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EFFECT OF DISULFIRAM/COPPER GLUCONATE COMBINATION ON OXIDATIVE STRESS MARKERS IN THE TESTIS OF RATS

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ABSTRACT

This study sought to determine the chronic toxicological effects of the disulfiram/copper gluconate drug combination in rats in a 90 day dose and time dependent study on spermatic parameters. A total of 88 rats weighing between 260-300g were used. The rats were divided into eleven groups consisting of 8 rats each with Groups 1 and 2 serving as control groups. The control groups received normal saline as placebo and 99.5% dimethyl sulfoxide (DMSO) (Solvent control). The drugs were administered as $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ of the LD_{50} of 373mg/kg and 75mg/kg for disulfiram and copper gluconate respectively. Dosing was done daily with that of the combination given 12hours apart. The post mitochondria fraction of the organs of the animal were washed in ice cold 1.15% KCL solution, blotted and weighed and homogenized with 0.1M phosphate buffer (pH 7.2). The organs were blended with laboratory sand (acid washed sand). The resulting homogenate was centrifuged at 2500rmp speed for 15mins and the supernatant was decanted, stored at -20°C and used to assay the following antioxidant enzymes spectrometrically, Superoxide dismutase (SOD), catalase, reduced glutathione(GSH) and malondialdehyde(MDA). The results revealed a decrease in reduced glutathione (GSH), superoxide dismutase(SOD), catalase(CAT) and an increase in malondialdehyde(MDA) levels in the testis of test rats. These results are indicative of the development of oxidative stress.

INTRODUCTION

Repurposing drugs is not novel as several examples abound. Two failed cancer drugs which have been repurposed include zidovudine (AZT), the first antiviral approved for HIV/AIDS in 1987 and farnesyltransferase inhibitor (FTI) which was recently used to successfully treat children with the rapid–aging disease Progeria in a 2012 clinical trial. Other drugs that have been repurposed for other indications include raloxifene which was initially developed to treat osteoporosis but was approved in 2007 for invasive breast cancer in post-menopausal women. Thalidomide was used as a sedative to reduce nausea in pregnant women in the late fifties and as treatment for leprosy in 1998. In 2006, it was approved for the treatment of multiple myeloma a bone marrow cancer. Tamoxifen originally used to treat metastatic breast cancer was approved for bipolar disorders in 2007.

Disulfiram (antabuse) and copper gluconate are also undergoing research for possible use in cancer chemotherapy. A number of researchers have worked on cancer cell lines (Chen *et al,* 2011, Grossmann *et al,* 2011, papaioannou *et al*, 2013) have suggested that based on the apoptotic effect of the drug combination observed in cancer cell lines, clinical trials could be undertaken. This research study therefore set out to assess the chronic toxicological implications of repurposing disulfiram and copper gluconate on spermatic parameters.

METHODOLOGY

88 male albino rats weighing between 260g- 300g obtained from the department of Pharmacology animal house were used for this study. The rats were bred and maintained under suitable conditions, allowed an acclimatization period of two (2) weeks, housed in hygienic cages in groups of five and allowed free access to feed obtained from vital feeds UAC PLC and water *ad libitum*. The beddings were changed and cages cleaned out on alternate days. Animals were handled according to Helsinki declaration on animal care. The animals were divided into 11 groups, each consisting of 8 rats each. The groups included those for treatment and the control groups. Drugs were administered orally via a 1ml syringe.

CHRONIC TOXICITY TESTS

This study spanned 3 months and was domiciled in the Department of Pharmacology, University of Port Harcourt, Animal house and Laboratory. A dose and time dependent toxicological evaluation of the effects of these individual drugs and their combinations on the liver function of rats was evaluated. The rats were divided into eleven groups consisting of 8 rats each. Groups 1 and 2 served as control groups and the rats received normal saline as placebo and 99.5% DMSO (Solvent control) respectively. Drugs were administered orally via a 1ml syringe.

The test group rats were divided into groups 3, 4 and 5 consisting of 24 rats in each group. Drug administration was done orally for 90 days as follows;
Control group 1 rats received 1ml of normal saline orally daily for 90 days
Solvent control group 2 received 0.5ml of DMSO orally daily for 90 days
Group 3a rats received *15mg/kg of copper gluconate daily orally
Group 3b rats received *7.5mg/kg of copper gluconate daily orally
Group 3c rats received *3.75mg/kg of copper gluconate daily orally
*doses were $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ of the LD_{50} of Copper gluconate
Group 4a rats received °74.6mg/kg of DSF and *15mg/kg of copper gluconate daily orally
Group 4b rats received °37.3mg/kg of DSF and *7.5mg/kg of copper gluconate daily orally
Group 4c rats received °18.65mg/kg of DSF and *3.75mg/kg of copper gluconate daily orally
° Doses were $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ of the LD_{50} of disulfiram (DSF)
* Doses were $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ of the LD_{50} of copper gluconate
N/B The drug combination was given following the protocol of Grossman *et al*, 2011 .
Group 5a rats received °74.6mg/kg of DSF daily orally
Group 5b rats received °37.3mg/kg of DSF daily orally
Group 5c rats received °18.65mg/kg of DSF daily orally
° Doses were $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ of the LD_{50} of disulfiram (DSF)

