

**GONADAL HISTO-MORPHOLOGIESMORPHOLOGYAND ENHANCED
FERTILITY POTENTIALIN MALE STZ-INDUCED DIABETIC RATS TREATED
WITHOF CURCUMA LONGACurcuma LongaIN MALE STZ-INDUCED DIABETIC
RATS**

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ABSTRACT

The study was aimed at investigating the effects of ethanolic extract of *Curcuma longa* (Turmeric) on fertility profile and gonadal histo-morphology of ~~Streptozotocin~~ streptozotocin induced hyperglycemic male Wistar rats.

Semen parameters (count, motility and viability) and testicular histology of streptozotocin (STZ) -induced hyperglycemic male Wistar rats were investigated following the administration of *Curcuma longa* rhizome extract. Twenty-four rats weighing averagely 110 g-180g were randomly assigned into four groups of six rats each. Group A animals served as the normal control and they were given rat pellets and distilled water. Group B served as the diabetic control and the animals were ~~administered~~ induced to diabetes with 65mg/kg body weight of STZ intraperitoneally. Group C served as the STZ + Metformin treated group and the animals were administered 250mg/kg body weight of ~~M~~metformin. Group D served as the STZ + *Curcuma longa* treated group given 500mg/kg body weight of *Curcuma longa*. The administration of metformin and *C. longa* lasted for ~~twenty eight~~ twenty-eight days. Histo-morphology of the testis and semen analysis (sperm count, sperm motility, sperm viability) were carried out. The diabetic group showed seminiferous tubules with reduced germinal epithelium and distorted interstitial connective tissue with a significant ($p < 0.05$) reduction in sperm motility, count, viability compared to the normal group that showed normal histological features and normal sperm parameters (motility, count and viability). However, the administration of *C. longa* showed significant ($p < 0.05$) increase in levels of sperm parameters (motility, count and viability) and the progressive restoration of histological integrity within the testes.

In ~~Conclusion~~ conclusion, ethanolic extract of *C. longa* has the potential to reverse the damage caused by hyperglycaemia in male reproductive function.

Comment [Reviewer1]: Be consistent in your spelling and standardize to either British English or American English spelling.

Key words: *Curcuma longa*, ~~Semen-semen~~ analysis, testis, diabetes mellitus

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder with a characteristic high level of blood sugar (hyperglycemia) over a prolonged period of time¹ due to two major mechanisms which are either inability of the pancreas to produce insulin or the inability of the cells of the body to take up the insulin produced by the pancreas. Insulin is produced by the beta cells of the pancreas and its function is to regulate the uptake of glucose from blood into the different cells and tissues.²

Three major symptoms of Diabetes ~~Mellitus-mellitus~~ include frequent urination (Polyuria), increased hunger and appetite (~~p~~Polyphagia) and increased thirst (~~p~~Polydipsia).¹ DM is also associated with complications which include diabetic ketoacidosis, ~~Cardiovascular~~cardiovascular disease, foot ulcers, damage to nerves and eyes, encephalopathy, ~~Cardiomyopathy~~¹cardiomyopathy^{1,3,4} and reproductive dysfunction.⁵

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The diabetic complications on different cells, tissues and organs have been linked to the reactive oxygen species (ROS) that is generated due to hyperglycemia.⁶

Prolonged and poorly controlled diabetes has been reported to cause sexual dysfunction in males and females. Sexual dysfunction can also be an early sign of diabetes.⁷ In males, diabetes has effects on the endocrine control of spermatogenesis, causes erectile dysfunction and impaired ejaculation.⁸ It also negatively impacts sperm parameters such as volume, count, motility and morphology.⁹ Due to the ~~above-mentioned~~above-mentioned complications, it became imperative to find affordable solutions by scientists around the world.

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Studies have shown that to achieve a good metabolic control of diabetes and energy balance, a combination of lifestyle, nutrition, exercise and pharmaceutical treatment are important.¹⁰ And to some, they are the major factors in the management of diabetes.¹¹

Pharmaceutically, Metformin is considered to be the ~~first choice~~ first-choice agent for treatment of diabetes.¹² Another class of drugs of choice is Sulfonylureas¹³ These drugs however are seen to have undesirable side effects. On account of these side effects, there is advocacy for the use of medicinal plants¹⁴, because they have been found to have little or no side effects and are easily available and affordable. Some of these plants and herbs possess antioxidant properties. They contain ~~c~~Carotenoids, ~~f~~Flavonoids, alkaloids, glycosides and some are said to have anti diabetic effects.¹⁵ One of such plants is *Curcuma longa* commonly called ~~t~~urmeric.

Curcuma longa, the turmeric plant is ~~used as a spice~~ commonly used as a spice but has been recognized by the scientific community for its _____ property. In Asia, it has been used as a natural therapeutic medicine since ancient times.¹⁶ Its main constituent is curcuminoid¹⁷ which gives it a wide range of pharmacological properties including antioxidant, anti-protozoan, anti-venom, anti-inflammatory¹⁷ antibacterial, antidiabetic and antiviral and anticancer activities.¹⁵ It would therefore be interesting to evaluate its antidiabetic effects and its effects on the fertility profile of streptozotocin induced hyperglycemic male Wistar rats.

It has been estimated that 8-12% of couples worldwide during their reproductive life suffer some form of infertility, an estimated 50-80 million couples.¹⁸ Previously, infertility was thought to be a problem of the females but studies have now shown that 20-30% of infertility is linked to the ~~man~~¹⁹ males¹⁹ commonly due to poor semen quality or quantity²⁰

Comment [Reviewer2]: Paraphrase to give better meaning.

Diabetes has also shown an increase in prevalence over the years with an estimated 425 million persons worldwide and nearly 50% of that number is not diagnosed.²¹ Studies have shown that there is a decline in fertility in males with diabetes⁵ leaving a high percentage of them with reproductive dysfunctions including ~~a~~reduced libido and impotence.

The use of medicinal plants as an alternative medicine has in the last millennium, been accepted all over the world.²² In the United States of America for instance, about 38% of the population uses herbal medicine²³ and in Turkey, 48.8% of the population are said to use herbal medicines²⁴ to treat chronic diseases and illnesses. In Africa, the World ~~health~~ Health organization ~~Organization~~ states that at least 80% of the population relies on medicinal plants²⁵ ~~basically~~ due to low cost, availability and ~~apparently perceived~~ low toxicity²⁶

Diabetes Mellitus is said to cause reproductive dysfunctions in both males and females^{5,27} showed that diabetic men may observe a reduction in sperm count, motility and morphology due to hyperglycaemic induced Oxidative stress which causes damage to nuclear DNA of the sperm cells.^{27,28} In 2015, ⁸ found out that type 1 diabetic patients showed a lower level of spermatozoa with increased motility, altered mitochondrial function and post ejaculatory dysfunction of the epididymis.

Comment [Reviewer3]: Lacks continuity with the previous paragraph and the following paragraph.

