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Protective Effects of Cyclohexyl methyl dithiocarbamates Sodium Salts on Diclofenac Induced Reproductive Toxicity in Male Albino Rats

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Abstract

The present study investigates the effect of cyclohexylmethyldithiocarbamates sodium salt (a synthetic compound) and vitamin E against diclofenac-induced blood and testes of experimental Wistar male albino rats. The study consists of six groups of six rats each. Group I (control) received corn oil (3 ml/kg b.w), Group II received diclofenac (100 mg/kg b.w), Group III rats were treated with diclofenac and cyclohexylmethyldithiocarbamates sodium salt (30 mg/kg b.w), Group IV was administered diclofenac and Vitamin E (30 mg/kg b.w), Group V was given cyclohexylmethyldithiocarbamates sodium salt (30 mg/kg b.w) only and Group VI received vitamin E only. The results showed a significant ($p<0.05$) decrease in the daily sperm production in the diclofenac only treated rats, diclofenac with Na (HxMedtc), diclofenac with vitamin E treated rats and Na (HxMedtc) treated rats but no significant ($p>0.05$) in the vitamin E only treated rats when compared with the control. There was a significant ($p<0.05$) decrease in the Testicular sperm number (TSN) in the diclofenac only treated rats, diclofenac with Vitamin E treated rats and vitamin E only treated rats and no significant ($p>0.05$) decrease in the diclofenac with Na (HxMedtc) treated rats and Na (HxMedtc) only treated rats when compared with the control. Also, there was a significant ($p<0.05$) decrease in TSN in the diclofenac with Na (HxMedtc) treated rats and diclofenac with vitamin E treated rats when compared with diclofenac only treated rats. And a decrease ($p<0.05$) in the daily sperm production (DSP) in the diclofenac only treated rats, and diclofenac with cyclohexylmethyldithiocarbamate sodium salt only treated rats when compared with the control.

Keywords: Cyclohexyl methyl dithiocarbamates sodium salt; Diclofenac; Albino rats; Vitamin E

Introduction

The testis is the organ responsible for the production of spermatozoa and hormones which are required for maintenance of secondary sexual functions [1]. Exogenous compounds such as drugs and other foreign substances may interfere with the synthesis, secretion, transport, binding, action or elimination of hormones responsible for the reproduction and other physiological functions of the body [2], thereby disrupting spermatogenesis. Exposure to varying concentrations of these endogenous compounds has also been reported to cause adverse effects on several organs and alter several defense mechanisms in both animal and human models [3].

Diclofenac, a 2-arylacetic acid, (marketed as Voltaren), is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reducing pain in conditions such as arthritis or acute injury [4]. Recent evidence suggests that diclofenac metabolism involves the production of reactive oxygen species leading to oxidative stress and genomic DNA fragmentation [5,6]. Furthermore, there are indications that the mitochondrial inner membrane permeabilization and activity of caspases play a crucial role in the pathogenesis of diclofenac [7].

Furthermore, it has been reported that the extensive use of diclofenac increases the risk of acute myocardial infarction and several cases of severe local reactions associated with intramuscular injection of diclofenac have been reported [8]. Diclofenac was found to generate protein adducts in the livers of treated mice as well as in rat hepatocytes via protein acylation by the drug glucuronide [9]. *In vitro* experiments with cultured rat hepatocytes have shown, however, that the covalent binding of diclofenac is neither the only nor the major cause of acute cytotoxicity [10]. Moreover, previous work has suggested that diclofenac is cytotoxic to rat hepatocytes after cytochrome P-450 (CYP)-mediated metabolism [11].

Dithiocarbamates are the reduced forms of thiuram disulfides with strong complexing properties [11]. They exhibit very rich coordination chemistry with a large variety of transition metals and are used as vulcanizing (analytical agents) [12]. Thiuram disulfides (thiram), dithiocarbamate salts (nabam) or their complexes with iron (ferbam), manganese (maneb) and zinc (ziram, zineb, propineb, metiram) are well known as pesticides with an estimated annual global consumption of 25,000 – 35,000 metric tons [12].

Dithiocarbamates exert both antioxidant and pro-oxidant effects in cells [13]. Their antioxidant behaviour includes eliminating hydrogen peroxide and scavenging the superoxide radical, peroxynitrite and the hydroxyl radical [14] and lipid peroxidation products such as the peroxyl radical [13]. The reaction of dithiocarbamates with reactive oxygen and nitrogen species generates dithiocarbamate thiyl radicals which ultimately dimerize to form thiuram disulphides [13], the oxidized form of dithiocarbamates. The pro-oxidant consequences of dithiocarbamate action, including that of PDTC and diethyldithiocarbamate have recently been highlighted with respect to their effects on apoptosis [15]. However, there is limited information on the effects of dithiocarbamate sodium salts and diclofenac on reproductive system. The purpose of this study, therefore, is to investigate the effect of dithiocarbamate on diclofenac induced testicular damage in rats.

