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Original Research Article

Metabolic and hematological disruptions induced by Diphenyl Diselenide in male wistar rats

*Joseph A. Olagunju[1], Gabriel O. Ajayi[1,2], Babatunde A. Murtala[3], & Adejuwon A. Adeneye[4]

Affiliations

[1]Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria

[2]Department of Biochemistry, College of Basic and Applied Sciences, Mountain Top University, Prayer City, Ibafo, Ogun State, Nigeria

[3]Department of Physiology, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria

[4]Department of Pharmacology, Therapeutics and Toxicology, Faculty of Basic Clinical Sciences, Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria

***For correspondence**: **email:** joseph.olagunju@lasu.edu.ng, **tel:** +234 (0) 803 815 7392

Abstract

Diphenyldiselenide (DPDS), a potent antioxidant and enzyme inhibitor, exerts cellular toxicity mainly through interaction with thiol group of proteins. This study evaluates its toxicity on glycolysis and gluconeogenesis as well as on some hematological parameters in male Wistar rats. Animals were grouped into five with Groups I and II animals intrapenitoneally dosed with 1ml/kg body weight distilled water and olive oil serving as controls while Groups III, IV and V animals were treated with DPDS in olive oil once daily for 21 days at the dose of 50-, 100- and 200mg/kg body weight respectively. All rats were sacrificed on day 21, and plasma, livers and thigh muscles harvested for biochemical assays. Treatment with 200mg DPDS/kg body weight resulted to 100% mortality while 50- and 100mg/kg body weight caused significant reduction ($p<0.05$) in red blood cell counts, hemoglobin concentration and packed-cell volume. The activities of thigh muscle key glycolytic enzymes along with plasma lactate and liver key gluconeogenic enzymes along with alanine- and aspartate aminotransferases and glycogen were significantly increased ($p<0.05$) suggesting increased glycolysis and gluconeogenesis respectively in these organs. The increased glycolysis observed may be a consequence of decreased oxygen transport to the peripheral tissue due to reduced hemoglobin hence, increased reliance on anaerobic sources for energy generation. The resultant increased gluconeogenesis and hepatic glycogen storage could be a detoxification mechanism whereby excess lactate due to increased glycolysis is converted to a non-toxic and neutral glucose to maintain pH balance in order to preserve the animals against DPDS toxicity.

Keywords*: Diphenyl diselenide toxicity, gluconeogenesis, glycolysis, compensatory change*

Introduction

Selenium, a trace mineral element and a nutritionally important agent in preventing various degenerative pathologies (Pillai *et al*., 2014; Pereira *et al*., 2022), is a component part of most

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organoselenium compounds reputed for their antioxidant properties through which they also exert their therapeutic effects and are reputed in the management of oxidative stress-linked degenerative diseases (Arteel and Sies, 2001; Quispe *et al*., 2019; Zhang *et al*., 2021). One of such synthetic organoselenium compound with well-established and potent antioxidant property is diphenyldiselenide (Whilhem *et al*., 2009; Nogueira *et al*., 2021) whose antioxidant property have been linked to glutathione peroxidase- or thiol peroxidase-like activity (Noguiera and Rocha, 2010; Kade *et al*., 2013). Despite the therapeutic role of these organoselenium compounds, their administration particularly at high doses may be toxic due to the interaction between the organic selenium and essential thiol groups in many thiol-containing enzymes, especially those sensitive to prooxidant situations that abound in the physiological systems (Nogueira *et al*., 2021; Lu *et al*., 2021). The conjugation of these organoselenium compounds to the thiol-containing enzymes accompanied by concomitant free radical formation could compromise the functions of these enzymes, damage the cells and ultimately pose a serious threat to the survival of the organism (Rocha *et al*, 2012; Ehudin *et al*., 2022). Besides impacting toxicity on thiol-containing enzymes, it has also been established that these organoselenium compounds could have multiple targets thus making them capable of causing widespread toxicity in an organism (Maciel *et al*., 2000; Nogara *et al*., 2020).

Diphenyl diselenide (DPDS)has been reported to be a potent inhibitor of δ-aminolevulinic acid dehydratase, an important thiol-containing enzyme, in the liver of rodents and whose inhibitory effect has been attributed to the oxidation of its catalytically essential thiol group leading to a change in the configuration of the enzyme and consequent impairment in its activity and functions (Nogueira *et al*., 2004; Nogara *et al*., 2020). This enzyme is very important in the biosynthesis of heme, a major component of hemoglobin, as it catalyzes the condensation of two 5-aminolevulinic acid to produce porphobilinogen, an important compound required during hematopoiesis (Ogun *et al*., 2022). The inhibitory effect of δ-aminolevulinic acid dehydratase by DPDS can impair hematopoiesis and ultimately diminishes hemoglobin concentration (Mukasi *et al*., 2020). Diminished hemoglobin concentration is capable of impairing oxygen transport and delivery to the peripheral tissue, interfering with aerobic oxidation with the high possibility of causing tissue anoxia (Bergonia *et al*., 2015; Balegar*et al*. 2022). In addition to disruption in heme synthesis, DPDS has been reported to induce mitochondrial dysfunction *in vitro* by a mechanism associated with mitochondrial thiol group oxidation especially those thiols located in the inner mitochondrial membrane leading to mitochondrial protein aggregate formation via intermolecular disulfide crosslink and increasing cell apoptosis (Posser *et al*., 2011; Quispe *et al*., 2019; Macena *et al*, 2021).