Streptozotocin (STZ) is an alkylating agent that ~~produces~~ causes pancreatic islet β -cell destruction and is widely used experimentally to produce a model of type 1 diabetes mellitus (T1DM).²⁹ It is a cytotoxic glucose analogue which has been used as a chemotherapeutic agent in the treatment of metastasizing pancreatic islet cell tumours and other malignancies³⁰ and its effect can be seen within seventy-two hours after administration depending on doses administered.³¹ STZ has been one of the chemical agents used for the induction of diabetes in experimental animals.

Streptozotocin functions as DNA ~~(Deoxyribonucleic acid)~~ synthesis inhibitor in bacterial and mammalian cells.³² The selective pancreatic beta cell toxicity and diabetic condition, resulting from STZ induction, is related to the glucose moiety in its chemical structure which enables STZ to enter the beta cell via the low affinity glucose -2- transporter in the plasma membrane.³³

Semen analysis serves as a pivotal and indispensable procedure in the assessment of male fertility status, carrying substantial importance within the field.³⁴ When conducted meticulously and in an in-depth manner, it possesses the ability to elucidate the underlying causes contributing to male infertility, thereby offering valuable insights and diagnostic clarity.³⁵ The comprehensive evaluation of semen encompasses the analysis of diverse parameters, including the measurement of ejaculate volume, quantification of sperm count, assessment of sperm motility and movement patterns, evaluation of sperm morphology and structural integrity, as well as the investigation of the composition and makeup of seminal secretions.³⁵

Methods

Plant Collection and extract preparation

Rhizomes of *Curcuma longa* (Turmeric) were bought at ~~watt~~ market in Calabar, Cross River State. It was identified and authenticated by a taxonomist from the ~~department~~ Department of Botany, University of Calabar, Calabar, and a voucher number (Bot/Herb/UCC/201) was given.

The fresh turmeric rhizomes were cleaned, chopped into tiny pieces and air dried for 7 days after which they were grounded into powdered form. A measured amount of 950g of powdered rhizomes were extracted using 2 liters of 95% ethanol for 24 hours. The extract was first

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Comment [Reviewer4]: This paragraph lacks continuity with the previous paragraph.

Comment [Reviewer5]: wet?

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Comment [Reviewer6]: To include information on country.

Comment [Reviewer7]: To include information on country.

double filtered with Chess cloth, then with filtered paper (Whatman No.1 filter paper). The filtrate (extract) was concentrated under reduced pressure at 45°C in rotary evaporator to 10% volume and then to complete dryness using vacuum water bath yielding 58.9g (6.2%) of crude extract. The crude extract (paste) obtained was stored in a refrigerator until it was required.

Comment [Reviewer8]: cheese cloth?

Comment [Reviewer9]: Can the use of a vacuum water bath remove all of the water content from the crude extract? Is this a standard procedure?

Comment [Reviewer10]: indicate the temperature for storage.

Experimental Animals

~~Twenty-four~~ Twenty-four (24) adult male Wistar rats with average weight of 160g were used for this research. The rats were kept in clean cages and divided into four groups designated A, B, C and D with six rats in each group. The rats were allowed to acclimatize for two weeks in animal house, University of Calabar and allowed unrestricted access to commercially available chow (livestock feed) and water.

Experimental Design

Table 1 shows the experimental design of the research. ~~Twenty-four~~ Twenty-four animals were divided randomly into four groups containing six rats each. The groups as shown in the table includes the Normal control, the Diabetic control, the Metformin treated group and the Extract(*C.longa*) treated group.

Table 1: Experimental Design

S/N	N	GROUPS	TREATMENT
1.	6	A	Normal control (<u>d</u> Distilled water)
2.	6	B	Diabetic control 65mg/kg BW <u>bw</u> of STZ
3.	6	C	<u>65mg/kgbw of STZ</u> + 250mg/kg.bw metformin
4.	6	D	<u>65mg/kgbw of STZ</u> + 500mg/kg.bw Curcuma longa

Comment [Reviewer11]: indicate what is S/N before using the abbreviation

Comment [Reviewer12]: Number of rats per group

Induction of Hyperglycaemia

Streptozotocin (STZ) was administered intra-peritoneally after fasting for twelve hours in order to induce hyperglycaemia. STZ was reconstituted in 0.5M sodium citrate and administered at a dose of 65mg/kg-bw.³⁶

Confirmation of Diabetes Mellitus

Diabetes was confirmed three days after administration of STZ using Accu-Check glucometer with blood samples obtained from tails of the Wistar rats. The blood glucose levels (mg/dl) was checked (before and after) induction and every seven(7) days during administration of the ethanolic extract of *Curcuma longa* to ascertain hyperglycaemic state. It was observed that the blood glucose of all the animals in the diabetic groups were above 170 mg/dl as compared to that of the normal control which had values less than 91 mg/dl.

Administration of Extract

Curcuma longa extract administration commenced three days after induction of hyperglycaemia by oral gastric intubation and lasted for 28 days.

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Termination of Experiment

At the end of treatment period, the experimental animals were weighed and sacrificed through chloroform inhalation. The anterior abdominal wall was incised. The testis was excised, weighed and preserved in 10% formal saline for tissue processing using Haematoxylin and eosin staining method. Semen was extracted from the caudal epididymis for analysis using WHO Recommended method.

Comment [Reviewer13]: 1.Reference?
2.Indicate when the semen sample was taken;
before or after preservation.

Ethical clearance

According to the international guideline for handling of laboratory animals and the relevant University ethical clearance committee on laboratory animal use, all animal experiments were carried out in accordance with the approved institutional and WHO guidelines for the care and use of animals. Ethical clearance was obtained from the Faculty Animal Research Ethics Committee (FAREC-FBMS), Faculty of Basic Medical Science, University of Calabar, Calabar with the number: 220ANA2423

Comment [Reviewer14]: Requires paraphrasing to avoid repetitive statements.

Determination of weight

All the animals were weighed before and after induction of hyperglycaemia and every three days during the course of administration of the extract.

Analysis of sperm parameters

The epididymis was dissected out and placed in a physiological saline in a ratio of 1:10 weight (g) by volume (ml), and was then macerated using surgical blade to release the sperm cells. The suspension was then filtered with 80µm stainless mesh after pipetting according to the method of [37]. The following sperm parameters were estimated as follows:

Comment [Reviewer15]: use the proper symbol

Comment [Reviewer16]: mention author before the citation.

i) **Sperm motility (%)**: Two drops of sperm suspension were ~~put~~ placed on a clean and labelled microscope slide and covered with a coverslip. This was mounted on light microscope and the number of motile cells divided by the total number of sperm cells counted and was expressed in percentage.

ii) **Sperm viability (%)**: This was done using the eosin-nigrosin staining technique. The sperm suspension was mixed with equal volume of the stain and smeared on glass slides. Live sperm excluded the stain and appeared lightly coloured, while dead sperm took up the stain and appeared pink in colour. The counts of live sperm were divided by the total number of sperm cells and multiply by 100 expressed as percentage.

iii) **Sperm count (x10⁶/ml)**: Improved nueberhaeocytometer was used for the sperm count. A capillary tube was used to pipette the sperm suspension into the counting chamber of the nueberhaeocytometer. The nueberhaeocytometer was placed on a light microscope and the counting was estimated by multiplying the number of cells counted by the dilution factor and by the haemeocytometer volume.