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Materials and Methods

Materials

Chemicals and reagents: *N*,*N*-dibenzylamine (Boheringer), *N*-benzyl-*N*ethylamine (Aldrich), *N*-benzyl-*N*-isopropylamine (Boheringer), *N*-benzyl-*N*-methylamine (Aldrich), *N*-ethyl-*N*methylamine (Aldrich) and *N*-benzyl-2-phenethylamine (Boheringer).

Animal ethics

All of the animals received humane care according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy Science and published by the National Institute of Health (USA). The ethic regulations have been followed in accordance with national and institutional guidelines for the protection of animals' welfare during experiments. The experiment was carried out at the Drug Metabolism and Toxicology Laboratory, Department of Biochemistry, University of Ibadan, Oyo State, Nigeria.

Preparation of test compounds (Dithiocarbamates)

Synthesis of Cyclohexyldithiocarbamate salt: Thiophosgene, chlorothioformates and isothiocyanates were used in the synthesis of dithiocarbamates (DTCs) according to the method described by [12]. Amines were mixed with dithiocarbamate and sodium salts without any solvent and then maleic anhydride was slowly added to this mixture at room temperature. The combination produced dithiocarbamate derivatives of about 70% yields at room temperature. The product is stable even under reflux in toluene for 12 h.

Chemicals and reagents preparation

All chemicals were if an analytical grade and are supplied from sigma chemical co. USA. Distilled water was used in all biochemical assays.

Experimental Animals

Thirty-six (36) adults Laboratory breed male wistar albino rats weighing between 120-150 g were purchased from covenant Farm limited, Gbolasire, Iwo Road, Ibadan, and Oyo state. The animals were maintained under standardized environmental conditions in a well ventilated rat house in cages, in the departmental animal house at room temperature (22-28°C), under controlled light cycle of 12 hrs light/ 12 hrs dark with free access to standard rat feed purchased from Ladokun Feeds Nig. Limited, Ibadan, Nigeria and water supplied *ad libitum*.

Experimental Design

The thirty-six rats used for the experiment were randomly assigned to six (6) groups at the end of acclimatization period. The animals in group 1 serve as control and received corn oil throughout the treatment period. Animals of groups 2, 3, 4, 5 and 6 received diclofenac only, diclofenac and Cyclohexylmethyldithiocarbamate sodium salt, Diclofenac and vitamin E, Cyclohexylmethyldithiocarbamate sodium salt only and vitamin E only respectively. The animals in the test group were pretreated with vitamin E (groups 5 and 6) and the test compound (groups 3 and 4) for one week. Appropriate dose dilutions were made with distilled water and corn oil for test compound and vitamin E respectively based on body weight (100 mg/kg) to provide for a total volume of 0.3 ml. Thus, 0.3 ml of dose dilution was similarly administered orally through gavage to each rat. All the surviving animals were sacrificed after 24 hrs of diclofenac.

Semen collection and characteristics

Daily feed intake and body weight were recorded weekly. Total sperm output calculated by multiplying semen ejaculate volume and semen concentration. Assessment of live and abnormal spermatozoa was performed using an eosin– nigrosine blue staining mixture [12]. The percentages of motile sperm were estimated by visual examination under low-power magnification (10×) using a phase-contrast microscope with heated stage. Total number of motile sperm calculated by multiplying percentage of motile sperm and total sperm outputs. Reaction time for the buck is calculated as the time needs for mounting a doe until complete ejaculation; it measured in seconds using a stopwatch. Initial hydrogen ion concentration (pH) of semen samples was determined immediately after collection using a pH cooperative paper (Universal indikator pH 0-14 Merck, Merck KgaA, 64271 Darmstadt, Germany). Packed sperm volume (PSV) was recorded. Total functional sperm fraction (TFSF) parameter was also calculated as (total sperm output × motility (%) × normal morphology (%) [16].

