

Full-Length Research Article

Insulin and Zinc Co-Administration Ameliorates Diabetes Mellitus-Induced Reproductive Dysfunction in Male Rats

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Summary: Impaired male reproductive function is a major complication associated with diabetes mellitus (DM). Whether or not insulin, when co-administered with zinc will reverse or ameliorate reproductive dysfunction in male diabetics is not known. This study thus sought to establish if co-administration of insulin and zinc reverses or ameliorates male reproductive dysfunction in DM better than either insulin or zinc. Five (5) normal and twenty (20) diabetic sexually mature rats were assigned into five groups of five animals each. Group A consisted of normal rats and had access to only food and water. Group B consisted of diabetic animals with no treatment and served as DM control. DM was induced by a single intraperitoneal injection of 60mg/kg of streptozotocin after an overnight fast. Groups C and D consisted of diabetic animals that received insulin and zinc respectively. Group E consisted of diabetic animals that received both insulin and zinc. All diabetic animals had free access to food and water. Insulin in all cases was given subcutaneously twice daily in the morning and evening at 1 unit and 4 units respectively. Zinc (10mg/kg) was given orally once daily. Treatments in all cases commenced two weeks after DM was confirmed. The treatment lasted ten days. Samples were thereafter collected for analyses. DM decreased sperm count, sperm motility, sperm viability, normal sperm cells, semen pH, Serum Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and testosterone, while increasing sperm cells with defective tails. DM also impaired testicular morphology. Insulin and zinc co-administration improved sperm viability, Sertoli cell count, Johnsen's score, serum FSH, LH and testosterone. Co-administration also improved semen pH towards normal. Insulin or zinc ameliorated several aspects of DM-induced male sexual dysfunction. However, the co-administration of insulin and zinc provided better results.

Keywords: Diabetes mellitus, Insulin, Zinc, Co-administration, Reproductive dysfunction, Ameliorates

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INTRODUCTION

Diabetes mellitus (DM) is a chronic and complex metabolic condition that is characterized by increased blood glucose, otherwise known as hyperglycaemia. It is the result of aberrations in either insulin secretion or insulin action. DM could also result from a combination of aberrations in both insulin secretion and action (American Diabetes Association, 2011). Today, DM has become a disease of major public health concern. The 2017 International Diabetes Federation report estimates that there are 425 million diabetics worldwide. This figure will increase to 693 million people by 2045. At present, 79% of diabetic cases are in low and middle-income countries (IDF, 2017). DM is associated with a number of complications including cardiovascular disorders, neuropathy, nephropathy, retinopathy and infertility (Atkinson and Maclaren, 1994; Melendez-Ramirez *et al.*, 2010). A number of studies have shown that DM impairs male reproductive capacity at various levels. DM impairs steroidogenesis (Baccetti *et al.*, 2002; Schoeller, *et al.*, 2012), alters glucose metabolism in testes (Alves *et al.*, 2013), induces changes in testicular morphology (Ricci *et al.*, 2009), lowers sperm quality and quantity (Scarano *et al.*, 2006), reduces libido, causes

ejaculatory dysfunction (Sexton and Jarow, 1997) and impairs spermatogenesis (La Vignera *et al.*, 2012). DM impairs male reproductive capacity by increasing oxidative stress through the generation of reactive oxygen species (ROS) (Tabak *et al.*, 2011; La Vignera *et al.*, 2012). Since DM is mainly characterized by hyperglycaemia, the management of the glycaemic state is crucial to reducing the complications associated with it. Insulin is the most powerful antihyperglycaemic agent commonly used for glycaemic control in diabetics (Peyrot *et al.*, 2010). Diabetes mellitus (DM) is usually diagnosed in the advanced phase, thus allowing the progression of functional deterioration in cells that may not be reversed with insulin therapy alone (Donner and Munoz, 2012). About half of the people living with DM are unaware they have the disease (IDF, 2017). Diabetes mellitus is usually accompanied by reduced zinc levels (Kelly, 1998) and increased oxidative stress levels (La Vignera *et al.*, 2012), both of which impair male sexual function. Zinc is a trace element that is essential for testosterone production (Giugliano *et al.*, 1996). It also has antioxidant effects (Stumvoll *et al.*, 2005). Whether or not zinc, when co-administered with insulin in male diabetics, who already present with complications, will ameliorate

reproductive impairments better than insulin administration alone is what this study hopes to establish.

MATERIALS AND METHODS

Streptozotocin (S0130), Insulin (Humulin) and Zinc gluconate were obtained from Sigma-Aldrich (St. Louis, MO, USA), Novo Nordisk A/S (Denmark) and Pharmedic (Vietnam) respectively. All other chemicals and reagents were commercially available and of analytical grade.

Experimental animals: Twenty-five (25) male Wistar rats aged between 8 and 10 weeks and weighing between 150 – 230g purchased from the Animal House of the Anatomy Department of the University of Benin, Benin, Nigeria were used for this experiment. These animals were housed in standard animal cages with sawdust as beddings in the Animal House of Physiology Department, University of Calabar, Calabar, Nigeria. The animals were allowed a period of two weeks to acclimatize. Animals were kept in standard conditions and had free access to rat feed and water.

Experimental design/Drug administration: Five (5) normal and twenty (20) diabetic rats were assigned into five (5) groups of five (5) animals each (The animals were assigned into groups such that the mean differences in weight before the start of the experiment were similar): Group 1 – Normal control, Group 2 – Diabetes mellitus (DM) control, Group 3 – Diabetes mellitus (DM) + Insulin, Group 4 – Diabetes mellitus (DM) + Zinc, Group 5 – Diabetes (DM) + Insulin + Zinc. Diabetes mellitus (DM) was induced after overnight fast by single intraperitoneal injection of 60mg/kg of streptozotocin (STZ) (De Young *et al.*, 2004). Fresh 0.1M citrate buffer (pH 4.5) was used as vehicle. Blood from tail vein prick was assayed for fasting glucose forty eight hours after DM induction. Animals with fasting blood glucose of 250 mg/dL and above were considered diabetic (Cao *et al.*, 2012). Animals were then allowed a period of two weeks, with free access to just normal rat feed and water before commencement of treatments. Insulin was subcutaneously administered twice daily to animals in group 3, at 1 and 4 units in the morning and evening respectively (Pinheiro *et al.*, 2011). Oral zinc (10mg/kg) was given once daily to animals in group 4 (Shidfar *et al.*, 2010). Animals in group 5 received both insulin and zinc in the doses and routes as above. The treatments lasted for ten (10) days after which samples were collected for analyses.