Taking together the mitochondrial dysfunction occasioned by toxicity of DPDS coupled with the adverse effect resulting to the inhibition of δ-aminolevulinic acid dehydratase with the attendant impairment in hematopoiesis, oxygen transport and delivery to the peripheral tissue and the negative effect on aerobic respiration, oxidative phosphorylation and energy transduction processes in the mitochondria, this study aims at evaluating the consequences of these findings on the possibility of the organism shifting its reliance on anaerobic sources of energy production in the peripheral tissue for survival in the face of DPDS intoxication; hence, the evaluation of DPDS toxicity on glycolysis and the fate of the end product of glycolysis under this condition.

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Materials and Methods

Animals

After necessary institutional ethical approval Ref. No.: AREC/2023/054 was obtained from the Animal Research Ethics Committee of the Lagos State University College of Medicine, Ikeja, seventy-five (75) male Wistar rats weighing between 150 and 250g previously allowed to grow and acclimatize before commencement of study were used and they were housed individually in stainless steel cages and maintained under a standard natural photoperiodic condition of 12 hours of light alternating with 12 hours of darkness with room temperature of between 25ºC and 26ºC and humidity of 65±5%.

Table 1: Composition of Normal Rat Chow

Energy Composition (%)	
Carbohydrate	63.9
Protein	20.3
Fat	15.8
Ingredients (g/kg)	
Casein	200
L-Cystine	3
Corn Starch	397
Maltodextrin	132
Sucrose	100
Cellulose	50
Soybean Oil	70
t-Butylhdroquinone	0.014
Mineral mixture	35
Vitamin mixture	10
Choline Bitartrate	2.5

Rats were fed with rat chow whose composition is as indicated in Table 1 above and allowed free access to drinking water until they were randomly allotted into the various experimental groups.

Experimental design and grouping of animals

The animal model employed in this study conformed to the guiding principles for research involving animals as recommended by the American Physiological Society (2002) and National Committee for Research Ethics in Science and Technology (NENT) (2018). Rats were randomly divided into five groups (I-V) of fifteen rats per group such that the weight differences within and between groups did not exceed ±20%. Group I consist normal rats not treated with any drug or vehicle (olive oil) to rule out the effects of these substances and mode of administration while Group II rats were intraperitoneally treated with 1ml olive oil per kg weight of rat. Groups III, IV and V were intraperitoneally dosed once daily with DPDS in olive oil (stock 1.5g in 30ml olive oil) at selected graded doses of 50mg, 100mg and 200 mg/kg body weight respectively, for twenty-one days for lethality studies as well as for the evaluation of toxicity on carbohydrate metabolism. Throughout the period of experimentation, experimental procedures involving the animals and their care were conducted in conformity with the guidelines for the care of Laboratory Animals in Biomedical Research as earlier cited. Prior to sacrifice, animals that survived the treatments (ten animals per group) were fasted overnight before they were anesthetized with controlled light inhalation of diethyl ether. Following anesthesia, blood samples were obtained directly from the chambers through cardiac puncture with 21G needle mounted on 5 ml syringe and kept in

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heparinized tubes and centrifuged at 3000rpm (Uniscope Laboratory Centrifuge Model SM 902V, Surgifriend Medicals, England) for 5 minutes so as to separate out the plasma. The livers and the thigh muscle were identified and dissected out *en bloc* and stored wet-frozen in a Revco Freezer (Model RDE30086FA, ThermoFisher Scientific) at -60^{o}C until ready for use.

Hematological analyses

Red blood cells count, hemoglobin concentration, and packed-cell volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were determined by Haemogram Full Automation Analysis using the Sysmex KX-21N Hematology Analyzer (LabX), a three-part differential autoanalyzer requiring approximately 20µL of blood and running on cell pack diluents, stromatolyzer (lyse solution) and cell clean (wash solution) with automated results display.

Preparation of hepatic tissue and thigh muscle homogenates

The hepatic tissue and thigh muscle samples ascetically removed after animal sacrifice were weighed, minced and washed several times with cold (4^{o}C) normal saline solution to remove all traces of blood. Specifically for the muscle before homogenization, the weighed, minced and washed sample was first ground into a paste using a mortar and a pestle that had been previously chilled in ice following which the paste was homogenized for one minute in 10 volumes of a solution containing glycylglycine buffer, pH 7.5, 5mM EDTA and 20mM NaF to make a 10% ($^{w}/_{v}$) thigh muscle homogenate. On the other hand, the weighed, minced and washed liver tissues were homogenized in ice-cold 1.15% ($^{w}/_{v}$) KCl in a previously cooled all glass pyrex Potter-Elvehjem Tissue Homogenizer (Thomas Scientific) fitted with Teflon pestle attached to a rotating rotor for one minute to make a 10% ($^{w}/_{v}$) liver homogenate. The various homogenates were first centrifuged at 4^{o}C in a refrigerated centrifuge (High-Speed Centrifuge Model CFG-21J, Bioevopeak) at 2,500rpm to sediment the nuclei and other cell debris while the pellet was discarded. The supernatant was then centrifuged at 10,000rpm at 4^{o}C for 15 minutes following which the pellet was discarded and the supernatant saved. The supernatant which constitutes the post-mitochondrial fraction was saved in test tubes and stored wet-frozen in a Revco Freezer (Model RDE30086FA, ThermoFisher Scientific) at -60^{o}C until ready for use for the various enzyme assays.

