gaps in the sections and appear to radiate in arrays in the direction of the central vein. Staining makes the nuclei of these cells very obvious. Rats given the lower dose of 20 mg/kg DiNP exhibit increased sinusoidal gaps, infiltrations, altered hepatocyte cytoarchitecture, and localised congestion in the central veins in histologic sections. In rats given 200 mg/kg DiNP, there were notable and pervasive abnormalities in the hepatocellular organisation, including nuclear condensation, pyknosis, and regions with vacuolar changes that could signify fibrosis or a lack of structural support for the liver.

DISCUSSION

A large variety of typical MDCs, which have an impact on many facets of energy balance, are exposed to by humans. The most important metabolic tissues involved in energy storage are the liver, white adipose tissue, skeletal muscle, and the endocrine pancreas. MDCs change the insulin-dependent glucose metabolism in the liver, skeletal muscle, and adipocytes and prevent the pancreatic cells from producing insulin. MDCs also have an

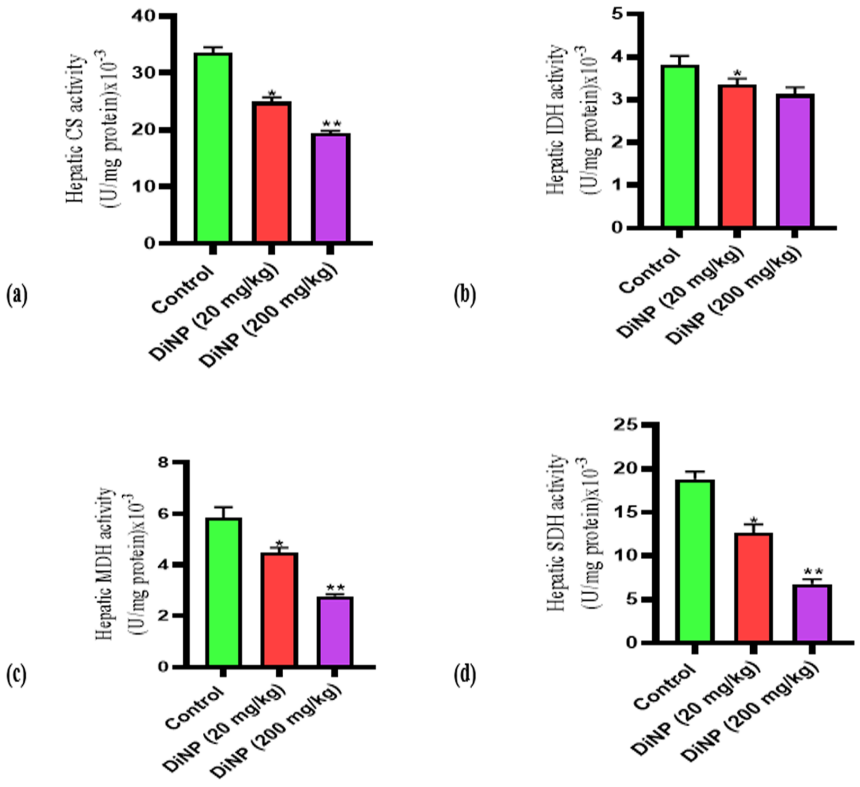

Figure 3 Effects of DiNP on the activities of hepatic ETC enzymes in rats. The results are presented as mean±SEM of six rats in each group. * indicates a significant difference from the control (P<0.05). ** indicates a significant difference from the DINP (20mg/kg) group (P<0.05).