Collection of blood samples and Serum Preparation: Under chloroform anaesthesia, 5mls of blood was collected by means of cardiac puncture into plain sample bottles and then made to stand for thirty (30) minutes. The blood was then centrifuged for ten (10) minutes at 4000g for the collection of serum for hormonal assay.

Preparation of testicular homogenates: Testes (Right) were each homogenized separately in cold 0.1M phosphate buffer (pH 7.4) with Heidolph homogenizer and Teflon pestle. The homogenate thereafter was centrifuged at 3500g for ten (10) minutes at 4°C. The supernatants were then assayed for biochemical parameters.

Measurement of body weight and testicular weight: Total body weight of rats was measured on a weekly basis throughout the duration of the study. The testis was harvested, cleared of adjoining tissue and also weighed. Digital weighing scale was used for both measurements.

Hormonal assay: Testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in serum were determined using ELISA kits following manufacturer's protocol.

Semen analysis: The epididymis together with the vas deferens were harvested and sperm count, viability, morphology and semen pH assessed from caudal portion of the epididymis (Ujah *et al.*, 2021). Sperm motility was determined from cells in the vas deferens. Method was as in a previous study (Nna *et al.*, 2017).

Histopathological techniques: Haematoxylin and eosin (H and E) technique was used for testicular histopathology. The left testis was harvested under chloroform anaesthesia and immediately placed in Bouin's fluid for fixation. It was then dehydrated and embedded in paraffin blocks. The tissue blocks were sectioned and stained with H and E on glass slides. The slides were appropriately labelled and photomicrographs taken at x40 magnification under a light microscope. Tissue sections were evaluated for Sertoli cell count, seminiferous tubular diameter, number of germ cell layers, leydig cell count, seminiferous epithelial height and Johnsen's score. Seminiferous tubular diameter and seminiferous epithelial height were measured by ocular micrometer calibrated with stage micrometer (D'Souza, 2004). The Johnsen's score was evaluated using the Johnsen scoring method as shown in table 1 (Johnsen, 1970).

Table 1
Johnsen's Score

Score	Features
10	Complete spermatogenesis with perfect tubules
9	Many spermatozoa present but disorganized spermatogenesis
8	Few spermatozoa present
7	No spermatozoa but many spermatids present
6	Only a few spermatids present
5	No spermatozoa or spermatids present but many spermatocytes present
4	Only a few spermatocytes present
3	Spermatogonia alone present
2	Germ cells absent
1	No germ cells no Sertoli cells present

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Statistical analysis

Graph pad prism 7.0 (Graph Pad Software Inc., La Jolla, CA, USA) was used for data analysis. The One-way analysis of variance (ANOVA) followed by post-hoc test (turkey) was used to analyse data. Results were presented as mean \pm standard error of mean. A p value below 0.05 was considered significant.

RESULTS

Fasting blood glucose: Fig. 1 shows a significant increase ($P<0.001$) in fasting blood glucose concentration in the DM control group (602.80 ± 0.73 mg/dL), DM + insulin group (129.40 ± 3.20 mg/dL) and DM + Zinc group (582.20 ± 20.05 mg/dL) when compared to the normal control group (99.20 ± 1.24 mg/dL). Results further showed that fasting blood glucose concentration was significantly higher ($P<0.001$) in the DM + Zinc group when compared to the DM + Insulin group. There was a significant decrease in fasting blood glucose concentration in the DM + Insulin + Zinc group (116.00 ± 1.18 mg/dL) when compared with the DM control ($P<0.001$) and DM + Zinc ($P<0.001$) groups.

Semen analysis: There was a significant decrease ($P<0.001$) in sperm count in DM control ($0.16 \times 10^6 \pm 0.04$ /ml), DM + insulin ($0.16 \times 10^6 \pm 0.02$ /ml), DM + zinc ($0.12 \times 10^6 \pm 0.02$ /ml) and DM + insulin + zinc ($0.20 \times 10^6 \pm 0.03$ /ml) when compared with the normal control group ($19.08 \times 10^6 \pm 0.63$ /ml) (Table 2).

There was a significant decrease ($P<0.001$) in sperm motility in DM control ($0.00 \pm 0.00\%$), DM + insulin ($12.00 \pm 1.22\%$), DM + zinc ($0.00 \pm 0.00\%$) and DM + insulin + zinc ($10.00 \pm 0.00\%$) groups when compared to normal control group ($77.00 \pm 4.36\%$). When compared to DM control however, sperm motility increased significantly in the DM + insulin group ($P<0.01$) and DM + insulin + zinc group ($P<0.05$). Sperm motility decreased significantly ($P<0.01$) in the DM + zinc group when compared with the DM + insulin group. There was a significant increase ($P<0.05$) in sperm motility in the DM + insulin + zinc group when compared with the DM + zinc group. (Table 2).

Sperm viability decreased significantly ($P<0.001$) in DM control ($12.00 \pm 1.22\%$), DM + insulin ($24.60 \pm 0.24\%$), DM + zinc ($24.00 \pm 0.55\%$) and DM + insulin + zinc ($25.00 \pm 0.32\%$) groups when compared to normal control group ($84.20 \pm 0.49\%$). Results further show a significant increase ($P<0.001$) in sperm viability in DM + insulin, DM + zinc and DM + insulin + zinc groups when compared to DM control group. (Table 2).

Abnormal sperm morphology increased significantly in DM control ($15.20 \pm 0.73\%$, $P<0.001$) and DM + zinc group ($4.80 \pm 0.66\%$, $P<0.05$) groups when compared to normal control group ($1.80 \pm 0.37\%$). There was however a significant decrease ($P<0.001$) in abnormal sperm morphology in DM + insulin group ($3.60 \pm 0.40\%$), DM +

zinc group and DM + insulin + zinc group ($3.80 \pm 0.49\%$) when compared to the DM control. (Table 2).

