OPEN ACCESS

Saudi Journal of Medicine
Abbreviated Key Title: Saudi J Med
ISSN 2518-3389 (Print) | ISSN 2518-3397 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com

Original Research Article

The Co-Administration of Insulin and Zinc Ameliorates Diabetes Mellitus-Induced Oxidative Stress in Testicular Tissue

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DOI: 10.36348/sjm.2022.v07i07.003 | **Received:** 14.05.2022 | **Accepted:** 29.06.2022 | **Published:** 05.07.2022

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Abstract

Oxidative stress is often associated with diabetes mellitus (DM). It has been reported to cause infertility in males. Diagnosed at an advanced phase, DM-associated complications may not be reversed by standard therapy alone. This study therefore investigated whether zinc given alongside insulin may reverse or ameliorate oxidative stress induced by DM. Five normal rats was assigned into the normal control group. Twenty diabetic rats were randomly assigned into four groups of five rats each. The first group had no treatment throughout the experiment. The second group were administered insulin two times daily at one and four units in the morning and evening. The third group had oral zinc (10mg/kg). The fourth group had a combination of insulin and zinc at doses as above. Treatments in all cases commenced after two weeks of DM induction and lasted ten days. Testes were harvested and assayed for parameters. DM decreased SOD, GPx, GSH and increased MDA. While insulin or zinc increased SOD, GPx, GSH and decreased MDA, insulin and zinc in combination had better results.

Keywords: Diabetes mellitus, Insulin, Zinc, Oxidative stress, Reactive oxygen species, Testicular tissue.

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INTRODUCTION

Oxidative stress is a well-known cause of male infertility (Amaral *et al*., 2008). It is a product of either increased reactive oxygen species (ROS) generation or impaired antioxidant defence system (Dickinson *et al*., 2002). Oxidative stress induces lipid peroxidation in tissues; the end product of which is malondialdehyde (MDA) (Mohasseb *et al*., 2011). Superoxide dismutase (SOD) and glutathione peroxidise (GPx) are the two main ROS-scavenging enzymes in the testis (Fujii *et al*., 2003).

Testicular tissue is particularly susceptible to oxidative injury because its membrane is richly endowed with polyunsaturated fatty acids (Aitken *et al*., 1989; Peltola *et al*., 1992). Generally, aerobic cells use oxygen for the generation of energy. ROS are however by–products of this process (Cutler, 1991). These are harmful to tissues and as such, under normal conditions, the antioxidant defence system comprising SOD, GPx, GSH, catalase, vitamins C and E (Yao *et al*., 2007) eliminates them (Hu *et al*., 2005). Any upset in the defence system will expose tissues to ROS-mediated oxidative injury (Tian *et al*., 1998).

Diabetes mellitus (DM) is a metabolic pathology that is chronic, complex and characterized by elevated blood glucose. It results from defects in either insulin secretion or insulin action. It could also be the result of both (American Diabetes Association, 2011).

DM is reported to generate ROS, increasing oxidative stress thereby and by that impair male reproductive capacity (Amaral *et al*., 2008; Tabak *et al*., 2011; La Vignera *et al*., 2012). Increased MDA and reduced SOD, GPx and GSH in the testis have been reported following DM (Ricci *et al*., 2009; Mohasseb *et al*., 2011; Afifi *et al*., 2015).

The prevalence of DM is rising worldwide (IDF, 2007) and it is expected to cause an increase in male fertility (Alves *et al*., 20013). Given that DM is diagnosed in the advanced phase, especially in developing countries, cellular functional deterioration may not be reversed by insulin therapy alone. Also,

with oxidative stress being a critical culprit in the pathogenesis of DM and zinc being reported to have antioxidant effects (Stumvoll *et al*., 2005), this study therefore sought to establish if zinc in combination with insulin could reverse or ameliorate oxidative stress in testicular tissues.

MATERIALS AND METHODS

Materials

Insulin was bought from Novo Nordisk A/S, Denmark, Streptozotocin from Sigma-Aldrich, St. Louis, MO, USA and Zinc gluconate from Pharmedic, Vietnam). All other reagents/chemicals used were of analytical grade and commercially available.

Ethical approval

Approval for this study was granted by the Faculty of Basic medical sciences research ethics committee.