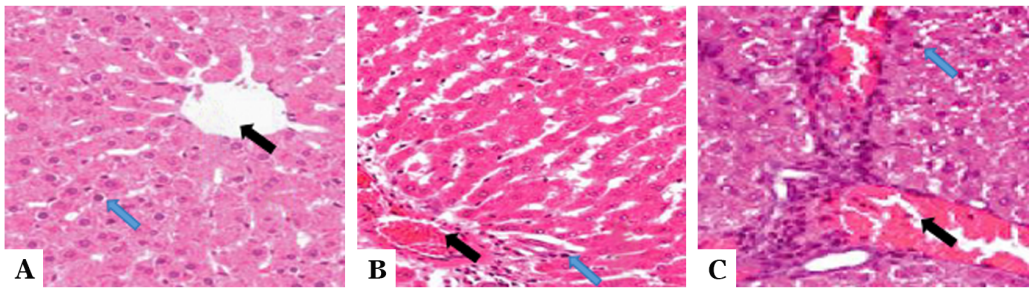

Figure 4 Histopathology of hematoxylin and eosin-stained sections of hepatic tissue from DiNP-exposed rats (X200). Blue arrow= hepatocytes, black arrow= central vein. A) Control group: histological appearances looks normal and clear, normal hepatocytes and clearly visible and well stained hepatocyte nuclei. B) 20mg/kg DiNP: Distortion of normal cytoarchitecture of hepatocytes and nuclei. C) 200 mg/kg DiNP: Loss of hepatic architectural support.

Table 1 **Abnormalities in liver histopathology of rats exposed to DiNP.**

Histopathological abnormalities	Control	20 mg/kg DiNP	200 mg/kg DiNP
Focal congestion	Nil	++	+++
Widened sinusoidal spaces	Nil	+	+++
Nuclear condensation	Nil	++	++
Cytoarchitecture of hepatocytes	Nil	++	+++
Pyknosis	Nil	++	+++
Infiltrations in the sinusoidal spaces	Nil	+	++
Vaocular Changes	Nil	++	+++

Nil= none, (+)= mild, (++)= moderate, (+++)= severe.

impact on the gut flora and neurons in the hypothalamus. Energy balance is impacted by MDCs' change of the metabolic cues that connect the various metabolic organs.[21]

Glucose imports and export in liver purely depends on GLUT2, hepatocytes can choose between glucose and/or fatty acids as their preferred source of energy. Nutrient and hormonal signals both influence the choice of fuel. In the fed state, where there is a surplus of glucose, glycolysis predominates. In addition to being fully oxidized to produce ATP, glycolytic intermediates and products are also employed to manufacture lipids, amino acids, and other significant compounds. Hepatocytes convert to fatty acid β oxidation for energy supply while they are fasting because their blood glucose levels are low.[2] Data from this study showed that DiNP-treated rats experienced inhibition of the enzymes involved in the glycolytic pathway relative to the control. From previous study, enhanced gluconeogenesis has been demonstrated to be the reason for decreased glucose uptake and oxidation in DEHP-exposed rats.[22] With an inhibition of the glycolytic enzymes; HK, ALD, PFK-1, LDH and NADase observed from the DiNP-treated rats, it may suggest that there is an increased actions of the gluconeogenesis enzymes relative to the glycolytic enzymes, hence, the conversion of glucose to pyruvate; a substrate for the other pathways of ATP generation, is inhibited. HK, PFK-1, ALD, LDH and NADase are involved in the process of glycolysis where glucose is phosphorylated (first step that commits glucose to further metabolism), catalyses the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate through phosphorylation, which is the initial regulatory step that commits glucose into glycolytic route, catalyses reversibly the cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, convert pyruvate to lactate and catalytic hydrolysis of NAD^+ to generate nicotinamide and ADP-ribose respectively.[5]

Tricarboxylic acid cycle involves a series of chemical reactions taking place in cells of all aerobic organisms to release energy which is stored in the form of ATP by conversion of Acetyl CoA derived from carbohydrates, fats, and proteins and it takes place in mitochondria in the second phase of cellular respiration. DiNP from this research decreased the activities of some key TCA cycle enzymes such as citrate synthase (CS), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH) and succinate dehydrogenase (SDH). Activities of these enzymes decreased with increase in DiNP concentration relative to the control. The citric acid cycle's succinate, a component, has the power to contribute electrons

to the electron transfer chain. Mitochondria depend on the enzyme succinate dehydrogenase (SDH), which is component of both the Krebs cycle and the respiratory chain. The n-butylmalonate-sensitive dicarboxylate carrier, also known as the phenylsuccinate-sensitive dicarboxylate carrier, may easily import succinate into the mitochondrial matrix in exchange for inorganic phosphate or another organic acid, such as malate.[23] DiNP has been shown to reduce TCA cycle enzyme activity, implying that DiNP may influence energy metabolism pathways.[6] The down-regulation of the activities of the TCA cycle enzymes, particularly SDH, a key enzyme that contributes electrons needed by the ETC indicated electron transfer to the ETC will be inhibited and subsequently resulting into no generation of ATP needed for the hepatocytes functions.