Comment [Reviewer17]: Check spelling (sp.)

Comment [Reviewer18]: sp.

Comment [Reviewer19]: sp.

Gonadal histo-morphology was assessed using Haematoxylin& Eosin

Comment [Reviewer20]: Requires further details. Incomplete.

Statistical Analysis

Data obtained from the experiment was analysed using one-way Analysis of Variance and Duncan post hoc test using a Statistical Package for Social Science, SPSS version 26.0 for Windows. The results were presented as mean \pm standard error of mean and considered statistically significant at $p < 0.05$

RESULTS

Assessment of Blood Glucose

Table 2 shows the effect of STZ on fasting blood sugar levels. At day zero, blood glucose level in all experimental groups was considered normal ranging from about 70 mg/dl to 107 mg/dl. Elevated blood glucose concentration was seen in all the groups that were induced for

diabetes (B, C and D) following administration of 65mg/kg-bw of streptozotocin. Blood glucose levels in these groups were significantly higher when compared with the normal control ($p < 0.05$).

Along the course of the experiment through to its termination (Day 28), all diabetic animals still had values of blood sugar level increased significantly higher (± 19.53) compared with normal Control group ($p < 0.05$). Groups C and D animals placed on standard anti-diabetic drug (Metformin) and extracts of *curcuma longa* showed a significant decrease (± 2.98) in blood glucose level compared to both diabetic control group and normal control group ($p < 0.05$) (Tables 2a and 2b).

Comment [Reviewer21]: units?

Table 2a: Daily blood glucose concentration of the different experimental groups (mg/dl).

Comment [Reviewer22]: to combine tables 2a and 2b into a single table

Group	Day				
	1	4	7	10	13
Normal Control	82.20 ± 5.45	87.60 ± 4.39	90.60 ± 4.39	86.80 ± 3.84	82.20 ± 4.02
Diabetic Control	274.80 $\pm 8.73^*$	260.00 $\pm 6.52^*$	260.40 $\pm 7.78^*$	219.40 $\pm 12.32^*$	235.00 ± 10.03
Metformin Treated	310.60 $\pm 24.43^*$	264.60 $\pm 10.41^*$	215.20 $\pm 9.72^*$	199.60 $\pm 5.31^*$	177.80 $\pm 7.66^{*,a}$
Extract group	337.40 $\pm 19.68^{*,a}$	285.80 $\pm 17.62^*$	246.80 $\pm 11.91^*$	213.40 $\pm 4.23^*$	174.80 $\pm 8.82^{*,a}$

Values are expressed in Mean \pm SEM. N = 4.

* = Values are significantly decreased when compared to Normal Control at $p < 0.05$.

a = Values are significantly increased when compared to Diabetic Control at $p < 0.05$.

Table 2b: Continuation of daily blood glucose concentration of the different experimental groups(mg/dl).

Group	Day					Change
	16	19	22	25	28	
Normal Control	89.40 ± 4.53	86.40 ± 4.84	89.60 ± 2.48	95.40 ± 3.57	94.80 ± 5.47	12.60 ± 8.23
Diabetic Control	222.60 $\pm 18.24^*$	264.67 $\pm 5.34^*$	249.33 $\pm 19.53^*$	268.33 $\pm 5.68^*$	279.67 ± 6.49	-24.40 ± 26.61
Metformin	142.00 $\pm 5.49^{*,a}$	123.60 $\pm 6.95^{*,a}$	107.80 $\pm 6.90^{*,a}$	101.20 $\pm 4.35^a$	83.00 $\pm 4.35^a$	-227.60 $\pm 24.32^{*,a}$
Extract group	129.20 $\pm 6.61^{*,a}$	115.00 $\pm 2.98^{*,a}$	134.20 $\pm 18.33^{*,a}$	114.40 $\pm 9.53^a$	100.80 $\pm 5.67^a$	-236.60 $\pm 25.14^{*,a}$

Values are expressed as mean \pm SEM, n = 5.

* = significantly different from control at $p < 0.05$

a = significantly different from diabetic control at $p < 0.05$

Figure 1 & 2 show the blood glucose levels of all groups throughout the experimental period. Elevated blood glucose concentration was seen in all diabetic groups (B, C and D) following administration of 65mg/kg.bw of streptozotocin. Blood glucose level in these groups was remarkably higher ($p < 0.05$) when compared with the normal control. At termination of the experiment (Day 28), the diabetic control animals still had values of blood sugar level significantly higher ($p < 0.05$) when compared with normal control. The blood glucose of the Metformin treated group and extract treated group however reduced significantly and had values which had no statistical significance when compared with normal control group.

Comment [Reviewer23]: Both figures are showing the same set of data. Suggestion to retain only Figure 1.

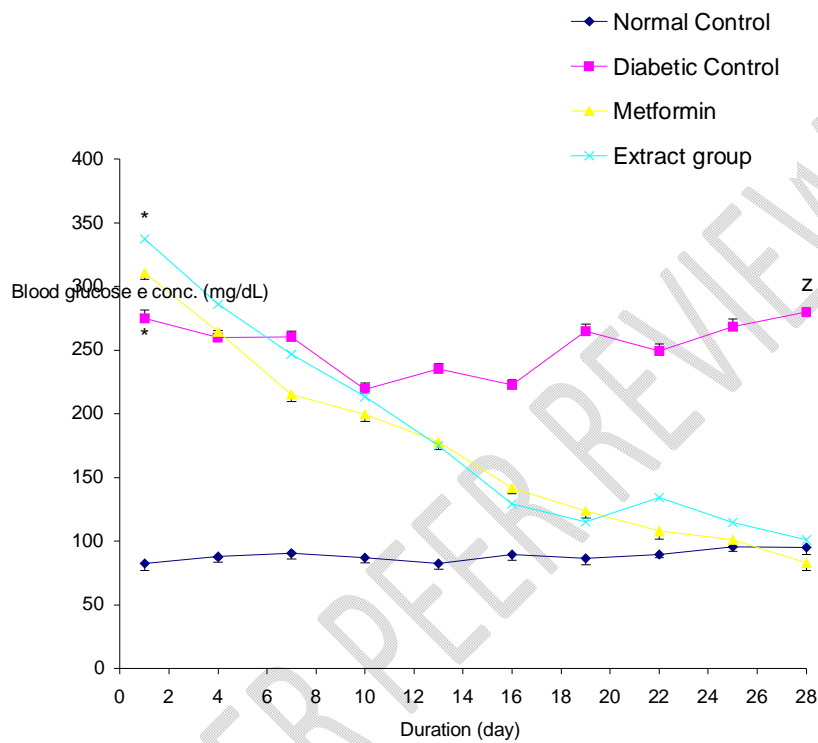


Figure 1: Daily blood glucose level of the different experimental groups.