Enzymatic assays

Assay of muscle hexokinase activity

Muscle hexokinase activity assay of treated rats was done spectrophotometrically based on the reduction of NAD^{+} through a coupled reaction with glucose 6-phosphate dehydrogenase as described by Biochemical Merck (1970a) but modified by McFarlane and Murray (2020). The reaction medium (3ml) contained 0.05M Tris-HCl buffer, pH 8.0, containing 13.3mM $MgCl_2$, 0.67M glucose, 16.5mM ATP, 6.8mM NAD^{+}, and glucose 6-phosphate dehydrogenase from *Leuconostoc mesenedriodes* pipetted into two cuvettes with one cuvette serving as the blank. The two cuvettes were incubated at 30^{o}C for 6 – 8 minutes to achieve temperature equilibration. To the blank cuvette, 100µL of homogenizing buffer was added and used to set the spectrophotometer to zero. At zero time, 100µL of diluted homogenate was added to the reaction mixture in the second cuvette and the absorbance was read at 340nm in the spectrophotometer (Jenway Model 7205, UV/VIS) at 30 seconds interval for 3 minutes following which the change in absorbance per minute was determined and the enzyme activity calculated based on the millimolar extinction coefficient of NADH. One unit of hexokinase activity is defined as the amount of enzyme that reduced one µmole of NAD^{+} at 30^{o}C per minute at pH 8.0

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Assay of muscle phosphofructokinase activity

This enzyme activity was measured according to the method described by Sharma *et al*. (1995) which was based on the coupled assay reaction system involving the reactions catalyzed by triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase while the decrease in absorbance of NADH was measured at 340nm in the spectrophotometer (Jenway Model 7205, UV/VIS) to indirectly express phosphofructokinase activity. The incubation medium made up of 50mM Tris-HCl buffer, pH 8.0, 3mM fructose 6-phosphate, 0.1mM ATP, 3.3mM $MgCl_2$, 0.04mM NADH, 0.66 U/ml glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle and 5.6 U/ml triosephosphate isomerase from rabbit muscle were pipetted into two cuvettes with one serving as the blank. The blank received 100µL of the homogenizing buffer instead of the homogenate. At zero time, 100µL of diluted homogenate was added to the reaction mixture and the content mixed thoroughly and the absorbance was read at 340nm at 30 seconds interval for 3 minutes against the blank following which the change in absorbance per minute was determined and the enzyme activity calculated based on the millimolar extinction of NADH. One unit of pyruvate kinase activity is the amount of enzyme that oxidized one µmole of NADH per mg protein at 38^{o}C per minute at pH 8.0.

Assay of muscle pyruvate kinase activity

Muscle pyruvate kinase activity was assayed spectrophotometrically by a coupled assay reaction system with lactate dehydrogenase by the method described by Biochemica Merck (1970b) and partly utilizing the modification described by Malcovati and Valentini (1982). The reaction mixture (3ml) consisted of 0.05M imidazole-HCl buffer, pH 7.6 containing 0.12M KCl, 0.062M $MgSO_4$, 45mM ADP, 6.6mM NADH, 45mM phosphoenolpyruvate and 1400U/ml lactate dehydrogenase from bovine heart were pipetted into two cuvettes with one serving as the blank and incubated at 25^{o}C for 5 minutes to achieve temperature equilibration. To the blank cuvette, 0.1ml homogenizing buffer was added to the reaction mixture and the resulting mixture was used to set the spectrophotometer (Jenway Model 7205, UV/VIS). At zero time, 100µL of diluted homogenate was added to the reaction mixture in the second cuvette and the content was mixed thoroughly following which absorbance was read at 340nm at intervals of 30 seconds for 3 minutes. The change in absorbance was calculated and enzyme activity was estimated using the millimolar extinction coefficient for NADH. One unit of pyruvate kinase activity is the amount of enzyme that oxidized one µmole of NADH per mg protein at 25^{o}C per minute at pH 7.6.

Assay of muscle lactate dehydrogenase activity

Muscle lactate dehydrogenase activity was assayed according to the method of Bergmeyer (1963) by employing the modifications suggested by Vanderlinde (1985). The incubation medium (3ml) made up of 0.02M Tris-HCl buffer, pH 7.3, 6.6mM NADH and 30mM sodium pyruvate were pipetted into two cuvettes with one serving as the blank and they were incubated at 25^{o}C for 5 minutes to achieve temperature equilibration. To the blank cuvette, 0.1ml homogenizing buffer was added and the thoroughly mixed content was used to set the spectrophotometer (Jenway Model 7205, UV/VIS) to zero. At zero time, 100µL of diluted homogenate was added to the reaction mixture and absorbance was read at 340nm at 30 seconds interval for 3 minutes and the change in absorbance per minute was determined. The enzyme activity was calculated using the millimolar extinction coefficient of NADH. One unit of lactate dehydrogenase activity is the amount of enzyme that oxidized one µmole of NADH per mg protein at 25^{o}C per minute at pH 7.3.