Sperm head defect: Table 2 shows comparison of sperm head defect between normal control ($0.00 \pm 0.00\%$), DM control ($1.00 \pm 0.77\%$), DM + insulin ($0.60 \pm 0.24\%$), DM + zinc ($0.60 \pm 0.24\%$) and DM + insulin + zinc ($0.40 \pm 0.24\%$) groups. Results show no significant change ($P>0.005$) in sperm head defect between the different groups.

Sperm neck defect: Table 2 shows results for sperm neck defect in normal control ($0.40 \pm 0.24\%$), DM control ($1.20 \pm 0.49\%$), DM + insulin ($0.60 \pm 0.24\%$), DM + zinc ($0.40 \pm 0.24\%$) and DM + insulin + zinc ($1.40 \pm 0.24\%$) groups. There was no significant difference ($P>0.05$) between the groups.

Sperm tail defect: There was a significant increase in sperm tail defect in DM control group ($12.80 \pm 0.37\%$, $P<0.001$) and DM + zinc group ($3.60 \pm 0.51\%$, $P<0.05$) when compared to normal control group ($1.40 \pm 0.24\%$). There was however a significant decrease in sperm tail defect in DM + insulin ($2.60 \pm 0.40\%$, $P<0.001$), DM + zinc ($P<0.001$) and DM + insulin + zinc ($2.00 \pm 0.63\%$, $P<0.001$) when compared to the DM control (Table 2)

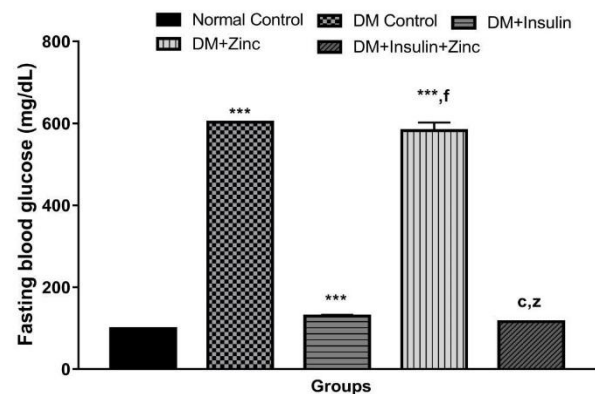


Figure 1:

Comparison of fasting blood glucose concentration between the different groups.

Values are mean \pm SEM, $n = 5$.

*** vs Normal control ($p<0.001$); c vs DM control ($p<0.001$); f vs DM +

Insulin ($p<0.001$); z vs DM + Zinc ($p<0.001$)

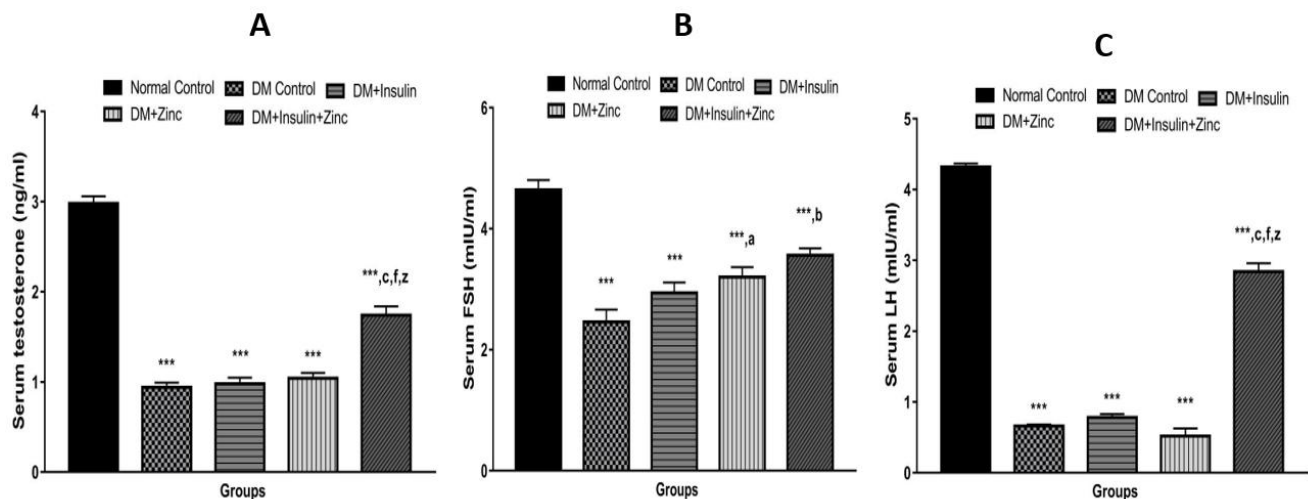
Table 2
Semen Analysis

	Normal control	Diabetes mellitus	Diabetes mellitus+Insulin	Diabetes mellitus + zinc	Diabetes mellitus+insulin + zinc
Sperm count $\times 10^6$/ml	19.08 \pm 0.63	0.16 \pm 0.04***	0.16 \pm 0.02***	0.12 \pm 0.02***	0.20 \pm 0.03***
Sperm motility (%)	77.00 \pm 4.36	0.00 \pm 0.00***	12.00 \pm 1.22***,b	0.00 \pm 0.00***,e	10.00 \pm 0.00***,a,x
Sperm viability(%)	84.20 \pm 0.49***,c	12.00 \pm 1.22***,c	24.60 \pm 0.24***,c	24.00 \pm 0.55***,c	25.00 \pm 0.32***,c
Abnormal sperm morphology(%)	1.80 \pm 0.37	15.20 \pm 0.73***	3.60 \pm 0.40 ^c	4.80 \pm 0.66 ^{x,c}	3.80 \pm 0.49 ^c
Head defect(%)	0.00 \pm 0.00	1.00 \pm 0.77	0.60 \pm 0.24	0.60 \pm 0.24	0.40 \pm 0.24
Neck defect(%)	0.40 \pm 0.24	1.20 \pm 0.49	0.60 \pm 0.24	0.40 \pm 0.24	1.40 \pm 0.24
Tail defect(%)	1.40 \pm 0.24	12.80 \pm 0.37***	2.60 \pm 0.40 ^c	3.60 \pm 0.51 ^{x,c}	2.00 \pm 0.63 ^c
Semen pH	6.12 \pm 0.04	5.80 \pm 0.04***	6.22 \pm 0.08 ^c	6.30 \pm 0.00 ^c	6.06 \pm 0.02 ^{b,x}

Values are mean \pm SEM, $n = 5$.