Experimental animals

Animals were all male and of the Wistar strain. The twenty five rats used weighed between 150 – 230g and aged about 10 weeks. All rats were purchased and housed from Department of Anatomy, University of Benin, Benin City and in the Animal House of the Department of Physiology, University of Calabar, Calabar respectively. Standard animal cages were used to house the animals with sawdust as beddings. All animals were allowed to acclimatize for fourteen days. Animals were kept in standard conditions and had access to rat feed and water *ad libitum.*

Experimental design

Twenty five rats, twenty diabetic and five normal were randomized into five groups of five rats each as follows: Group A – Normal control group, Group B – Diabetic mellitus (DM) control group, Group C – Diabetes mellitus (DM) and Insulin, Group D – Diabetes mellitus (DM) + Zinc and Group E – Diabetes (DM) + Insulin + Zinc. The induction of diabetes mellitus (DM) was done after fasting the animals overnight by administering intraperitoneally 60mg/kg of streptozotocin (STZ) (De Young *et al.*, 2004). Vehicle was fresh preparation of 0.1M citrate buffer (pH 4.5). Forty eight hours later, DM was confirmed in blood from tail vein. Animals were considered diabetic with fasting blood glucose of 250 mg/dL and above (Cao *et al*., 2012). All rats were then allowed a period of fourteen days, with access to just normal feed and water before treatments commenced. Through subcutaneous route, insulin was given to animals in group C twice daily. 1 unit in the morning and 4 units in the evening (Pinheiro *et al*., 2011). Oral zinc was administered at 10mg/kg once daily to animals in group D (Shidfar *et al*., 2010). Group E rats received both insulin and zinc as above. The animals were sacrificed after ten days of treatment. Testes were collected for analyses.

METHODS

Preparation of testicular homogenates

Testes (left) were homogenized in cold 0.1M phosphate buffer (pH 7.4). A homogenizer and Teflon pestle were used. Homogenates were subsequently centrifuged for ten (10) minutes at 3500g. They were then assayed for parameters.

Determination of testicular superoxide dismutase (SOD) activity

Testicular SOD activity was assayed using SOD assay kit with reference to manufacturer's instructions. The principle involves the generation of superoxide radicals by xanthine and xanthine oxidase. This radical reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red dye. The degree of inhibition of this reaction is measured as SOD activity. One unit of SOD is equivalent to 50% inhibition of this reaction. SOD activity was then read-off at 505 nm using a spectrophotometer and as unit per milligramme of protein (U/mg protein).

Determination of testicular glutathione peroxidase (GPx) activity

Manufacturer's instructions were followed using GPx assay kit to measure testicular GPx activity. The principle is based on the oxidation of glutathione (GSH) by cumene hydroperoxide with GPx as catalyst. Oxidised glutathione (GSSG) in the presence of glutathione reductase and NADPH is promptly converted to its reduced form with a simultaneous oxidation of NADPH to $NADP^+$. Spectrophotometer was used to measure the decrease in absorbance at 340 nm. GPx activity was expressed as (U/mg protein).

Determination of testicular GSH activity

Glutathione assay kit was used to determine GSH activity in testis. The principle involves the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) and oxidized glutathione (GSSG) by GSH. Using spectrophotometer, the absorbance due to TNB formation was determined at 412 nm with GSH used as standard. It was expressed as U/mg protein.

Determination of testicular catalase (CAT) activity

Testicular tissue was homogenized in triton X-100 1% and diluted with phosphate buffer (pH 7.0). Hydrogen peroxide was added to the mixture to initiate a reaction. The ability of catalase in the tissue to decompensate hydrogen peroxide was used to quantify the level of its activity in the testis by observing the decrease in absorbance at 240 nm. Catalase activity was expressed as U/mg protein (Aebi, 1984).

Determination of testicular malondialdehyde (MDA) activity

At a ratio of 1-9 mls, testicular tissue was homogenized in KCL. A volume of homogenate was

mixed with 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 mol/l HCL. This was then heated in a boiling bath for fifteen minutes. Upon cooling, the solution was centrifuged at 1000g for ten minutes. Using a spectrophotometer, the absorbance of the supernatant was recorded at 535 nm and MDA values calculated and expressed as nmol/g protein (Kheradmand *et al.*, 2009).

Statistical Analysis

Graph pad prism was used to analyze all data. One-way analysis of variance (ANOVA) was used to analyze data. It was followed by Tukey post-hoc test and the results presented as mean ± standard error of mean. Statistically significance was considered at $p<0.05$.