Assay of hepatic glucose 6-phosphatase activity

Hepatic glucose 6-phosphatase activity was measured according to the modified method of Swanson *et al*. (1955) as described by Mallick *et al*. (2007) using ice-cold tissue homogenate at a concentration of 50 mg/ml. In a calibrated centrifuge tube, 0.1ml of 0.1 M glucose-6-phosphate solution and 0.3 ml of 0.5M maleic acid buffer (pH 6.5) were taken and brought to 37^{o}C in water bath and incubated for 15 min. The reaction was stopped with 1 ml of 10% ($^{w}/_{v}$) trichloroacetic acid (TCA) followed by chilling in ice and centrifuged at 3000rpm for 10 min. The pellet was discarded and the filtrate saved. Aliquots of 0.1ml were drawn from the filtrate for the determination of inorganic phosphate. One unit of activity is equivalent to one millimole of inorganic phosphorus formed per hour per mg protein at 37^{o}C at pH 6.5.

Assay of hepatic fructose 1,6-diphosphatase

The assay of hepatic fructose 1,6-diphosphatase was carried out by the modified method of McGilvery (1955) and the rate of catalysis was monitored by the release of inorganic phosphorus. Diluted liver homogenate (100µL) was incubated at 38^{o}C with 100µL of 0.1M fructose 1,6-diphosphate substrate in 0.3ml 0.1M tris-citrate buffer, pH 6.5 for 30 minutes and the reaction was stopped by the addition of 0.5ml of 10% ($^{w}/_{v}$) trichloroacetic acid following which the resulting mixture was centrifuged at 1200 rpm at 25^{o}C. The pellet was discarded while the filtrate was saved from which aliquots of 0.1ml was drawn for inorganic phosphorus analysis. One unit of activity is equivalent to one millimole of inorganic phosphorus formed at 38^{o}C per hour per mg protein at pH 6.5.

Liver and plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays

Assay of liver and plasma alanine aminotransferase and aspartate aminotransferase activities were done using the method described by Huang *et al*. (2006) which is essentially a modification of the method previously described by Reitman and Frankel (1957).

Other assays

Protein assay was done according to the method described by Kramer *et al*. (2015) while inorganic phosphate analysis was done by the modified method of Katewa and Katyare (2003) and blood lactate by the adaptation of the spectrophotometric method described by Brandt *et al*. (1980). Hepatic glycogen was isolated and purified by the procedure earlier described by Clark (1964) but as modified by Gonzalez *et al*. (2016) and Burst (2020) while the hepatic glycogen was quantified using the method described by Stingyl *et al*. (2006) which was based on the method previously described by Jermyn (1975).

Statistical analysis

Data analysis was performed using the Graph Pad Prism (Graph Pad Software – Version 5.0, Graph Pad Software Inc., La Jolla, California, USA). Data were expressed as Mean±S.E.M. for all assays. The data were analyzed using one-way ANOVA for comparison between the control and the treated groups and post hoc test conducted using Newman-Keuls' test. Level of statistical significance was considered at $p<0.05$.

Results

Experimental animal mortality

The untreated animals (Group I) and those treated with olive oil (Group II) remained active

throughout the period of experimentation while animals treated with graded intraperitoneal dose of DPDS in olive oil (Group III, Group IV and Group V) were not generally very active and inactivity usually preceded death with the group treated with 200 mg/kg body weight (Group V) recording 100% mortality after 21 days, while the group treated with 50 mg/kg body weight (Group III) and 100 mg/kg body weight (Group IV) recording 60% and 80% mortality respectively during the same period.

Effects of DPDS treatment on hematological parameters in normal and treated rats

The effects of DPDS on the hematological parameters of treated and untreated Wistar rats are as summarized in Table 2.

Table 2: Hematological parameters of normal rats and rats treated with various doses of DPDS

S/N	Biochemical Variable	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
1	RBC (x 10^6/mm^3)	9.8±0.9	9.5±0.7	6.9±0.5*	7.5±0.7*	ND
2	Hb (g/dL)	15.5±2.2	15.3±3.1	9.8±1.9*	10.6±0.9*	ND
3	PCV (%)	50.7±8.1	51.3±7.2	40.6±2.1*	41.6±2.7*	ND
4	MCH (pg)	17.5±2.8	16.2±1.6	17.1±2.1	16.8±1.9	ND
5	MCHC (g/dL)	27.4±5.2	24.2±4.1	24.8±3.3	24.4±3.7	ND

Group I – Untreated Rats; Group II – Rats treated with olive oil at 1ml/kg body weight; Group III – Rats treated with 50 mg/kg body weight DPDS; Group IV – Rats treated with 100 mg/kg body weight DPDS; Group V – Rats treated with 200mg/kg body weight DPDS; ND – Not Determined due to lethality. RBC = Red blood cell count; Hb = Hemoglobin concentration; PCV = Packed cell volume; MCH = Mean Corpuscular Hemoglobin; MCHC = Mean Corpuscular Hemoglobin Concentration; DPDS = Diphenyldiselenide. Each value represents mean ± SEM of five rats.