*** vs Normal control ($p<0.001$); a vs DM control ($p<0.05$), b vs DM control ($p<0.01$); c vs DM control ($p<0.001$); e vs DM + Insulin ($p<0.01$); x vs DM + Zinc ($p<0.05$)

Insulin and zinc ameliorate sexual dysfunction in diabetic males

**Figure 2:**

Comparison of serum reproductive hormone concentration between the different groups.

Values are mean \pm SEM, $n = 5$.

*** vs Normal control ($p < 0.001$); a ($p < 0.05$), b ($p < 0.01$) vs DM control. c vs DM control ($p < 0.001$); f vs DM + Insulin ($p < 0.001$); z = vs DM + Zinc ($p < 0.001$)

Semen pH: Table 2 also compares the semen pH across the different groups. There was a significant decrease ($P < 0.001$) in semen pH in the DM control group (5.80 ± 0.04) when compared to normal control group (6.12 ± 0.04). There was a significant increase ($P < 0.001$) in semen pH in DM + insulin (6.22 ± 0.08) and DM + zinc (6.30 ± 0.00) groups when compared to DM control. There was however a significant decrease ($P < 0.01$) in semen pH in the DM + insulin + zinc group (6.06 ± 0.02) when compared to the DM control. There was a significant decrease ($P < 0.05$) in semen pH in the DM + insulin + zinc group when compared to DM + zinc group.

Serum testosterone: Figure 2A shows comparison of serum testosterone between the different groups. There was a significant decrease in serum testosterone ($P < 0.001$) in the DM control group (0.94 ± 0.05 ng/ml), DM + Insulin group (0.98 ± 0.07 ng/ml), DM + zinc group (1.04 ± 0.06 ng/ml) and DM + insulin + zinc group (1.74 ± 0.10 ng/ml) when compared with the normal control (2.98 ± 0.08 ng/ml). Results also show a significant increase in serum testosterone in the DM + insulin + zinc group when compared with DM control ($P < 0.001$), DM + insulin ($P < 0.001$) and DM + zinc ($P < 0.001$) groups.

Serum follicle stimulating hormone (FSH): Serum FSH decreased significantly ($P < 0.001$) in DM control (2.46 ± 0.20 mIU/ml), DM + insulin (2.94 ± 0.17 mIU/ml), DM + zinc (3.20 ± 0.16 mIU/ml) and DM + insulin + zinc (3.56 ± 0.12 mIU/ml) groups when compared to the normal control group (4.64 ± 0.16 mIU/ml). serum FSH was significantly increased in DM + zinc ($P < 0.05$) and DM + insulin + zinc ($P < 0.01$) groups when compared to the DM control group (Fig. 2B)

Serum luteinizing hormone (LH): Figure 2C shows that serum LH was significantly decreased ($P < 0.001$) in DM control (0.66 ± 0.02 mIU/ml), DM + insulin (0.78 ± 0.05 mIU/ml), DM + zinc (0.52 ± 0.11 mIU/ml) and DM + insulin + zinc (2.84 ± 0.12 mIU/ml) groups when compared

to the normal control group (4.32 ± 0.05 mIU/ml). The serum LH level increased significantly in the DM + insulin + zinc group when compared with DM control ($P < 0.001$), DM + insulin ($P < 0.001$) and DM + zinc ($P < 0.001$) groups.

Sertoli cell count: Sertoli cell count significantly decreased ($P < 0.001$) in DM control (5.02 ± 0.25), DM + insulin (6.34 ± 0.06), DM + zinc (5.65 ± 0.21) and DM + insulin + zinc (7.00 ± 0.36) when compared to the normal control group (8.60 ± 0.14). There was a significant increase in sertoli cell count in DM + insulin ($P < 0.01$) and DM + insulin + zinc ($P < 0.001$) groups when compared to DM control group. A significant increase ($P < 0.01$) in Sertoli cell count was recorded in the DM + insulin + zinc group when compared to the DM + zinc group (Fig. 3A)

Leydig cell count: Results show a significant decrease ($P < 0.001$) of Leydig cells in DM control (3.64 ± 0.15), DM + insulin (3.69 ± 0.06), DM + zinc (3.71 ± 0.10) and DM + insulin + zinc (4.03 ± 0.08) groups when compared to the normal control group (5.30 ± 0.05). (FIG. 3B).

Germ cell layers: Fig. 3C shows a significant decrease ($P < 0.001$) in the number of germ cells in the DM control group (1.80 ± 0.20), DM + zinc group (2.60 ± 0.24), DM + insulin + zinc (3.00 ± 0.32) and DM + insulin group (4.20 ± 0.49 , $P < 0.05$) when compared to the normal control group (5.60 ± 0.24). There was a significant increase ($P < 0.001$) in the number of germ cell layers in DM + insulin group when compared to the DM control group. The number of germ cell layers significantly decreased ($P < 0.05$) in the DM + zinc group when compared with the DM + insulin group.

Seminiferous tubule diameter: There was a significant decrease ($P < 0.001$) in seminiferous tubule diameter in DM control (63.30 ± 0.45 μ m), DM + insulin (154.64 ± 1.42 μ m), DM + zinc (68.56 ± 0.65 μ m) and DM + insulin + zinc (83.78 ± 2.01 μ m) groups when compared to the normal control group (170.44 ± 1.01 μ m). Results further show a significant increase ($P < 0.001$) in seminiferous tubule

diameter in DM + insulin and DM + insulin + zinc groups when compared with DM control group. A significant decrease ($P < 0.05$) in seminiferous tubule diameter was seen in DM + insulin group when compared to DM control group. Seminiferous tubule diameter was significantly decreased ($P < 0.001$) in DM + zinc and DM + insulin + zinc groups when compared to DM + insulin group. Seminiferous tubule diameter significantly increased ($P < 0.001$) in DM + insulin + zinc group when compared to DM + zinc group. (Fig.3D).