RESULTS

Testicular superoxide dismutase (SOD)

SOD significantly decreased ($P<0.001$) in DM control (8.13 ± 0.04 u/mg), DM + insulin (11.44 ± 0.02 u/mg), DM + zinc (10.75 ± 0.08 u/mg) and DM + insulin + zinc (13.06 ± 0.04 u/mg) groups when compared to the normal control group (15.40 ± 0.05 u/mg). When compared to the DM control, SOD in testes increased significantly ($P<0.001$) in DM + insulin, DM + zinc and DM + insulin + zinc groups. Results further show a significant decrease ($P<0.001$) in DM + zinc group and a significant increase ($P<0.001$) in DM + insulin+ zinc group of testicular SOD when compared to DM + insulin group. Testicular SOD was also significantly increased ($P<0.001$) in DM + insulin + zinc group when compared to DM + zinc group (Fig 1).

Testicular glutathione peroxidase (GPx)

It was seen from Fig 2 that GPx in testes was significantly decreased ($P<0.001$) in DM control (6.89 ± 0.13 u/mg), DM + insulin (9.35 ± 0.09 u/mg), DM + zinc (8.18 ± 0.14 u/mg) and DM + insulin + zinc (11.52 ± 0.07 u/mg) groups when compared to the normal control group (16.98 ± 0.11 u/mg). Testicular GPx significantly increased ($P<0.001$) in DM + insulin, DM + zinc and DM + insulin + zinc groups when compared to DM control group. Also, when compared to DM + insulin group, there was a significant increase ($P<0.001$) in testicular GPx in DM + zinc and DM + insulin + zinc groups. In the DM + insulin + zinc group, testicular GPx increased significantly ($P<0.001$) when compared to DM + zinc group.

Testicular glutathione reductase (GSH)

Testicular GSH decreased significantly ($P<0.001$) in DM control (4.03 ± 0.06 u/mg), DM + insulin (7.03 ± 0.06 u/mg), DM + zinc (7.92 ± 0.04 u/mg) and DM + insulin + zinc (9.85 ± 0.03 u/mg) groups when compared to the normal control group (12.45 ± 0.02 u/mg). When compared to DM control group, there was a significant increase ($P<0.001$) in testicular GSH in DM + insulin, DM + zinc and DM + insulin + zinc groups. In DM + zinc and DM + insulin + zinc groups, testicular GSH increased significantly ($P<0.001$) when compared to DM + insulin group. Also, testicular GSH was significantly increased ($P<0.001$) in the DM + insulin + zinc group when compared to the DM + zinc group (Fig 3).

Testicular catalase (CAT)

Results from FIG. 4 show comparison of CAT between normal control (3.09 ± 0.07 u/mg), DM control (3.01 ± 0.06 u/mg), DM + insulin (2.97 ± 0.01 u/mg), DM + zinc (2.98 ± 0.01 u/mg) and DM + insulin + zinc (3.02 ± 0.06 u/mg) groups. There was no significant difference ($P>0.05$) between the groups.

Testicular malondialdehyde (MDA)

There was a significant increase ($P<0.001$) in testicular MDA in DM control (39.13 ±0.35 nmol/g), DM + insulin (35.56 ± 0.64 ± 0.64 nmol/g), DM + zinc (35.65 ± 0.19 nmol/g), DM + insulin + zinc (33.48 ± 0.26 nmol/g) groups when compared to the normal control group (30.54 ± 0.11 nmol/g). Testicular MDA decreased significantly ($P<0.001$) in DM + insulin, DM + zinc and DM + insulin + zinc groups when compared to the DM control. Testicular MDA in the DM + insulin + zinc group decreased significantly when compared to the DM + insulin ($P<0.01$) and DM + insulin + zinc ($P<0.01$) groups (Fig 5).