*Statistically significantly different ($p<0.05$) when compared with the controls (Groups I and II)

Results showed that the treatment with DPDS did not affect mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration as the values obtained were not significantly different ($p>0.05$) for all the treated groups when compared with the controls. Also, when the two controls (Group I versus Group II) were compared, there were no significant differences ($p>0.05$). Treatment with DPDS significantly reduced ($p<0.05$) the red blood cells count in both Group III and Group IV by 29.6% and 23.5% respectively while hemoglobin concentration was significantly reduced ($p<0.05$) in both Group III and Group IV animals by 35.5% and 31.6% respectively. Packed cell volume was also significantly reduced ($p<0.05$) in both Group III and Group IV animals by 19.9% and 17.9% respectively. For all these hematological parameters, there were no significant differences ($p>0.05$) between the Group I and Group II animals. No assays could be carried out on Group V animals owing to mortality.

Effects of DPDS on key glycolytic enzymes and plasma lactate

The results of the effects of graded intraperitoneal dosing of Wistar rats with DPDS are presented in Table 3.

Table 3: Thigh muscle glycolytic enzymes and plasma lactic acid in normal and rats treated with various doses of DPDS

S/N	Biochemical Variable	GROUP I	GROUP II	GROUP III	GROUP IV
1	Muscle Hexokinase (µmol NADH/mg Protein/min)	114.22±6.23	112.11±4.22	145.62±8.87*	171.31±8.11*
2	Muscle Phosphofructokinase (µmol NADH/mg Protein/min)	58.82±2.82	61.08±3.12	106.89±6.88*	116.22±7.11*
3	Muscle Pyruvate Kinase (µmol NADH/mg Protein/min)	370.22±12.19	365.22±10.11	592.08±12.12*	721.11±11.22*
4	Muscle Lactate Dehydrogenase (µmol NADH/mg Protein/min)	10.22±1.08	9.88±1.02	18.22±1.98*	21.08±1.75*
5	Plasma Lactic acid µmol/mL)	42.18±6.21	40.12±5.28	70.21±6.08*	71.88±6.02*

Group I – Untreated Rats; Group II – Rats treated with olive oil at 1ml/kg body weight; Group III – Rats treated with 50 mg/kg body weight DPDS; Group IV – Rats treated with 100 mg/kg body weight DPDS; Group V – Rats treated with 200 mg/kg body weight DPDS; Group V not assayed due to lethality. Each value represents mean±S.E.M. of five rats.

*Statistically significantly different ($p<0.05$) when compared with the controls (Groups I and II)

Results showed that the activities of all the key glycolytic enzymes assayed in the thigh muscle homogenate of the treated rats were significantly elevated ($p<0.05$) when compared with the controls. While the activities of controls (Group I and Group II) were comparable for all the key enzymes, the activity of hexokinase was elevated by 27.5% in the group treated with 50 mg/kg (Group III) and 49.9% in the group treated with 100 mg/kg (Group IV). A similar trend was also observed for phosphofructokinase and lactate dehydrogenase. An increase in activity of 59.9% and 94.8% was observed for phosphofructokinase in Group III and Group IV treated animals respectively while 78.3% and 106.3% were observed for lactate dehydrogenase in Group III and Group IV treated animals respectively. The increases were dose-dependent for all the enzymes assayed. Lactate, the end-product of glycolysis, was also significantly elevated ($p<0.05$) in all the treated groups. There was an elevation of 66.5% in the plasma lactate of Group III animals while an increase of 70.4% was recorded in Group IV animals. Plasma lactate was comparable in the controls.

Effects of DPDS on key gluconeogenic enzymes, some plasmaenzymes and liver glycogen

Table 4 summarizes the results of the effects of DPDS on some key enzymes, some plasma enzymes regulating gluconeogenesis and glycogen stored in the liver of the treated and untreated animals.

Table 4: Liver gluconeogenic enzymes, plasma and liver aminotransferases and liver glycogen of normal rats and rats treated with various doses of DPDS

S/N	Biochemical Variable	GROUP I	GROUP II	GROUP III	GROUP IV
1	Plasma Alanine Aminotransferase (U/mL)	152.22±9.88	158.22±8.72	155.22±9.15	151.29±9.07
2	Plasma Aspartate Aminotransferase (U/mL)	214.43±12.84	209.34±12.24	219.42±11.48	220.14±10.74
3	Liver Alanine Aminotransferase (U/mg protein/min)	25.52±2.21	22.59±2.27	45.22±2.11*	47.18±1.71*
4	Liver Aspartate Aminotransferase (U/mg protein/min)	10.22±0.92	11.01±1.08	17.15±1.95*	16.32±1.76*
5	Liver Glucose 6-Phosphatase (mmol P_i/mg protein/hr)	0.72±0.05	0.69±0.04	1.55±0.09*	1.62±0.08*
6	Liver Fructose 1,6-Diphosphatase (mmolP_i/mg protein/hr)	1.03±0.07	1.09±0.09	2.22±0.62*	2.35±0.68*
7	Liver Glycogen (mg/100g wet weight)	5.22±0.77	5.28±0.72	12.75±1.10*	13.28±1.12*