Values are expressed as mean +SEM, n = 5.

* = p<0.05 vs control

z = p<0.05 vs other groups

The blood glucose level of the diabetic control and metformin groups declined daily

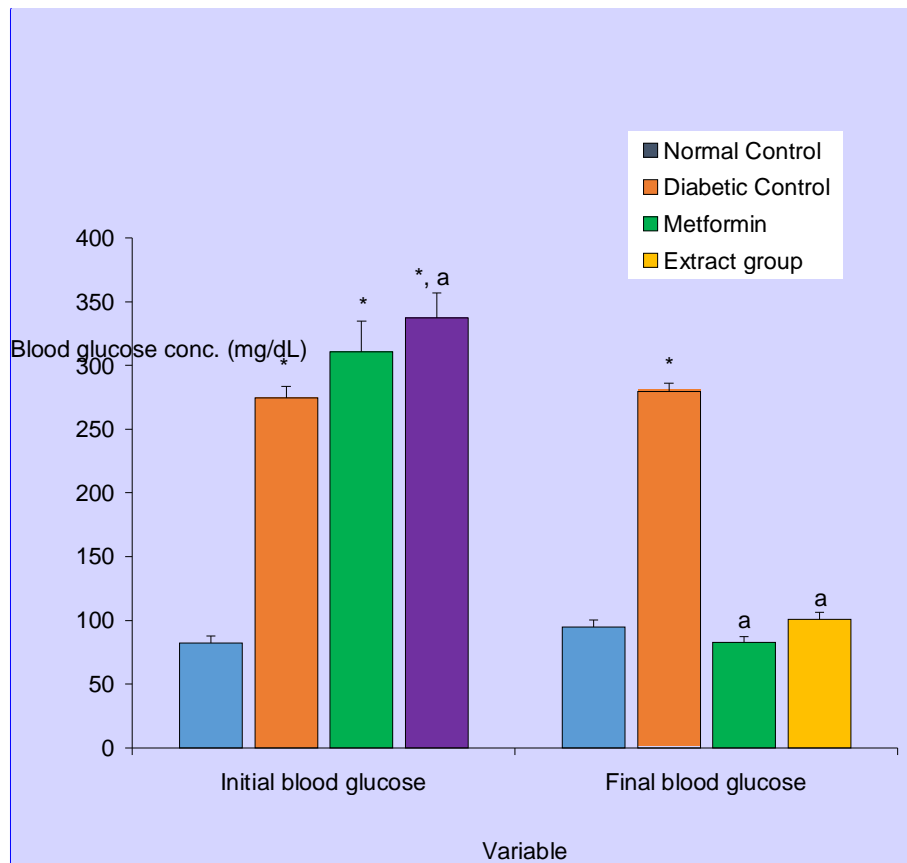


Figure 2: Initial and final blood glucose concentrations of the different experimental groups.

Values are expressed as mean +SEM, n = 5.

Comment [Reviewer24]: which group does the purple bar represent?

Assessment of Body Weight

There was a significant decrease in body weight of the experimental rats induced with diabetes (± 6.57) as compared to the normal control ($p < 0.05$). On administration of the standard drug (metformin), and extract of *curcuma longa*, results showed a significant increase (± 4.81) increase of body weight compared to diabetic control group ($p < 0.05$) and progressive change as observed from day 7 of treatment.

At termination of the experiment (Day 28), the diabetic control animals had values of body weight significantly lower ($p < 0.05$) when compared with normal control which also had no difference significantly from the metformin treated group and extract treated group (Tables 3a and 3b) (Figs 3 & 4).

Comment [Reviewer25]: 1. must include units for all measurements.
2. To include the initial and final value before stating the value for observed change

Comment [Reviewer26]: see above comment

Comment [Reviewer27]: Combine the tables into one single table

Comment [Reviewer28]: Be clear to which table or figure you are referring to in this sentence.

Table 3a: Daily body weights of the different experimental groups

Group	Day				
	1	4	7	10	13
Normal Control	158.80	160.20	162.60	164.20	166.80
	± 3.98	± 3.37	± 3.47	± 4.28	± 4.83
Diabetic Control	183.00	169.60	163.40	158.40	154.20
	$\pm 6.57^*$	± 5.35	± 4.48	± 4.96	± 4.33
Metformin	163.00	156.60	158.80	161.60	160.00
	$\pm 4.81^{*a}$	± 4.51	± 5.62	± 5.54	± 5.61

Extract group	165.40	166.40	161.40	164.00	167.20
	$\pm 5.34^*$	± 5.57	± 5.38	± 5.37	± 5.00

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Table 3b: Continuation of daily body weights of the different experimental groups

Group	Day					Change
	16	19	22	25	28	
Normal Control	169.60	172.20	174.40	176.80	178.80	20.00
	±4.58	±4.69	±4.50	±4.99	±4.14	±1.14
Diabetic Control	152.20	148.67	146.33	142.67	142.67	-37.00
	±4.08	±5.77*	±5.66*	±6.35*	±6.09*	±5.75*
Metformin	159.20	161.40	160.80	162.20	163.80	0.80
	±4.79	±5.37	±6.27	±6.40	±6.01	±1.80* ^a
Extract group	165.40	164.80	163.20	162.40	164.20	-1.20
	±6.28	±5.34	±5.82	±3.57	±4.07* ^a	±2.08* ^a

Values are expressed as mean ±SEM, n = 5.

* = significantly different from control at p<0.05

a = significantly different from diabetic control at p<0.05

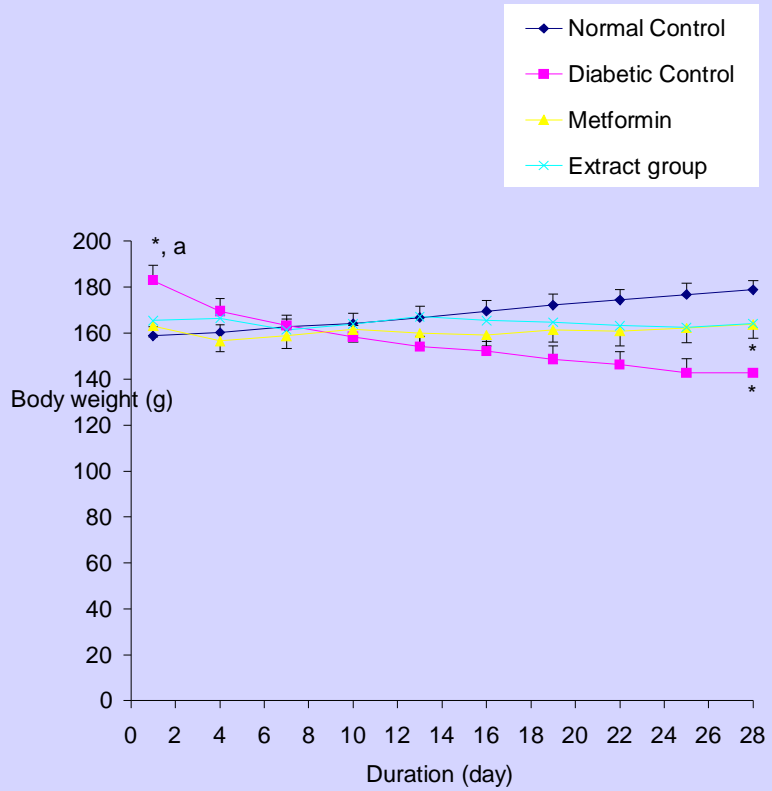


Figure 3.: Daily body weights of the different experimental groups.