Blood collection and testosterone determination

Blood samples were collected from the ear vein of each buck every other week and placed immediately on ice in heparinized tubes. Plasma was collected from blood by centrifuged at 860 g for 20 min and stored at -60°C. Testosterone concentration in plasma was measured by simple solid phase enzyme immunoassay utilizing horseradish peroxidase as a tracer (Equipar, via G. Ferrari, Saronno, Italy). Intra and interassay coefficient of variations were 3.9% and 6.2%, respectively. All rats were euthanized at the end of the experimental period (16 week). Weight of testis and epididymis was recorded.

Organ collection

The testes were collected, weighed and excised. Part of it was excised and stored in 50% formalin solution for Histology studies and the other was removed into ice-cold 0.25 M sucrose solution. It was then blotted with tissue paper and homogenized in ice-cold 0.25M sucrose solution (1:5 w/v) using Teflon homogenizer. This was kept frozen until required for the enzyme assay.

Statistical analysis

Results are presented as means ± SD. Effect of non-enzymatic antioxidants for different variables was analyzed by the analysis of variance (ANOVA). When the F–ratio was significant ($P<0.05$), Tukey's Honestly significant difference was used to compare the treatment mean.

Results

Results show no significant decrease ($P<0.05$) in the body weight of all animals administered with Diclofenac and cyclohexylmethyldithiocarbamates sodium salt (Na (HxMedtc)) as compared with control (Table 1).

Table 2 above is a significant ($p<0.05$) decrease in the sperm motility and count in the diclofenac only treated rats, diclofenac with Na (HxMedtc) treated rats, diclofenac with Vitamin E treated rats, Na (HxMedtc) only treated rats and vitamin E only treated rats when compared with the control. There is also a significant decrease ($p<0.05$) in the live/dead in the diclofenac with Na (HxMedtc) treated rats, diclofenac with Vitamin E treated rats, Na (HxMedtc) only treated rats when compared with the control. Also, no significant ($p>0.05$) decrease in the number of dead/live diclofenac with vitamin E treated rats and diclofenac with Na (HxMedtc) treated rats, but there is a

significant (p>0.05) in the sperm motility and sperm count in this treated rats when compared with the control. But there is no significant (p>0.05) decrease in the sperm motility, count and live/dead in the Na (HxMedtc) only treated rats, when compare with the diclofenac and Na (HxMedtc) treated rats. No significant (p>0.05) decrease in the sperm motility, live/dead and count in the vitamin E only treated rats when compared with vitamin E and diclofenac treated rats.

There was a significant (p<0.05) decrease in the daily sperm production in the diclofenac only treated rats, diclofenac with Na (HxMedtc), diclofenac with vitamin E treated rats and Na (HxMedtc) treated rats but no significant (p>0.05) in the vitamin E only treated rats when compared with the control. There is an increase (64%) inhibition in the diclofenac with Na (HxMedtc) treated rats when compared with the Na (HxMedtc) only treated rats against the control an increase (29%) inhibition in the daily sperm production in the diclofenac and vitamin E treated rats when compared with vitamin E only treated rats (Table 3).

There is a significant (p<0.05) decrease in the TSN in the diclofenac only treated rats, diclofenac with Vitamin E treated rats and vitamin E only treated rats and no significant (p>0.05) decrease in the diclofenac with Na (HxMedtc) treated rats and Na (HxMedtc) only treated rats when compared with the control. Also, there is a significant (p<0.05) decrease in Testicular sperm number (TSN) in the diclofenac with Na HxMedtc) treated rats and diclofenac with vitamin E treated rats when compared with diclofenac only treated rats (Table 4).

Discussion

Treatment with Diclofenac and Cyclohexylmethyldithiocarbamates Sodium Salts reduced testosterone levels, feed intake and body weight (BW) (Table 1). Moreover, previous studies also showed a decrease in these parameters in rabbits treated with cypermethrin [17,18]. Reported that the reduction of body weight of rabbits treated with carbofuran and glyphosate may be due to direct cytotoxic effect of the pesticides on somatic cells, and/or indirectly through the central nervous system which control feed and water intake and regulates the endocrine function. Also, the failure of different species exposed to environmental toxicant to gain body weight may be due to the decrease in feed intake, malabsorption of nutrients from the gastrointestinal tract and impaired feed conversion efficiency [19]. The decline in the BW of treated rabbits with appeared as a result of lesser intake of feed Cyclohexyl methyl dithiocarbamates Sodium Salts and Diclofenac (Table 1).