Seminiferous epithelial height: Result (Fig. 3E) show a significant decrease ($P < 0.001$) in seminiferous epithelial height in DM control ($19.38 \pm 0.32 \mu\text{m}$), DM + insulin ($28.70 \pm 1.23 \mu\text{m}$), DM + zinc ($27.36 \pm 1.62 \mu\text{m}$) and DM + insulin + zinc ($27.32 \pm 0.93 \mu\text{m}$) groups when compared to the normal control group ($65.14 \pm 0.38 \mu\text{m}$). There was a significant increase ($P < 0.001$) in seminiferous epithelial height in DM + insulin, DM + zinc and DM + insulin + zinc groups when compared to DM control group.

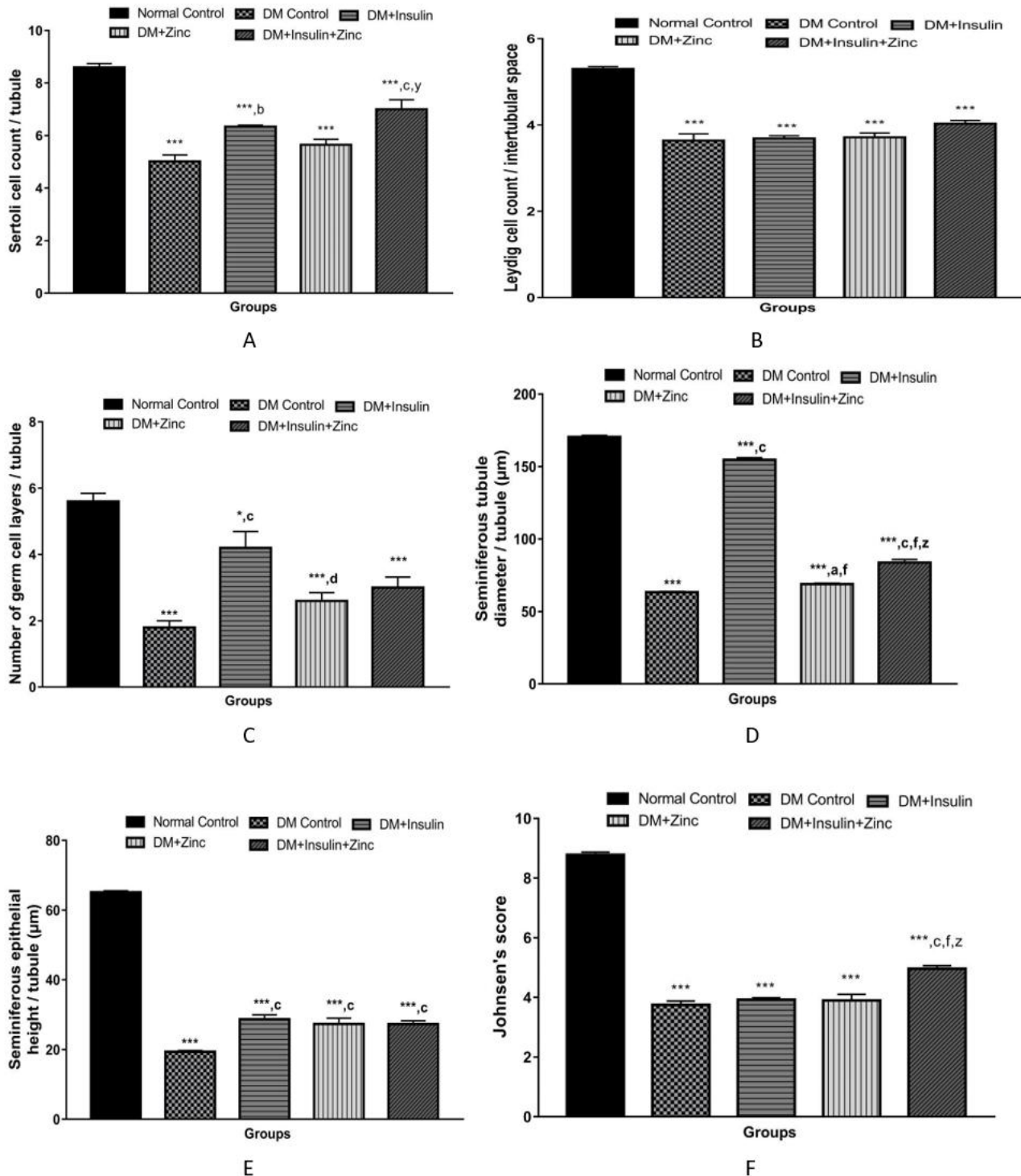


Figure 3

Comparison of histological indices between the different groups.

Values are mean \pm SEM, $n = 5$; *** vs Normal control ($p < 0.001$); b ($p < 0.01$), c ($p < 0.001$) vs DM control; d vs DM + insulin ($p < 0.05$); f vs DM + insulin ($p < 0.001$); y vs DM + zinc ($p < 0.01$); z vs DM + zinc ($p < 0.001$)

Johnsens's score: Johnsen's score decreased significantly ($P < 0.001$) in DM control (3.75 ± 0.13), DM + insulin (3.92 ± 0.06), DM + zinc (3.90 ± 0.20) and DM + insulin + zinc (4.96 ± 0.11) groups when compared to the normal control group (8.78 ± 0.09). There was a significant increase in Johnsen's score in the DM + insulin + zinc group when compared to DM control ($P < 0.001$), DM + insulin ($P < 0.001$) and DM + zinc ($P < 0.001$) groups. (Fig.3F).