Values are expressed as mean +SEM, n = 5.
 * = significantly different from control at p<0.05
 The body weight of the diabetic control declined daily

Comment [Reviewer29]: Both figures 3 and 4 are showing the same set of data. Suggestion to retain only Figure 3.

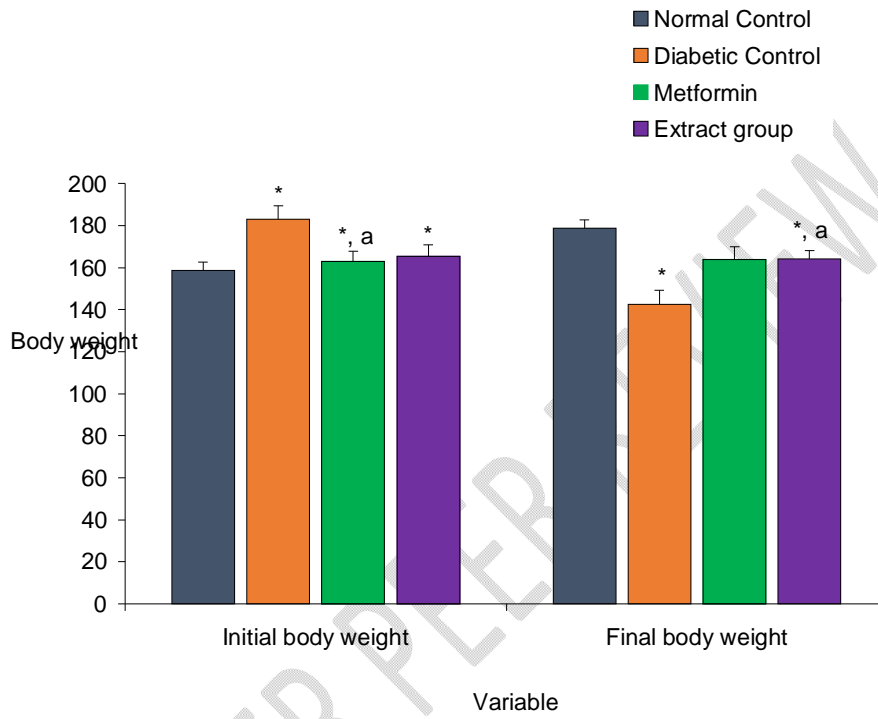


Figure 4: Initial and final body weights of the different experimental groups.

Values are expressed as mean +SEM, n = 5.
 * = significantly different from control at p<0.05
 a = significantly different from diabetic control at p<0.05

SEMEN ANALYSIS

Sperm Count

Results showed a significant ($p < 0.05$) decrease (37.70 ± 7.5) million/ml and (47.10 ± 1.7) million/ml of sperm count in the diabetic control and metformin treated groups respectively compared to the normal control group that had 69.6 ± 1.5 million/ml sperm count.

However, 65.40 ± 1.4 million/ml of sperm count was recorded in Group D animals (~~placed~~ ~~and~~ administered with 500mg/kg.bw of *C. longa*). This increase in sperm concentration was statistically significant when compared with the diabetic control ($p < 0.05$) (Table 4, Fig 5).

Comment [Reviewer30]: remove brackets

Comment [Reviewer31]: Table 4 and Fig 5 are showing the same set of data. Suggestion to retain only one.

Table 4: Sperm count of experimental groups expressed in million/ml.

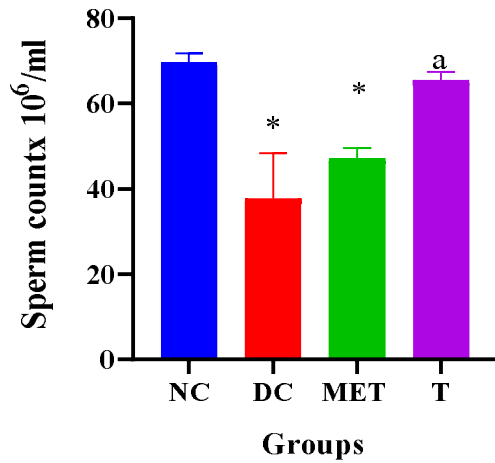
GROUPS	TREATMENT	Sperm Count (million/ml)
A	Normal Control	69.6 ± 1.5
B	Diabetic Control	37.70 ± 7.5
C	STZ + 250mg/kg b.w metformin	47.10 ± 1.7
D	STZ + 500mg/kg b.w <i>curcuma longa</i>	65.40 ± 1.4

Values are expressed in Mean ± SEM. N = 4.

Comment [Reviewer32]: check table format

Comment [Reviewer33]: initially n=6 per group. What happened to the other 2 rats?

UNDER PEER REVIEW



NC - Normal control
 DC - Diabetic control
 MET - Metformin treated group
 T - Tumeric treated group

Figure 5: Sperm count in the different experimental groups.

Values are expressed in Mean \pm SEM. N = 4.

* = Values are significantly decreased compared to Normal Control(p<0.05).

a = Values are significantly increased compared to Diabetic Control at p<0.05.

Sperm Motility

Table 5 and Fig 6 show the result of sperm motility for the experimental animals. Animals in the diabetic control group and the group placed on metformin recorded significant decrease in sperm motility values when compared with the normal control group ($p < 0.05$). However, Group D animals (placed on 500mg/kg.bw of *C. longa*), showed an increase in sperm motility which was statistically significant when compared with the diabetic control and the metformin treated group.

Comment [Reviewer34]: Table 5 and Fig 6 are showing the same set of data. Suggestion to retain only one.