COLLECTION OF SAMPLES

The post mitochondria fraction of the organs of the animal were washed in an ice cold 1.15% KCL solution, blotted and weighed. They were then homogenized with 0.1M phosphate buffer (pH 7.2), putting the organs each into the mortar; laboratory sand was added to it (acid washed sand) and it was blended. The resulting homogenate was centrifuge at 2500rmp speed for 15mins then it was removed from the centrifuge and the supernatant was decanted and stored at -20°C. The following antioxidant enzymes activities were determined spectrometrically as follows: Superoxide dismutase (SOD), catalase, reduced glutathione(GSH), malondialdehyde(MDA).

STOCK SOLUTIONS; Were prepared from 99.5% DMSO for disulfiram and distilled water for copper gluconate. Pure analytical grade samples, CAS No. 527-09-3(98%min

purity) and CAS No. 97-77-8(98%min purity) obtained from Shijiazhuang Aopharm Import and Export Co. Limited China were used for the study.

RESULT:

Table 1: The Effect of Low, Medium and High dose DSF, CG and DSF/CG combination on SOD in the testis of rats after 90 Days

TREATMENT GROUPS	DOSE		
	LOW	MEDIUM	HIGH
Control 1	2.04 ± 0.30	2.35 ± 0.20	2.33 ± 0.20
Control 2	2.05 ± 0.13	2.12 ± 0.12	2.51 ± 0.33
DSF	1.43 ± 0.01	1.35 ± 0.06 *	1.02 ± 0.12 *
CG	1.52 ± 0.02	1.34 ± 0.06 *	1.69 ± 0.41 *
DSF/CG	1.30 ± 0.14*	1.15 ± 0.07 *	0.82 ± 0.20 *

Results are expressed as mean ± SEM, the superscript (*) means significant difference with respect to control at $p<0.05$ (ANOVA).n =5

KEY: Control 1- Distilled water
Control 2- DMSO
DSF – Disulfiram Low, Medium, high doses - 18.65mg/kg, 37.3mg/kg, 74.6mg/kg
CG- Copper gluconate Low, Medium, High doses - 3.75mg/kg, 7.5mg/kg, 15mg/kg
DSF/CG- Low, Medium, High doses -(18.65mg/kg/3.75mg/kg), (37.3mg/kg/7.5mg/kg), (74.6mg/kg/15mg/kg)
All results are expressed as u/mg pro

Table 2: The Effect of Low, Medium and High dose DSF, CG and DSF/CG combination on GSH in the testis of rats after 90 Days

TREATMENT GROUPS	DOSE		
	LOW	MEDIUM	HIGH
Control 1	16.86 ± 0.21	16.83 ± 0.19	16.90 ± 0.24
Control 2	16.41 ± 0.34	16.07 ± 0.40	16.47 ± 0.39
DSF	14.24 ± 0.14*	13.54 ± 0.26*	12.29 ±0.37 *
CG	13.03 ± 0.10*	12.02 ± 0.10*	11.40 ±0.31 *
DSF/CG	12.44 ± 0.27*	10.68 ± 0.46*	5.25 ± 1.72 *

Results are expressed as mean ± SEM, the superscript (*) means significant difference with respect to control at $p<0.05$ (ANOVA).n =5

KEY: Control 1- Distilled water
Control 2- DMSO
DSF – Disulfiram Low, Medium, high doses - 18.65mg/kg, 37.3mg/kg, 74.6mg/kg
CG- Copper gluconate Low, Medium, High doses - 3.75mg/kg, 7.5mg/kg, 15mg/kg
DSF/CG- Low, Medium, High doses -(18.65mg/kg/3.75mg/kg), (37.3mg/kg/7.5mg/kg), (74.6mg/kg/15mg/kg)
All results are expressed as u/mg pro

Table 3: The Effect of Low, Medium and High dose DSF, CG and DSF/CG combination on CAT in the testis of rats after 90 Days

TREATMENT GROUPS	DOSE		
	LOW	MEDIUM	HIGH
Control 1	35.21 ± 0.89	35.33 ± 0.88	35.33 ± 0.88
Control 2	33.40 ± 0.70	33.00 ± 0.66	34.10 ± 1.46
DSF	28.47 ± 0.74*	26.67 ± 1.67*	24.33 ± 0.88*
CG	34.67 ± 0.33	34.00 ± 0.58	34.00 ± 0.58
DSF/CG	22.33 ± 0.88*	20.00 ± 1.16*	15.00 ± 1.73*