Histopathology of testicular tissue comparing seminiferous tubules and intertubular interstitium between the groups:

Figs 4a and 5a show histopathology of seminiferous tubules and intertubular interstitium respectively in the normal control group. The seminiferous tubules contain proliferating spermatogonia cells at various stages of maturation. These cells include spermatogonia A and B that are closer to the basement membrane, the primary spermatocytes, secondary spermatocytes, spermatid and spermatozoa. The cells have regular nuclei contour and there are 10 to 12 sertoli cells per tubules and the leydig cell population within the peritubular and intertubular interstitium is 4 to 6 cells.

Figs 4b and 5b show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM + insulin group. Section of the testis shows seminiferous tubules containing scanty spermatogonia with a thickened basement membrane. There is complete absence of spermatogonia in majority of the seminiferous tubules and some few scanty spermatogonia A and B. The spermatocytes, spermatids and spermatozoa are absent. The sertoli cell population is less than six per tubule and the leydig cell population is scanty and less than three per

cluster. The scanty spermatogonia are atrophic and few macrophages are seen in the lumen.

Figs 4c and 5c show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM + zinc group. Section of the testis shows atrophic seminiferous tubules containing sparsely populated spermatogonia. The basement membranes are thickened and the spermatogonia are atrophic. The spermatogonia are less than two cell layer thick and the intervening interstitium is scanty and contains less than three round to oval leydig cells. The supporting Sertoli cells are also reduced in number. The other cell lineage such as the spermatocyte, spermatid and spermatozoa are absent.

Figs 4d and 5d show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM + insulin + zinc group. Seminiferous tubules with thickened basement membrane are seen. The tubules contain sparsely populated spermatogonia at various stages of maturation. The cells are less than four layers thick and are atrophic. The luminal cavities are empty and the Sertoli cell population is less than seven and the Leydig cells are less than four per cluster. Some of the tubules contain only spermatogonia cells. The cells have deeply stained atrophic nuclei.

Figs 4e and 5e show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM control group. The tubules contain scanty spermatogonia cells with complete absence of the late series which includes spermatocytes, spermatid and spermatozoa. There is atrophy of the spermatogonia cells and reduction in the Sertoli cell population to less than five per tubule. The separating interstitium is scanty and contains less than six Leydig cells per cluster

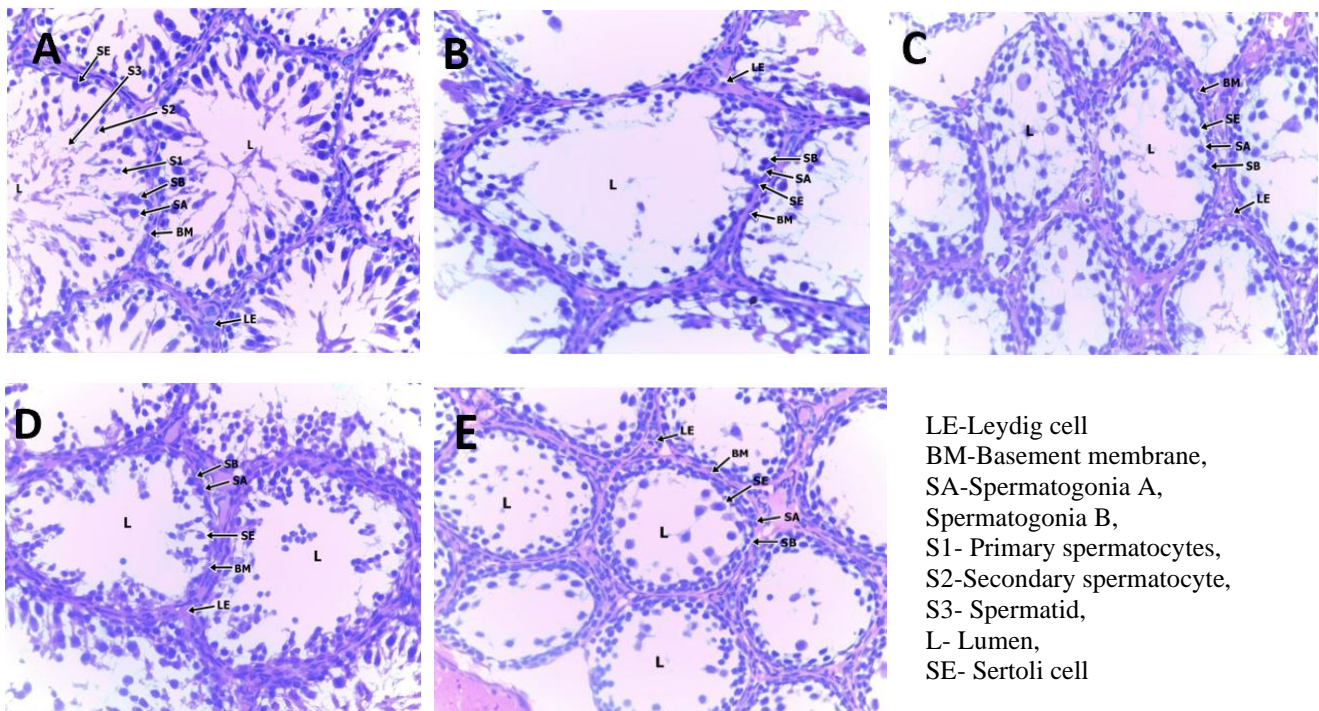


Plate 1

4a. Normal seminiferous tubules (ST) containing proliferating spermatogonia cells at various stages of maturation. F4b (DM + insulin group) shows ST with scanty spermatogonia. 4c (DM + Zinc group) shows atrophic ST containing sparsely populated spermatogonia. 4d (DM + insulin + zinc group) shows ST with sparsely populated spermatogonia. 4e (DM control group) shows ST containing scanty spermatogonia with complete absence of spermatocytes, spermatid and spermatozoa. All figures were viewed at x400 magnification