Group I – Untreated Rats; Group II – Rats treated with olive oil at 1ml/kg body weight; Group III – Rats treated with 50 mg/kg body weight DPDS; Group IV – Rats treated with 100 mg/kg body weight DPDS; Group V – Rats treated with 200 mg/kg body weight DPDS; Group V not Determined due to lethality. Each value represents mean±S.E.M. of five rats.

*Statistically significantly different ($p<0.05$) when compared with the control

Results showed that DPDS did not alter the activity of alanine aminotransferase and aspartate aminotransferase in the plasma at all the doses tested. In contrast, the activities of liver alanine aminotransferase and aspartate aminotransferase were significantly higher ($p<0.05$) in the treated animals when compared with the controls. At 50mg/kg, DPDS caused an increase of 77.2% (Group III) while at the higher dose of 100mg/kg (Group IV), an 84.8% increase was recorded. A similar trend was seen with aspartate aminotransferase where for Group III treated animals, a 67.8% increase was obtained while Group IV treated animals had the same enzyme activity increased by 59.7%. Except for aspartate aminotransferase, the increases were dose dependent.

The activities of both glucose 6-phosphatase and fructose 1,6-diphosphatase were significantly increased ($p<0.05$) in the treated animals as well. In the Group III treated animals, activity of glucose 6-phosphatase was significantly increased ($p<0.05$) by 115.3% while those of Group IV was increased by 125.0%. In the same manner, fructose 1,6-diphosphatase activity was increased significantly ($p<0.05$) in Group III animals by 115.5% while an increase of 128.2% was obtained in Group IV animals. Liver glycogen was also significantly increased ($p<0.05$) in Group III treated animals by 144.3% while Group IV animals had an increase of 154.4%. All the increases observed for glucose 6-phosphatase and fructose 1,6-diphosphatase activities as well as liver glycogen were dose dependent.

Discussion

This study aims at evaluating the possible toxic effects that DPDS has on specific key enzymes regulating glycolysis namely, hexokinase, phosphofructokinase, pyruvate kinase as well as on lactate dehydrogenase in rats. The fate of the end product of glycolysis were examined by evaluating the key enzymes regulating gluconeogenesis, namely, fructose 1,6-diphosphatase and glucose 6-phosphatase because the catabolites of glycolysis are major precursors for *de novo*

synthesis of glucose. Finally, an evaluation of the relationship between blood-formed elements and the metabolic activities occurring in peripheral tissues was conducted to corroborate the existing information in literature. In this study, DPDS at the dose of 50mg/kg, 100mg/kg and 200 mg/kg body weight was administered once daily for 21 days. A 100% mortality was recorded for the group treated with 200mg DPDS/kg body weight. The thiol group oxidation is a central molecular mechanism involved in the toxicity of organoselenium compounds and DPDS has been reported to induce mitochondrial dysfunction *in vitro* by a mechanism associated with mitochondrial thiol group oxidation particularly those located on the inner mitochondrial membrane (Puntel *et al*., 2010) causing mitochondrial protein aggregation and increased cell apoptosis (Posser *et al*., 2011; Radomska *et al*., 2021). The mitochondrial is the powerhouse of any living cell and any agent that compromises its functions may impair energy transduction and cause cell death. The 100% mortality recorded at the dose of 200mg DPDS/kg body weight may possibly be ascribed to events at the cellular and molecular levels associated with mitochondrial dysfunction leading to the death of these experimental animals and thereby lending credence to mitochondrial dysfunction possibly being the basis for DPDS toxicity.

Of significance to our study is the decrease in red blood cells count, packed-cell volume, as well as depressed hemoglobin concentration occasioned by treatment with 50mg and 100mg/kg DPDS. Several reports have established that the oxidation of thiols by organoselenium compounds mimics glutathione peroxidase activity (Kade *et al*., 2008; 2009; 2013) and that under *in vitro* condition, DPDS reportedly inhibits some thiol-containing enzymes (Nogueira *et al*., 2004; Kade *et al*., 2008; 2009; Nogueira *et al*., 2021). Of interest to our study is the report that this compound is a potent inhibitor of δ-aminolevulinic acid dehydratase, a thiol-containing enzyme, in different tissues of rodents, thus, destroying the essential thiol group through oxidation, which invariably changes the configuration of the enzyme, impair its catalytic activity and inhibit its catalytic action (Barbosa *et al*., 1998). In the biosynthetic pathway for heme synthesis, glycine condenses with succinyl-Co A to form 5-aminolevulinic acid. Two molecules of 5-aminolevulinic acid through the action of δ-aminolevulinic acid dehydratase condense to produce porphobilinogen which is converted enzymatically to coproporphyrinogen III which in turn produces protoporphyrinogen IX. Protoporphyrinogen IX is the precursor for the synthesis of protoporphyrin IX which is ultimately converted to heme, which itself, is an essential precursor for the biosynthesis of hemoglobin during hematopoiesis (Nogueira and Rocha, 2011; Medlock and Dailey, 2022). The inhibition of this enzyme may result to a metabolic block in the heme biosynthetic pathway, causing depressed heme synthesis and by extension, reduction in the number of the synthesized hemoglobin molecules with the net effect being reduction in hemoglobin concentration, reduction in the red blood cells count as well as reduced packed-cell volume (Medlock and Dailey, 2022) as obtained in this study. The metabolic block also causes accumulation of 5-aminolevulinic acid, a pro-oxidant, that could increase the presence of reactive oxygen species in the animal resulting to increased oxidative damage to tissues including hematopoietic pluripotent cells. The resultant cumulative effect is the hematopoiesis suppression which is characterized by reduction in the red blood cell counts, reduced hemoglobin concentration and reduced packed-cell volume all reported in this study.