UNDER PEER REVIEW

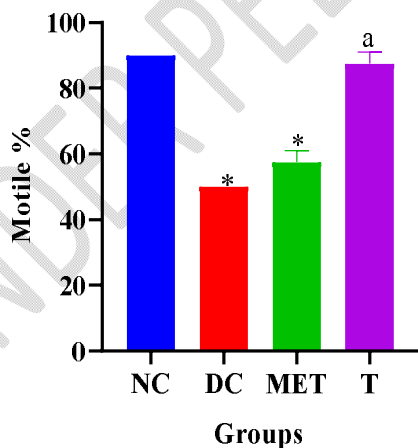
Table 5: Sperm motility of different experimental groups

GROUPS	TREATMENT	SPERM MOTILITY
A	Normal Control	90.0 ± 0.0
B	Diabetic Control	50.0 ± 0.0
C	STZ + 250mg/kg b.w metformin	57.50 ± 2.50
D	STZ + 500mg/kg b.w <i>wcurcuma longa</i>	87.50 ± 2.50

Comment [Reviewer35]: check table format

Values are expressed in Mean ± SEM. N = 4.

Comment [Reviewer36]: initially n=6 per group. What happened to the other 2 rats?



NC - Normal Control
 DC - Diabetic Control
 MET - Metformin treated group
 T - Turmeric Treated group

Figure 6: Sperm motility in the different experimental groups.

Values are expressed in Mean \pm SEM. N = 4.

* = Values are significantly decreased compared to Normal Control(p<0.05).

a = Values are significantly increased compared to Diabetic Control(p<0.05).

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Sperm Viability

Experimental animals in the diabetic control group and the group placed on metformin recorded low sperm viability values which was significantly decreased when compared with the normal control group ($p < 0.05$). However, Group D animals (placed on 500mg/kg.bw of *C. longa*), showed an increase in sperm viability of which was statistically significant when compared with the diabetic control (Table 6 & Fig 7).

Comment [Reviewer37]: same as previous comments. redundant.

UNDER PEER REVIEW

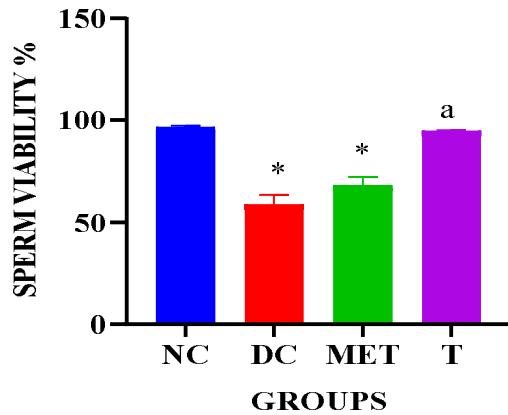
Table 6: Sperm Viability of different experimental groups

GROUPS	TREATMENT	Sperm Viability
A	Normal Control	96.5 ± 0.5
B	Diabetic Control	58.5 ± 3.5
C	STZ + 250mg/kg b.w metformin	68.0 ± 3.0
D	STZ + 500mg/kg b.w <i>curcuma longa</i>	94.50 ± 0.5

Values are expressed in Mean ± SEM. N = 4.

Comment [Reviewer38]: check

UNDER PEER REVIEW



NC- Normal control
 DC- Diabetic control
 MET- Metformin Treated group
 T- Tumeric treated group

Figure 7: sperm Viability in the different experimental groups.

Values are expressed in Mean \pm SEM. N = 4.

* = Values are significantly decreased when compared to Normal Control at $p < 0.05$.

a = Values are significantly increased when compared to Diabetic Control at $p < 0.05$.

HISTOLOGICAL OBSERVATIONS

Histological study on sections of testes in the normal control (X400) showed prominent seminiferous tubules with regular and consistent cellular arrangements on the germinal epithelium. The lumens of seminiferous tubules were filled with ~~tails~~ (flagella) of newly formed spermatozoa. Basement membrane was intact and the intervening interstitium which contained blood vessels and clusters of Leydig cells showed regular pattern (Plate 1).

Section of testes from the diabetic control group (Group B) showed significant alteration in histological patterns in the testes when compared with the normal control. Irregular shaped seminiferous tubules with shrunken lumen and decreased tubular diameter were observed (Plate 2).

Section of testes in diabetic animals treated with Metformin (Group C) showed normal seminiferous tubules although its germinal epithelium was distorted with differentiating cells arranged in a coarse pattern. The interstitial connective tissue was distorted (plate 3).

For the group placed on 500mg/kg.bw of *C. longa*, Seminiferous tubules were prominent and intact with basement membrane although some alterations were still observed, there is a progressive restoration observed.

Comment [Reviewer39]: indicate the alterations and restoration to which structures.

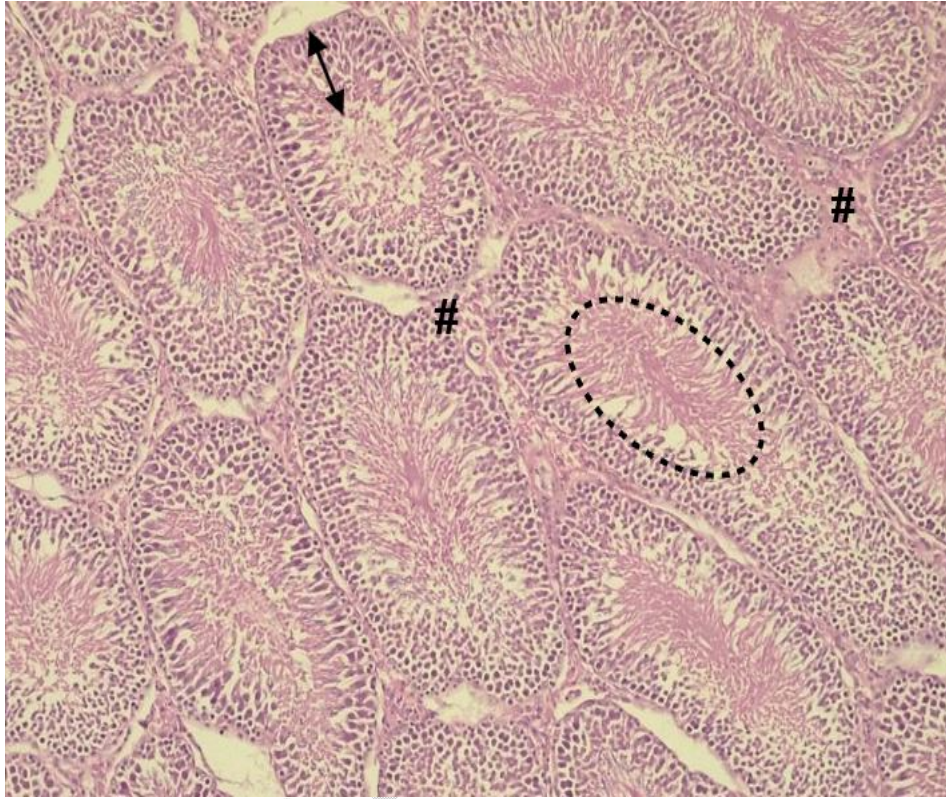


PLATE 1: TESTIS X400-NORMAL CONTROL X400

Section of testes showing normal seminiferous tubules with germinal cell layers (**double-headed arrow**) on the basal lamina to spermatocytes filled lumen (**dotted circular lines**).