Results are expressed as mean ± SEM, the superscript (*) means significant difference with respect to control at $p<0.05$ (ANOVA).n =5

KEY: Control 1- distilled water
Control 2- DMSO
DSF – Disulfiram Low, Medium, high doses - 18.65mg/kg, 37.3mg/kg, 74.6mg/kg
CG- Copper gluconate Low, Medium, High doses - 3.75mg/kg, 7.5mg/kg, 15mg/kg
DSF/CG- Low, Medium, High doses -(18.65mg/kg/3.75mg/kg), (37.3mg/kg/7.5mg/kg), (74.6mg/kg/15mg/kg)
All results are expressed as u/mg pro

Table 4: The Effect of Low, Medium and High dose DSF, CG and DSF/CG combination on MDA in the testis of rats after 90 Days

TREATMENT GROUPS	DOSE		
	LOW	MEDIUM	HIGH
Control 1	4.72 ± 0.03	4.75 ± 0.06	4.75 ± 0.06
Control 2	4.61 ± 0.03	4.65 ± 0.04	4.67 ± 0.09
DSF	5.77 ± 0.25 *	6.27 ± 0.41 *	8.33 ± 0.16 *
CG	5.69 ± 0.09*	6.73 ± 0.44*	8.72 ± 0.02*
DSF/CG	6.99 ± 0.30 *	7.89 ± 0.36 *	13.12 ± 0.45*

Results are expressed as mean ± SEM, the superscript (*) means significant difference with respect to control at $p<0.05$ (ANOVA). n=5

KEY: Control 1- Distilled water
Control 2- DMSO
DSF – Disulfiram Low, Medium, high doses - 18.65mg/kg, 37.3mg/kg, 74.6mg/kg
CG- Copper gluconate Low, Medium, High doses - 3.75mg/kg, 7.5mg/kg, 15mg/kg
DSF/CG- Low, Medium, High doses -(18.65mg/kg/3.75mg/kg), (37.3mg/kg/7.5mg/kg), (74.6mg/kg/15mg/kg)
All results are expressed as u/mg pro

DISCUSSION: Other studies had found that this drug combination was toxic to quite a few organs. In a bid to determine the possible mechanism of toxicity, stress markers were assayed in the testis of the test rats to determine the capability of the therapeutic agents to predispose to oxidative stress. Reduced glutathione (GSH) levels are used to assay the antioxidant status of a cellular system. The GSH molecule is a non-enzymatic antioxidant capable of scavenging free radicals and it could serve as a substrate to other antioxidant enzymes like the glutathione peroxidase. Akanji *et al*, (2009), postulated that low levels of GSH may be attributed to increased usage in order to scavenge free radical species or consumption as substrate by antioxidant enzymes which function to protect against oxidative damage.

Oxidative stress/ damage is an imbalance between free radical generation and antioxidant system. When subjected to stress, antioxidants such as SOD and CAT mop up the free radicals (ROS). However, when stress is overwhelming, the free radicals suppress the antioxidant system resulting to decreased antioxidant levels with consequent formation of malondialdehyde and damage to the cell, tissue or organ system. This may explain the results obtained in this study.

Some researchers postulate that the oxidation potential of copper may be responsible for some of its toxicity in excess ingestion cases. Copper at high concentrations is known to produce oxidative damage to biological systems, including peroxidation of lipids or other macromolecules (Bremner, 1998)

Delmaestro *et al,* 2000 studied the relationship between disulfiram (DSF) toxicity, lipid peroxidation, and copper in order to further elucidate the mechanisms of DSF toxicity in male Sprague-Dawley rats. Their results revealed an increased accumulation of copper, increased production of malondialdehyde (MDA), increased activity of glutathione peroxidase along with alterations in glutathione and glutathione disulfide concentrations indicative of oxidative stress in the hippocampus and cerebellum. The production of MDA was directly related to the level of copper in both areas of the brain indicating that excess copper may be the primary cause of disulfiram neurotoxicity. Forman *et al*, 1980 concluded in their research work on the possible mechanism of disulfiram toxicity, that it is likely that disulfiram administration potentiates oxygen toxicity via in vivo reduction to diethyldithiocarbamate and subsequent inhibition of superoxide dismutase. These findings were in agreement with the results

obtained in this study which revealed decreased GSH,SOD, CAT and increased MDA which indicate that the likely mechanism of toxicity is via release of free oxygen radicals.

CONCLUSION

The assay of oxidative markers revealed a decrease in reduced glutathione (GSH), superoxide dismutase(SOD), catalase(CAT) and an increase in malondialdehyde levels in the testis of test rats. These results are indicative of the development of oxidative stress.

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