Aside from δ-aminolevulinic acid dehydratase, other enzymes such as lactate dehydrogenase, nitric oxide synthase, inosine monophosphate dehydrogenase, lipooxygenases, uridine phosphorylase, thymidylate synthase, tyrosine kinase and iodothymidine deiodinase have also been reported to be affected by toxicity of organoselenium compounds (Oshita *et al*., 1994; Nogara *et al*., 2020). The toxicity may range from impaired biosynthesis of these enzymes to reduction in

activity or total loss of activity of these enzymes thereby compromising the physiological functions and roles of these enzymes. In this study, the toxic effect of DPDS on the activities of the glycolytic enzymes such as hexokinase, phosphofructokinase, pyruvate kinase and its end-point catabolite, lactate dehydrogenase, were evaluated. The nearly two-fold increase in the plasma lactate concentration recorded in all the treated rats suggested a profound increase in the flux across the glycolytic pathway leading to the excess production in the end-product, lactate. Since the pathway from glucose to lactate in the glycolytic sequence is controlled at the irreversible points catalyzed by hexokinase, phosphofructokinase and pyruvate kinase with the reaction catalyzed by phosphofructokinase being the committed step in the pathway, an increase in the activities of these enzymes particularly, phosphofructokinase, is a clear evidence that there is an increase in glycolysis in the peripheral tissues as a result of exposure to DPDS. The accumulation of lactate (lactic acidosis) can also be ascribed to the reversible conversion of pyruvate to lactate with the catalytic enzyme being lactate dehydrogenase whose activity has also been increased thereby causing the reaction equilibrium to be shifted to the right hence, lactic acid accumulation.

In a similar manner, the key enzymes of gluconeogenesis were significantly increased in this study. The pathway for the *de novo* synthesis of glucose is controlled by pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase and glucose 6-phosphatase because they catalyze irreversible steps in the pathway. The increase in the activities of the latter two, fructose 1,6-diphosphatase and glucose 6-phosphatase, is suggestive of an increase in the flux across the gluconeogenic pathway also. It is also interesting to note that the increases in the activities of the aminotransferases (alanine aminotransferase and aspartate aminotransferase) in the liver is suggestive of increased transamination of alanine to pyruvate and aspartate to oxaloacetate with both α-keto acids precursors for the *de novo* glucose synthesis via the gluconeogenic pathway. The increases observed in the activities of these enzymes also support the claim that gluconeogenesis must have been enhanced as a result of DPDS treatment. Since gluconeogenesis primarily occurs more in the liver and in the kidney, the fact that serum alanine aminotranferase and aspartate aminotransferase activities remained unaltered despite the treatment with DPDS is suggestive that enhanced gluconeogenesis occurred in an intact liver. Impairment in liver function is characterized by increase in plasma or serum aminotransferases particularly, alanine aminotransferase among other liver function indicators. Since the levels of the two aminotransferases assayed in this study remained comparable between the animals treated with DPDS and the untreated ones, the livers of the treated animals appear to have remained unaffected despite the treatment. The increase in liver glycogen, the end-product of gluconeogenesis, in the treated rats further underscore the fact that gluconeogenesis was enhanced in the treated animals.

The oxidation of the essential thiol of δ-aminolevulinic acid dehydratase by DPDS could result in impaired hematopoiesis which in turn could lead to reduced hemoglobin concentration and red blood cells count and subsequently tissue anoxia and tissue necrosis from reduced oxygen transport and delivery to the peripheral tissues (Mistry *et al*., 2018; Della Rocca *et al*., 2022). These situations are prevalent in this study. Anemia coupled with previously reported impairment in the mitochondrial functions resulting from DPDS treatment are possible scenarios that could have resulted in the 100% mortality of the 200mg/kg DPDS-treated rats. In rats treated with lower DPDS doses, it is possible that the increase in glycolysis, evolved as a compensatory change to seek alternative means of generating energy for the rats' survival in the face of peripheral tissue hypoxia such that energy could be generated using this alternative pathway for survival. There is, however, a possible link between enhancements in glycolysis as observed in the muscle and increased