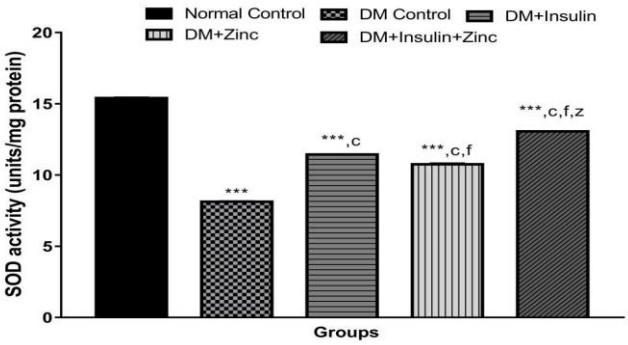

Fig 1: Comparison of testicular SOD activity between the different groups

Values are mean ± SEM, n = 5.

***p<0.001 vs Normal control; c = p<0.001 vs DM control; f = p<0.001 vs DM + Insulin; z = p<0.001 vs DM + Zinc.

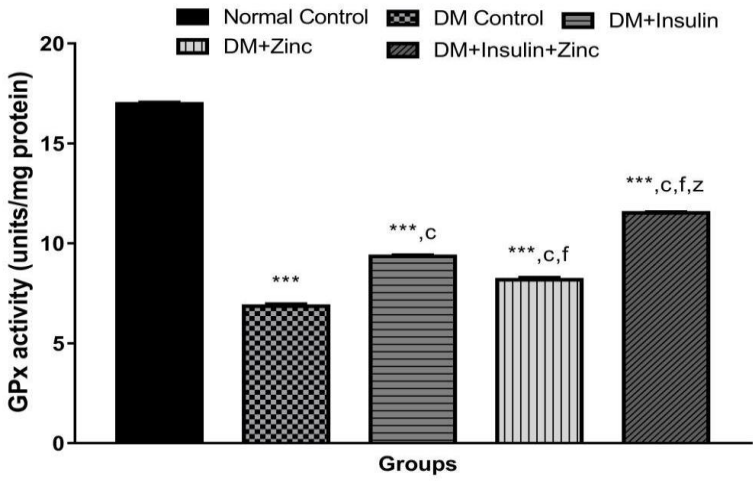

Fig 2: Comparison of testicular GPx activity between the different groups

Values are mean ± SEM, n = 5.

***p<0.001 vs Normal control; c = p<0.001 vs DM control; f = p<0.001 vs DM + Insulin; z = p<0.001 vs DM + Zinc

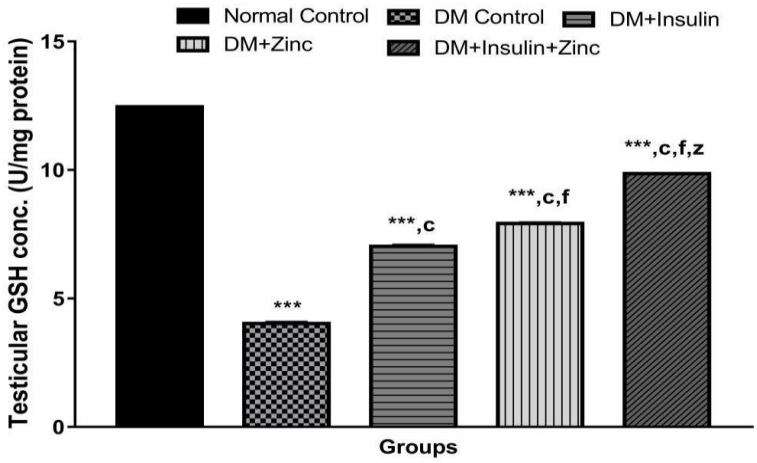

Fig 3: Comparison of testicular GSH concentration between the different groups

Values are mean ± SEM, n = 5.

***p<0.001 vs Normal control; c = p<0.001 vs DM control; f = p<0.001 vs DM + Insulin; z = p<0.001 vs DM + Zinc.

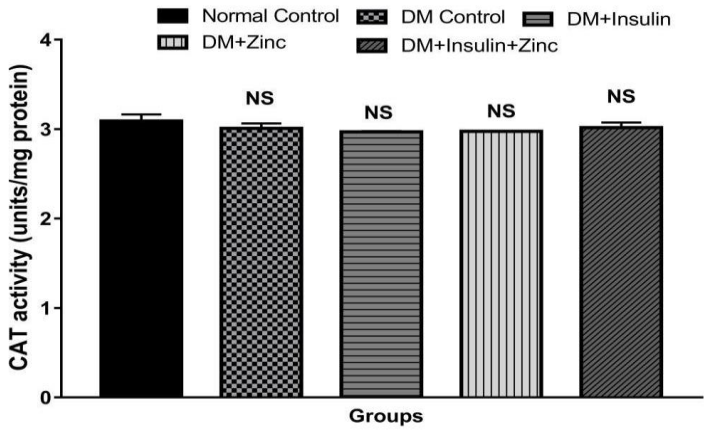

Fig 4: Comparison of testicular CAT activity between the different groups

Values are mean ± SEM, n = 5.