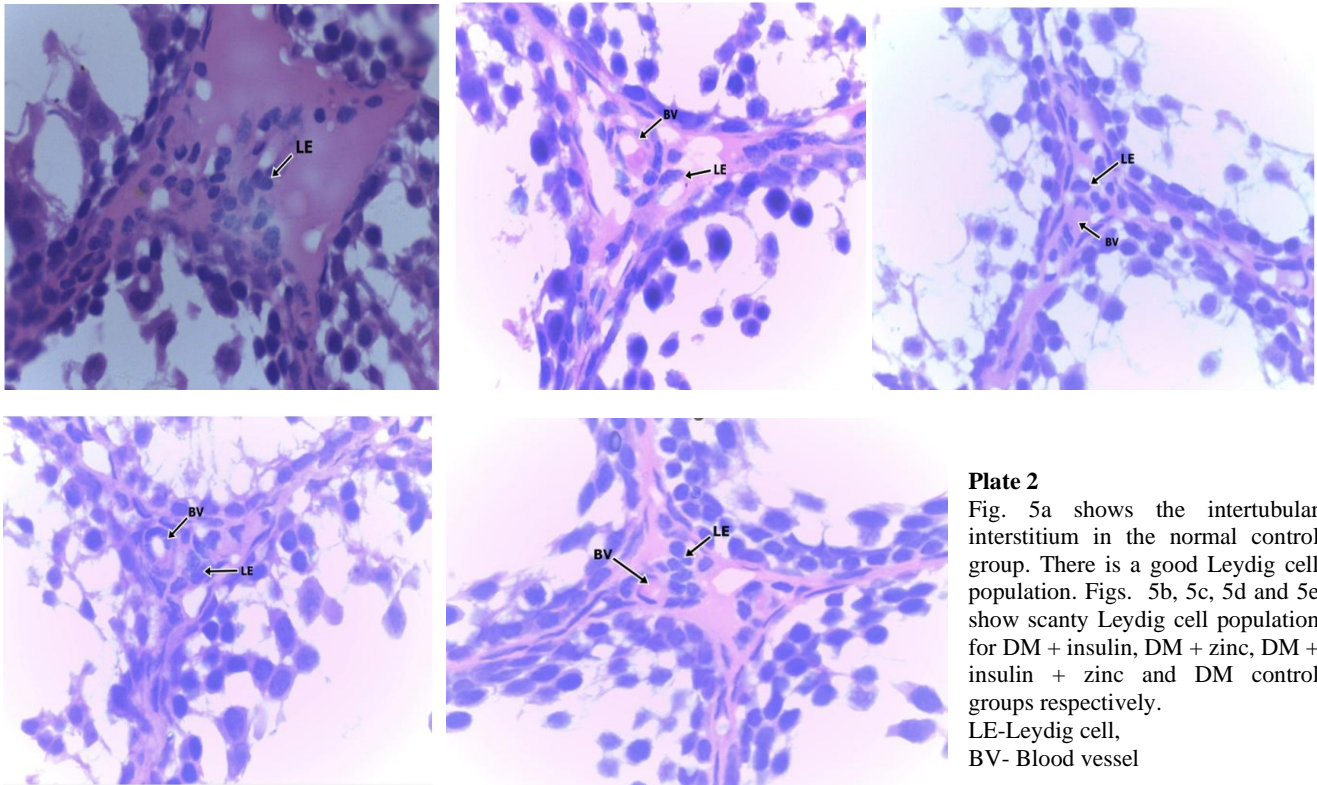
**Plate 2**

Fig. 5a shows the intertubular interstitium in the normal control group. There is a good Leydig cell population. Figs. 5b, 5c, 5d and 5e show scanty Leydig cell population for DM + insulin, DM + zinc, DM + insulin + zinc and DM control groups respectively.

LE-Leydig cell,
BV- Blood vessel

DISCUSSION

This study sought to establish if zinc and /or insulin could reverse or ameliorate diabetes mellitus (DM) – induced reproductive impairments in male rats.

True to its antihyperglycaemic effect, insulin decreased fasting blood glucose level in DM. the decrease was better when zinc was co-administered with insulin. It thus appears that while zinc may not have an antihyperglycaemic effect, it enhances insulin action by either prolonging its duration of action (Sharma and Divya, 2020) or increasing its tissue sensitivity (Thoen et al., 2019).

Results from the study showed that both body weight and testicular weight were decreased in diabetic conditions. This is consistent with other works (Scarano et al., 2006; Cao et al., 2012). The decrease in weight has been reported to be a consequence of metabolic alterations occasioned by insulin absence or insufficiency (Arduino, 1980). Also, testicular weight is a reflection of the size of the testis. Sertoli cells (SC) are important components of the testis, and the number of SCs is usually linked to testicular size (Sharpe et al., 2003). Insulin administration however improved body weight and its co-administration with zinc improved both general body weight and testicular weight in diabetic state. It appears that the improvement in body weight was mostly due to insulin effects. This is likely due to the improvement in metabolic activities following insulin therapy as insulin promotes cell division and growth (Wilcox, 2005). For testicular weight however, only in combination with zinc was insulin able to improve weight. This is likely due to the improvement in SC count in same group as shown by results.

Testosterone is a hormone that is critical to male fertility (Walker, 2010). Testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) are key

requirements for spermatogenesis, and together with gonadotropin-releasing hormone (GnRH) form the hypothalamus-pituitary-testicular axis (Ramaswamy and Weinbauer, 2015). In this study, the diabetic state decreased testosterone, FSH and LH. This is in line with a number of studies (Djursing et al., 1983; Scarano et al., 2006; Ricci et al., 2009; Mohasseb et al., 2011; Rato et al., 2015). The decreased levels of FSH and LH could be the result of diabetic neuropathy that may have affected the hypothalamus and/or pituitary (Dinulovic and Radonjic, 1990; Chao et al., 2022). The low level of testosterone in the diabetic state is likely the result of a decrease in Leydig cells (Rehman et al., 2001). Leydig cell count is usually directly proportional to testosterone concentration, as they are responsible for testosterone secretion (Nna et al., 2017). While insulin has been reported to have stimulatory effect on leydig cells (Ballester et al., 2004) and zinc to be essential for testosterone synthesis and release of FSH and LH (Al- Ani et al., 2015), neither the single administration of insulin nor zinc increased the level of any of these hormones in the diabetic states, except for FSH, where zinc administration increased its level in comparison to the diabetic untreated group. In combination however, insulin and zinc increased the levels of these hormones. There appears to be a synergy of actions of insulin and zinc when co-administered. Insulin binding to tissues is reported to be greater when co-administered with zinc (Thoen et al., 2019). Insulin sensitivity has been shown to be increased by zinc (Alkaladi et al., 2014). Its action and duration of action are also known to be potentiated by zinc (Dodson and Steiner, 1998; Owens, 2011). Zinc on the other hand is a co-factor in over 300 enzymes and hormones (Rink and Gabriel, 2000) and required for testosterone synthesis (Bhowmik et al., 2010). Insulin may have created an environment that enhanced zinc actions.