The interstitial connective (#) tissue was well preserved. H&E stain, x400 Magnification.

Comment [Reviewer40]: combine Plate 1-4 into a single figure to provide better comparison between groups.

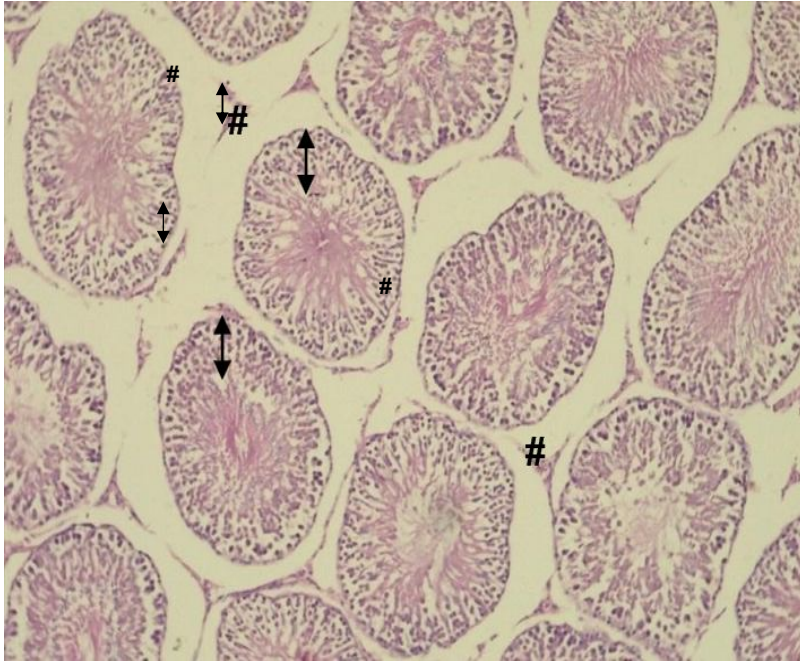


PLATE 2: H&E section of testis of Diabetic control (X400)

Section of testes showing seminiferous tubules with reduced germinal epithelium (double-headed arrow), distorted interstitial connective tissue (#).H&E stain, X400 magnification



PLATE 3: H& E section of TESTIS DC+METFORMIN X400

Section of testes showing normal seminiferous tubules with germinal cell layers (**doubleheads arrow**) starting from spermatogonia (**arrowhead**) on the basal lamina and spermatocytes filled lumen (**dotted circular lines**). The interstitial connective (**#**) tissues was distorted. H&E stain, X400 Magnification

Comment [Reviewer41]: double-headed

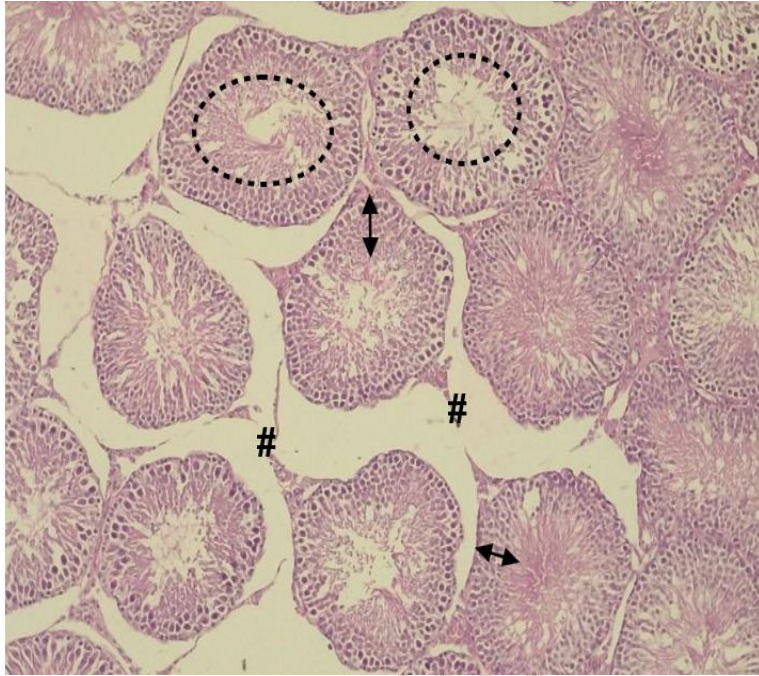


PLATE 4: H& E section of TESTIS X400 *C.longa* ONLY (500mg/kg.bw)

Section showed different stages of seminiferous tubules and the interstices. The seminiferous with germinal cell layers (**doubleheads arrows**) showed lumen partly & completely filled (**dotted circular line**) with mature spermatocytes. Although alterations are still noticed, there is a progressive restoration observed. H&E, X400 magnification

Comment [Reviewer42]: double-headed

DISCUSSION, SUMMARY AND CONCLUSION,

~~This discussion of the work, conclusion, contribution to knowledge and recommendation for further studies are well presented in this chapter~~

Discussion

Complex interplay of various pathological processes, prominently characterized by oxidative stress damage, inflammation, and apoptosis are involved in diabetic testicular damage.³⁸ Curcumin, a bioactive compound derived from *Curcuma longa*, has emerged as a potential therapeutic agent with protective properties against diabetic testicular injury. However, the precise underlying mechanisms through which curcumin exerts its beneficial effects in this context remain a subject of ongoing investigation. The present study examined the effect of *C. longa* crude extract on the histology of the testis and semen parameters of streptozotocin induced hyperglycemic male rats.

The sustained increase in the blood glucose levels of diabetic animals observed at the end of the study was corroborated by studies by ³⁹ and ⁴⁰ which revealed hyperglycemic response in STZ treated animals due to impaired glucose oxidation, which causes NAD⁺ depletion, ultimately culminating in the inhibition of insulin biosynthesis and secretion. The reduction in blood glucose levels in the metformin and extract-treated groups, aligns with the study by ⁴¹, who reported a significant reduction in fasting blood glucose levels in animals administered curcumin. Studies by ⁴², ¹⁷ have corroborated the anti-diabetic efficacy of the extract, attributing it to its antioxidant and anti-inflammatory properties. Findings reported by ⁴³ also revealed the potential of the methanol fraction of *C. longa*, to significantly reduce in blood glucose levels in experimental animals.

Comment [Reviewer43]: Sentence too long. consider breaking the sentence.

Comment [Reviewer44]: how was the findings from your study using ethanolic extract compare to the study using methanolic extract?

The final bodyweight of the experimental animals in the extract-treated group showed a significant increase when compared to both the diabetic and control groups. The overall changes in bodyweight statistically indicated that both the metformin and extract-treated groups experienced significant increases in their respective bodyweights when compared to both the control and diabetic groups. These findings are consistent with a study conducted by ⁴⁴ and ⁴⁵ which reported a significant decrease in bodyweight in streptozotocin-injected rats. Furthermore, the observed decrease in bodyweight of the diabetic rats aligns with the findings of ^{46, 47} who observed a similar effect on diabetic animals induced with streptozotocin. The underlying mechanisms contributing to the reduction in bodyweight within the diabetic group can be attributed to the intricate interplay of factors such as the degradation of structural proteins and muscle wasting, as reported by ⁴⁸. Conversely, the ameliorative effects of *Curcuma longa* on bodyweight is supported in the work of ⁴³ where the oral administration of the plant extract demonstrated remarkable improvement in the bodyweight of experimental animals.