Group	Initial weight (g)	Final weight (g)
Control	115 ± 5.48	116 ± 4.18
Diclofenac only	153 ± 5.16	132 ± 14.38
Diclofenac and Na(c-HxMedtc)	118 ± 11.69	116 ± 11.40
Diclofenac and Vitamin E	132 ± 4.08	107 ± 5.77
Na(c-HxMedtc) only	140 ± 0.00	135 ± 8.95
Vitamin E only	118 ± 11.69	116 ± 11.40

Values are expressed as means ± SEM of six independent experiments. Means in the same column not sharing the same letter(s) are significantly different (p < 0.05).

Table 1: Effects of administration on the initial and final body weight of experimental rats.

Semen quality (Table 2) deteriorated following treatment with Cyclohexylmethyldithiocarbamates Sodium Salts and Diclofenac and these results are in agreement with the previous studies [20,21]. Exposure to Diclofenac caused sexual dysfunction in male rats [19]. The decline in ejaculate volume, sperm concentration, total sperm output, and packed sperm volume (PSV), and increased reaction time can be partly attributed to the Diclofenac-induced reduction in testosterone levels (Table 3). The effects of certain drugs on spermatogenesis may be mediated through their effects on hormonal balance.

Previous studies showed reduced semen quality in men occupationally exposed to various pesticides [22] and in animals [23]. Additionally, [24] found that treatment with cypermethrin caused reduction in the fertility of male rats. Also, the epididymal and testicular sperm counts as well as daily sperm production were significantly decreased and the number of implantation sites was significantly reduced in females mated with males that had ingested cypermethrin. The decrease in sperm packed volume of immobile sperms. Fructose synthesis and secretion by the accessory glands is dependent upon the secretion of testosterone by the testes [25,26] reported that generation of reactive oxygen species and peroxidation of sperm membranes could bring negative effects on motility, midpiece abnormalities and sperm-oocyte fusion [27]. Suggested that pesticide's disruption of reproductive processes might be in part due to adverse effects on sperm cell function. In general the effect of Diclofenac on sperm quality may be due to the decrease in plasma testosterone concentration and/or indirectly by reducing feed intake.

Vitamin E treatment

Treatment with vitamin E alone caused a slight significant (P<0.05) decrease in body weight and relative testes and epididymis weights (Table 1) [28] found that supplementation of vitamin E to California and New Zealand White rabbits increased body weight gain and improved feed efficiency compared to the control group. Our previous studies showed that vitamin E supplementation stimulated weight gain in rats and rabbits [29] which is in agreement with the present results. The beneficial effects of vitamin E noted in the present study can be attributed to the antioxidant effects of this vitamin; it is scavenger of oxygen-free radicals which are toxic byproducts of many metabolic processes [19,30,31]. Vitamin E protects critical cellular structures against damage caused by oxygen-free radicals and reactive products of lipid peroxidation. It has been reported that lipid peroxidation was prevented by vitamin E [29,30]. Vitamin E inhibits peroxidation of membrane lipids by scavenging lipid peroxyl radicals, as a consequence

Treatment Group	Motility	Live/Dead	Count
Control	93.75 ± 2.50	98.00 ± 0.00	91.75 ± 7.67
Diclofenac only	76.00 ± 5.48*	93.5 ± 5.10	67.80 ± 4.44*
Diclofenac and Na(c-HxMedtc)	56.67 ± 5.77**	90.00 ± 5.00*	53.00 ± 2.65**
Diclofenac and Vitamin E	45.00 ± 7.07**	90.00 ± 5.00*	45.00 ± 4.58**
Na(c-HxMedtc) only	60.00 ± 8.94*	90.5 ± 5.24*	50.83 ± 7.00*
Vitamin E only	65.00 ± 5.77*	93.60 ± 3.51*	46.00 ± 2.65*

Values are expressed as means ± SEM of six independent experiments. Means in the same column not sharing the same letter(s) are significantly different (p < 0.05)

Table 2: Effect of cyclohexylmethyldithiocarbamates sodium salt , vitamin E, and Diclofenac on Spermatozoan of Albino rats

Group	Daily Sperm Production (10^5)
Control	4.83 ± 0.21
Diclofenac only	1.80 ± 0.24*
Diclofenac and Na(c-HxMedtc)	7.90 ± 0.94**
Diclofenac and Vitamin E	3.42 ± 0.62**
Na(c-HxMedtc) only	3.03 ± 0.35**
Vitamin E only	3.89 ± 0.49

Values are expressed as means ± SEM of six independent experiments. Means in the same column not sharing the same letter(s) are significantly different (p < 0.05).