gluconeogenesis as seen in the DPDS-treated rat livers. Lactate is a precursor for the *de novo* synthesis of glucose via the Cori Cycle and this compound is produced in excess as a result of enhanced glycolysis in the thigh muscle of the treated animals. It is common knowledge that lactate can disrupt the pH balance of the physiological system of the treated animals and result to acidosis. To avoid acidosis, the organism might opt for a mechanism converting surplus lactate, an acidic compound, into a substance that mitigates acidity within the physiological system, thus preventing acidosis. One such alternative is converting lactate into glucose, a neutral compound, to achieve this balance. Enhanced gluconeogenesis in the livers of the rats coupled with enhanced glycolysis in the thigh muscle of the rats occasioned by treatment with DPDS appears complementary processes and maybe compensatory changes to prevent cell and animal death on exposure to DPDS.

These recorded observations are consistent with findings of previous studies where compensatory changes occur to preserve cellular integrity and other physiological functions of the cell in situations where cell homeostasis was disturbed. Iron-deficiency anemia, a condition characterized by a disturbance in iron balance and metabolism, is often accompanied by a significant drop in hemoglobin concentration, hematocrit levels and red blood cells count similar to what was obtained in this study. The biochemical compensatory mechanisms or change appears to be at the red cell level. Nagababu *et al*. (2008) reported increased uptake of glucose which appears a consequence of higher rate of glycolysis with concomitant elevation of potassium accompanying increased levels of gluconeogenic amino acids in an anemic condition. This was supported by the observations of Ramot *et al*. (1969) and Alrahamdani *et al*. (2022) who reported double fold increases in the mean total blood hexokinase, glucose 6-phosphate dehydrogenase with concomitant increase in the activities of phosphofructokinase, pyruvate kinase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase in the anemic state. These changes were consistently shown to increase 2,3-diphosphoglycerate markedly in anemic subjects (Chan *et al*., 2017) and studies have shown that 2,3-diphosphoglycerate changes oxygen dissociation curve of hemoglobin to ensure improved cooperative binding of oxygen molecules to hemoglobin and at the same time, also ensure quick release of oxygen molecules in the peripheral tissues where oxygen tension must have reduced considerably in order to prevent tissue anoxia (Balcerek *et al*., 2020; Płoszczyca *et al*., 2021). These changes which culminated in increased 2,3-diphosphoglycerate may have occurred as compensatory changes in anemia to prevent extreme anoxia from decreased hemoglobin levels by efficiently aiding and ensuring supply of both oxygen and glucose to various peripheral tissues for oxidation to prevent cell death that could have arisen from extreme anoxia. These findings lend credence to our proposition that the changes observed in our study may be compensatory in nature.

Lactic acidosis was also reported in iron-deficiency anemia occasioned by increases in the key enzymes of the glycolytic pathway (Olagunju, 1990) with the detoxification of the acidic lactate leading to hyperalaninemia which was considered a compensatory detoxification process by converting toxic lactate to alanine, a non-toxic alternative to lactate (Olagunju, 1992a). Since alanine is a gluconeogenic amino acid earlier reported to have direct linear correlation between its circulating concentration and pyruvate, a precursor for lactate synthesis in basal man (Martino *et al*., 2022), it was not surprising that increased gluconeogenesis was also reported in iron-deficiency anemia (Olagunju, 1992b). These chains of metabolic events were postulated to be compensatory changes to keep the animals alive in the anemic state where lactic acidosis have been reported. However, in the reported studies, the increased gluconeogenesis could be thought to be a

consequence of hyperalaninemia whose production resulted from detoxification of lactate (lactic acidosis), a consequence of increased glycolysis. The increased glycolysis with a consequent increase in gluconeogenesis via the production of alanine is a compensatory mechanism to detoxify lactate and thus prevent acidosis. This observation is also in support of the findings in the present study. It is therefore being speculated that enhancement of both glycolysis in the thigh muscle and enhanced gluconeogenesis in the livers of the treated animals with both processes complementing each other, may be compensatory changes to prevent cell and animal death on exposure to DPDS.

Conclusion

Our study revealed that DPDS is generally toxic to the rat especially at a very high dose where 100% mortality was recorded. The toxicity leading to mortality as reported in this study has lent credence to the earlier reported mitochondrial thiol oxidation known to cause mitochondrial protein aggregation and increased cell apoptosis which is considered to be the central molecular mechanism for organoselenium toxicity. In addition to this, the observed increased glycolysis as a result of DPDS intoxication may evolve as a compensatory change to seek alternative means of generating energy for the rats' survival in the face of peripheral hypoxia while the concomitant increased gluconeogenesis may be a further and complementary sparing mechanism to avoid acidosis resulting from surplus lactate produced due to increased glucolysis by converting lactate to glucose, a neutral compound, in order to prevent acidosis, cell and animal death on exposure to DPDS. It is not known whether exposure to DPDS affects other forms/phases of metabolism or whether there are compensatory changes that could occur in the metabolism of other biomolecules as this study was restricted to its effects on carbohydrate metabolism. Efforts should be made to investigate the effect this organoselenide may have on the metabolism of other metabolites.

Conflict of interest

The authors declared no conflict of interest.

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