NS = not significant vs Normal control (p>0.05).

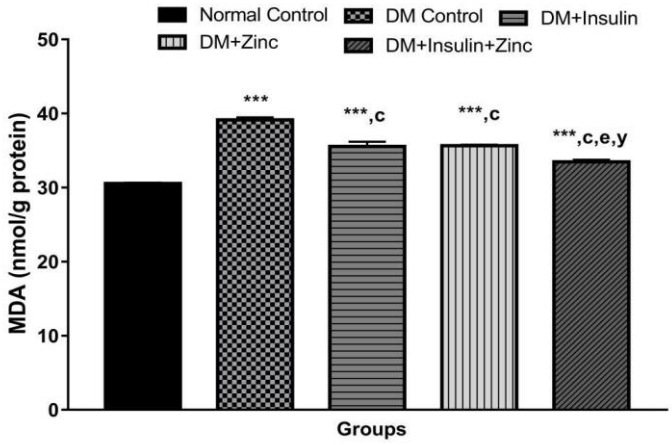

Fig 5: Comparison of testicular MDA concentration between the different groups

Values are mean ± SEM, n = 5.

***p<0.001 vs Normal control; c = p<0.001 vs DM control; e = p<0.01 vs DM + Insulin; y = p<0.01 vs DM + Zinc.

DISCUSSION

Oxidative stress is heavily implicated in the pathogenic role DM plays in inducing male reproductive impairment. It does this by generating reactive oxygen species (ROS) and decreasing antioxidant defence systems (Ahmed, 2005). Superoxide dismutase (SOD) and glutathione peroxidise (GPx) are the main antioxidant defence systems in the testes (Fujii *et al.*, 2003). SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and molecular oxygen, and thus forms the first line of defence against ROS (Bannister *et al.*, 1987). GPx on the other hand, uses GSH to reduce hydrogen peroxides, and hence protects cells against oxidative stress (Brigelius, 1999). In this study, there was significant oxidative stress in the diabetic rats' testes as evidenced by increased lipid peroxidation manifested as high malondialdehyde (MDA) level and a concomitant decrease in SOD, GPx and GSH testicular activities. These findings are in consonance with other studies (Mohasseb *et al.*, 2011; Sawiress *et al.*, 2011; La Vignera *et al.*, 2012; Afifi *et al.*, 2015). The decrease in these enzymes is likely due to the formation of glycation products as is the case in DM. These glycation products have been shown to inactivate antioxidant enzymes by reacting with the amino acid close to their active sites (Gillery, 2006). Testicular tissue and sperm cells are particularly susceptible to oxidative damage because of the high amount of unsaturated fatty acids in their membranes. Sperm cells lose their integrity and consequently become less motile (Aiteken *et al.*, 1989; Robinson *et al.*, 1992; Tramer *et al.*, 1998). The co-administration of insulin and zinc decreased lipid peroxidation and correspondingly increased SOD, GPx and GSH activities better than either insulin or zinc. Insulin may have decreased the accumulation of advanced glycation end products (AGE) by its antihyperglycaemic effect, thus decreasing the amount of ROS formed. On the other hand, zinc protects sulfhydril groups and displaces transition metals like iron and copper from catalytic sites, thus limiting lipid peroxidation (Aiteken and Roman, 2008). Working together, both insulin and zinc appeared to have synergized their actions.

Catalase (CAT) activity in the testes showed no significant change. Other studies (Ihrig *et al.*, 1974; Kheradmand *et al.*, 2009) have also shown similar testicular CAT activity as in this study. Evidence exists that CAT activity is usually low in the testis as compared to other tissues (Peltola *et al.*, 1992).

CONCLUSION

Diabetes mellitus causes oxidative stress in testes. This can cause injury, hence limiting function. Insulin or zinc ameliorates oxidative stress in testes. The combination of insulin and zinc however provided better results.

ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance of Mr Umoh Ededet

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