Semen analysis revealed a significant reduction in the total count of sperm cells in the diabetic control group, compared to the normal control group. This observation underscores the detrimental impact of diabetes mellitus, regardless of whether it is type 1 or type 2 diabetes, on male fertility. The findings of ⁴⁹ demonstrated the adverse effects of experimentally induced diabetes on sperm parameters. Also a study conducted by ⁵⁰ showed significantly reduced sperm count in diabetic animals. While the scientific basis underlying the pathophysiological mechanisms linking diabetes to semen parameters, particularly sperm count, remains diverse in the literature, prolonged hyperglycemia, triggers the body's oxidative stress response and leads to endothelial injury in blood vessels, including those

Comment [Reviewer45]: Discuss the relationship between body weight and diabetic status ie glucose levels

within the testis and epididymis⁵¹ Moreover, elevated blood glucose levels can disrupt the regulatory function of the hypothalamic-pituitary-gonadal axis, resulting in alterations in the number and morphology of testicular interstitial cells, degeneration of Sertoli cells, reduced synthesis and secretion of testosterone, impaired sperm development and maturation, and ultimately, compromised reproductive functions. However, the administration of the extract in the treated group led to a notable improvement in the total sperm count, which was comparable to that observed in the normal control group. These findings are in line with the research conducted by⁵²who demonstrated that replacing a ketogenic diet with curcumin supplementation improved semen quality. Additionally,⁵³ reported an increase in total sperm count among infertile men participating in a randomized clinical trial after receiving Curcumin. Together, these studies provide further support for the positive effects of curcumin and its potential in ameliorating the adverse impacts of diabetes on semen parameters, including sperm count.

Comment [Reviewer46]: only sperm count? Discuss on the effects of turmeric on the other variables that your experiment measured and how they compared to previous reports.

The experimental findings revealed a significant decline in the percentage concentration of motile sperm cells in the diabetic group compared to the control group, however-However both the extract-treated and metformin-treated groups showed an increase which was not statistically significant($p < 0.05$) compared to the normal control animals. These observed outcomes are consistent with the outcomes of prior investigations conducted by^{54, 55}who reported that the addition of curcumin in frozen-thawed Angora goat semen improved both progressive motility and acrosome integrity, while also enhancing the progressive motility and functional integrity of sperm plasma membrane in frozen bull semen. Similarly,⁵⁵ reported a higher percentage of progressive motile sperm cells associated with the supplementation of curcumin.

Comment [Reviewer47]: Explain or provide reasons on why it was not significant even in drug control group. Is there a connection between measuring total motility and only comparing specific types of motility (ie progressive motility)

Sperm viability values which decreased significantly in the DC and metformin groups compared with the normal control group. In the extract treated group there was an increase in sperm viability which was statistically significant when compared with the diabetic control. This finding aligns with reports by⁵⁶ who observed that the percentage of sperm viability in STZ-induced diabetic rats improved mildly in *Curcuma longa* treated group.

Comment [Reviewer48]: which group is this referring to, Group A, B, C or D. Be consistent.

Remarkable histological alterations were explicitly observed in the tissue sections of the experimental rats belonging to the diabetic group. These histopathological manifestations were characterized by irregularly shaped seminiferous tubules exhibiting a shrunken lumen and decreased tubular diameter, which were indicative of severe edema and pronounced hyperemia within the veins. These findings align harmoniously with the seminal research conducted by⁵⁷ who expounded upon the frequent occurrence of abnormal histology within the testes of diabetic animals, with discernible alterations observed in the cytoarchitecture of the seminiferous epithelium as well as disruptions in the occlusive distribution pattern.

Comment [Reviewer49]: Is your study just a repetition of previous work? What other findings does your study contribute regarding sperm viability?

Comment [Reviewer50]: This is expected. Focus on the findings for the group receiving *C. longa* extract

Conversely, the tissue sections derived from the extract-treated group exhibited remarkable therapeutic efficacy, showcasing prominent seminiferous tubules characterized by prominent basement membranes. Studies by^{58, 59} documented the positive influence of *Curcuma longa* on the cytoarchitecture of the testes. This remarkable therapeutic outcome can be attributed to the myriad of phytochemical compounds present within the extract, including saponins, flavones, tannins, and terpenes (reference). The collective action of these bioactive compounds, either individually or synergistically, confers an enhanced functionality to the testes. Notably, the extract's insulin-like action, as well as its proficiency in inducing DNA repair systems owing to its potent antioxidant activities, effectively curtails or prevents the

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Comment [Reviewer51]: Suggestion to use simple terms to retain better understanding of the observable changes that you are referring to in the group.

Comment [Reviewer52]: insulin sensitizer or reduce insulin resistance effect

generation of deleterious free radicals, thus exerting a protective effect on the testis and semen parameters.

Summary

The study examined the potential hypoglycemic effect of *Curcuma longa* (*C. longa*), known as turmeric, on the histology of the testis and reproductive parameters in male Wistar rats with streptozotocin-induced hyperglycemia. The study was aimed at investigating the effects of ethanolic extract of *Curcuma longa* (Turmeric) on fertility profile of Streptozotocin induced hyperglycemic male Wistar rats.

Twenty four male Wistar rats were divided into four groups of six rats each. The groups included: normal control group given normal chow and distilled water only, diabetic group administered with 65mg/kg.bw of STZ, metformin treated group which was given 250/kg.bw of metformin and the extract treated group to which 500mg/kg.bw of *curcuma longa* was administered. The study lasted for about forty five days of two weeks acclimatization of the animals, three days for confirmation of DM after administration of STZ and twenty eight days for administration of metformin and *C. longa* to the experimental groups. The research investigated various parameters related to testicular histology and reproductive function, such as sperm count, sperm motility, sperm viability. The findings from the study showed alterations in the cytoarchitecture of the testis with a significant decrease in sperm motility, count and viability in the diabetic group compared to the Normal control group. However, administration of *C. longa* as a therapeutic intervention exhibited promising

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Comment [Reviewer53]: This should be in methodology section. Remove from summary

outcomes, as evidenced by the increase in levels of sperm parameters (motility, count and viability) and the progressive restoration of histological integrity within the testes. This underscores the potential of *C. longa* to effectively counteract the detrimental effects induced by hyperglycemia.

Conclusion

The findings of the study suggests that ethanolic extracts of *Curcuma longa* has the potential to improve fertility in diabetic male animals as well as protect against the detrimental effects of hyperglycemia on the male reproductive systems.

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