Table 3: Effect of cyclohexylmethyldithiocarbamates sodium salt, vitamin E, and Diclofenac on daily sperm production of Albino rats

Group	Control	Diclofenac only	Diclofenac and Na (c-HxMedtc)	Diclofenac and Vitamin E	Na (c-HxMedtc) only	Vitamin E only
Tailess tail	0.83 ± 0.14	1.16 ± 0.14*	1.37 ± 0.20*	1.24 ± 0.25*	1.24 ± 0.25*	1.16 ± 0.14*
Headless tail	0.74 ± 0.01	1.24 ± 0.01*	1.13 ± 0.18**	0.83 ± 0.14*	0.92 ± 0.144	0.83 ± 0.14
Rudiment	0.66 ± 0.14	0.7 ± 0.01	0.75 ± 0.00**	0.5 ± 0.25	0.67 ± 0.14	0.67 ± 0.14
Bent tail	1.64 ± 0.12	2.24 ± 0.02*	2.47 ± 0.00**	2.66 ± 0.13**	2.57 ± 0.154**	2.42 ± 0.14*
Curved tail	1.73 ± 0.03	2.24 ± 0.02*	2.49 ± 0.02**	3.07 ± 0.60**	2.22 ± 0.03**	2.75 ± 0.00*
Bent mid-Pierce	1.63 ± 0.11	2.15 ± 0.25*	2.12 ± 0.19*	3.07 ± 0.16**	2.63 ± 0.15**	2.57** ± 0.13
Curved mid Pierce	1.48 ± 0.02	2.31 ± 0.11*	0.61 ± 0.16*	2.74 ± 0.02**	2.58 ± 0.14	2.67 ± 0.14*
Looped tail	0.40 ± 0.13	0.41 ± 0.14*	0.63 ± 0.18	0.5 ± 0.25	0.74 ± 0.01*	0.67 ± 0.14*
Total	9.11 ± 0.21	12.50 ± 0.51*	13.55 ± 0.60*	14.61 ± 0.31*	13.58 ± 0.36*	13.73 ± 0.03**

Values are expressed as means ± SEM of six independent experiments. Means in the same column not sharing the same letter (s) are significantly different ($p < 0.05$)

Table 4: Effect of cyclohexylmethyldithiocarbamates sodium salt, vitamin E, and Diclofenac on Testicular sperm number (TSN) of Albino rats

of which it is converted into a-tocopheroxyl radical. This radical is thought to be either recycled to a-tocopherol by interacting with soluble antioxidants, such as ascorbic acid, or irreversibly oxidized to a-tocopherylquinone. In fact, a-tocopherylquinone may act as a potent anticoagulant and as an antioxidant through its reduction to hydroquinone [31]. Also, [32] reported that the protective role of vitamin E against the toxicity of oxidants may be due to the quenching of hydroxyl radicals.

Similarly, [33] reported that vitamin E supplementation reduced ROS generation and protected spermatozoa from loss of motility [34] found that oral treatment with 200 mg vitamin E daily decreased reactive oxygen species significantly and increased fertilization rate of fertile normospermic human male after one month of treatment. Also, [35] reported that treatment with vitamin E decreased the formation of TBARS and improved semen quality of rabbits. In addition, in vitro study using rabbit sperm by [36] showed that vitamin E decreased TBARS and increased antioxidant enzymes (superoxide dismutase and catalase). They also reported that supplementation with vitamin E was more effective in improving sperm characteristics and in reducing the production of reactive oxygen species than Vitamin C [37] reported that in vivo experiments, seven weeks of oral vitamin E (1000 IU/d/animal) administration in boar caused a significant fall in the level of seminal plasma TBARS from 2.2 to 1.2 nmol/ml and significantly increased the number of spermatozoa. Therefore, the improving effect of vitamin E on semen characteristics may be due to the reduction in lipid peroxidation potential. The ameliorating effect of vitamin E (Table 2) against the toxicity of lambda-cyhalothrin on semen quality may be due to their role as antioxidant through quenching 1O2 or free radical and reacting with peroxyl radicals [38].

From the present results, it can be concluded that concurrent administration of Cyclohexylmethyldithiocarbamates Sodium Salts and Vitamin E to Diclofenac-treated animals ameliorated the induced sperm quality damage, significantly improved the sperm parameters and reduced the induction of seminal plasma free radicals. This is consistent with a vital role of vitamin E in antioxidant systems that protect against Diclofenac damage, possibly by preventing oxidative damage to sperm.

The present study suggests therapeutic effects of Cyclohexylmethyldithiocarbamates Sodium Salts Vitamin E to minimize the reproductive toxicity of Diclofenac exposure.

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