Furthermore, the mitochondria serve as the principal sites of cellular aerobic respiration, and their main function is the oxidation of organic molecules into ATP to produce energy. If mitochondria are destroyed, cells die from a lack of energy, suggesting that ATP level is a direct indicator of mitochondrial activity.[24] The ETC consists of a series of complexes (Complex I, II, III, and IV) that convey electrons from electron donors to electron acceptors through a redox process. Electrons are transferred together with protons (H+) when they cross a membrane. The electrochemical gradients of protons that are created as a result of their passage across the membrane power the production of ATP.[25] It was observed that DINP down-regulated the activities of all the complexes of the ETC in a dose-dependent manner when compared to the control. This further justify that the down-regulation of the ETC complexes by DiNP is a function of the inhibited SDH enzymes of the TCA that contributes to the electron transfer from TCA to the ETC.

Normal hepatocytes line sinusoidal gaps in the sections of control rats and virtually radiate in arrays toward the central vein. Hepatocyte nuclei are clearly visible with staining. In histologic sections, rats given the lower dose of 20 mg/kg DiNP show localized congestion in the central veins, altered hepatocyte cytoarchitecture, enlarged sinusoidal gaps, and infiltrations can also be found in the sinusoidal spaces. There were significant and widespread abnormalities in the hepatocellular arrangement in rats administered 200 mg/kg DiNP, including nuclear condensation, pyknosis, and areas with vacuolar alterations that could indicate fibrosis or a lack of structural support for the liver.

CONCLUSIONS

In this study, DiNP hepatotoxicity in rats was studied and investigated via indices of energy transduction. The two doses of DiNP (20 and 200mg/kg) significantly down-regulated hepatic glycolytic enzymes (HK, PFK, ALD, LDH, and NADase), tricyclic acid cycle enzymes (IDH, CS, SDH, and MDH), and electron transport chain enzymes (Complex I-IV). This suggests that DiNP disrupts hepatic energy metabolism by inhibiting enzymes involved in energy transduction, preventing the production of cellular energy (ATP) in sufficient quantities to carry out cellular functions.

ABBREVIATION

DiNP: Dissononyl Phthlates, TCA: Tricyclic acid cycle, ETC: Electron transport chain, HK: Hexokinase, ALD: Aldolase, LDH: Lactate dehydrogenase, PFK: Phosphofructokinase, NADase: NAD Glycohydrolase, CS: Citrate synthase, IDH: Isocitrate dehydrogenase, MDH: Malate dehydrogenase, SDH: sucinate dehydrogenase, CPLX I: Complex I, CPLX II: Complex II, CPLX III: Complex III, CPLX IV: Complex IV.

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DECLARATIONS

Authors' contributions

Conceptualization and funding acquisition: SAK. Data curation: SAK and ATO. Formal analysis, investigation, methodology, software, supervision, validation, and writing (original draft, review and editing): SAK, ATO, ETO, AO. Project administration: SAK, ATO, AO. Resources: SAK, AO, ATO. Visualization: SAK and ETO. All the authors review and approve the final draft before publication.

Conflict of interest

The authors declare no conflict of interest.

Ethical approvals

The Committee on Animal Ethics at the Faculty of Natural Sciences, Ajayi Crowther University granted clearance and authorization to use the animals (FNS/ERC/2021/006).

Data availability

The data that support the findings of this study is available from the corresponding author, upon reasonable request.

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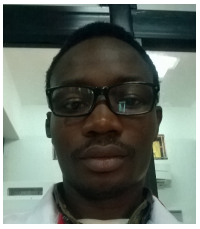

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