Insulin and zinc ameliorate sexual dysfunction in diabetic males

Leydig cell count decreased in all the diabetic groups and this was associated with a corresponding decrease on testosterone level in all the diabetic groups, except in the diabetic group treated with both insulin and zinc, where testosterone was increased. It is important to note that an increase (though not significant) in Leydig cell count in the insulin and zinc treated diabetic group was accompanied by a significant increase in LH in same group. LH is known to stimulate testosterone synthesis by Leydig cells (Ramaswamy and Weinbauer, 2015). This increased LH and the previous finding that zinc inhibits the conversion of testosterone to oestrogen (Al-Ani et al., 2015) may have been responsible for this phenomenon. It is also possible that the combination of insulin and zinc may have stimulatory effect on adrenal testosterone synthesis in ways that their separate administrations could not. The adrenal gland is known to synthesize testosterone (Nakamura et al., 2009) and this study assessed testosterone level in the serum.

Semen analysis is an essential tool in determining male fertility status. Sperm count, motility and viability were all decreased and abnormal sperm morphology increased in the diabetic groups. This is corroborated by several other studies (Scarano et al., 2006; Afifi et al., 2015; An et al., 2018; Laleethambika et al., 2019). Sperm quality is the end product of reproductive and other systems working together (Dinulovic and Radonjic, 1990) and hence a number of factors may have been responsible for the poor sperm quality observed in this study. Alterations in the hypothalamic-pituitary-gonadal axis, as manifested in decreased FSH, LH and testosterone is one way the sperm quality may have been compromised. These hormones are required for effective sperm production (Ramaswamy and Weinbauer, 2015). Other mechanisms may include; germ cell depletion and Sertoli cell deterioration (Bruning et al., 2000; Baccetti et al., 2002), all of which are evident in this study. Treatments in all cases seemed not to have improved sperm count compared to the untreated animals even though treatments improved a number of parameters (hormones, germ cell layer, Sertoli cell count, amongst others) influencing sperm count. It may be that the duration of study did not allow for full completion of the spermatogenic cycle which is averagely six weeks in rat (De Krester and Kerr, 1988). There was an increase in viability following treatments.

The increase in sperm motility in the diabetic untreated group following insulin and insulin and zinc co-treatment is mainly due to insulin as zinc when administered alone showed no significant change. Insulin being an antihyperglycaemic agent may have decreased oxidative stress by reducing the formation of glycation products. There is evidence that insulin interacts with sperm cells (Schoeller et al., 2012). Abnormal sperm morphology decreased to near normal level with treatments. It thus appears that zinc's antioxidant role (Sacan et al., 2016) and insulin's antihyperglycaemic effect reversed defects in sperm cells. Zinc concentration has been reported to be low in seminal fluid in DM (Dinulovic and Radonjic, 1990). Thus supplementation may have increased zinc levels to influence the observed changes. DM seems to have adverse effect on sperm morphology by mostly causing tail defects as this study shows. The head and neck of sperm cells were largely unaffected. This may also explain how DM decreases motility.

Semen pH was slightly decreased in diabetic untreated rats. DM is a metabolic disease that has been shown to be associated with high concentration of certain amino acids and their derivatives in the seminal fluid, believed to be the result of defective kreb's cycle (Kaemmerer and Mitzkat, 1985). This is likely how semen pH is decreased. This could also be implicated in the impairment of sperm motility and formation of defective sperm cells (Kaemmerer and Mitzkat, 1985). With either insulin or zinc treatment, semen pH was increased. The best result was however with insulin and zinc co-administration, as pH was brought to near normal levels. Zinc is essential for metabolism (Prasad, 2009) and it may have, especially in combination with insulin, reduced the amount of amino acids formed in the seminal fluid.

Effective male reproductive function is fundamentally dependent on a healthy testicular tissue. Testicular morphology was compromised by DM. Thickened basement membrane of the seminiferous tubules, sparsely populated spermatogonia and a scanty intertubular interstitium are some of the morphological changes that were seen from photomicrographs. Some of these changes have been similarly reported by Wankeu-Nya et al., (2013). A quantitative assessment of the testicular tissue revealed more morphometric deviations. Sertoli cell count, Leydig cells and germ cell layers were all depleted by DM. The SCs are key testicular cells that play a major role in spermatogenesis by nourishing the germ cells as has been stated earlier. Insulin and insulin co-treatment with zinc increased the SC count. The trend was similar with the number of germ cell layers except in the co-treatment group. While it could be said that the increase in the number of germ cell layers following insulin treatment is a consequence of the increase in SCs, same cannot be said for co-treatment. It may be that zinc has a yet unknown role in limiting germ cell proliferation, as even when administered alone; the number of germ cell layers was significantly lower than in insulin treatment. This wasn't the case with the SC count.

Seminiferous tubular diameter and seminiferous epithelial height are parameters that tell of testicular integrity (Nna et al., 2017). Oxidative stress causes testicular atrophy which manifests as a decrease in both tubular diameter and tubular epithelial height (Nna et al., 2017). The increased formation of glycation products is DM causes oxidative stress (Ahmed, 2005). Treatments improved epithelial height in equal measure while insulin treatment offered improvement in tubular diameter.

The Johsen's score is an index on a scale of 1-10 that reflects spermatogenesis (Johnson, 1970). DM impaired spermatogenesis. Insulin and zinc co-treatment improved spermatogenesis. This didn't however reflect in the sperm count. While the Johsen's score evaluates and scores all stages of spermatogenesis, sperm count measures only spermatozoa.

In conclusion, insulin or zinc ameliorates several aspects of DM-induced male reproductive dysfunction. However, the co-administration of insulin and zinc provides better results.

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