

Correlates of sperm quality parameters and oxidative stress indices in diabetic rats exposed to cold stress: Role of *Moringa oleifera* leaf extract

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ABSTRACT

Spermatogenesis is extremely sensitive to fluctuations in the environment, particularly temperature and hormones. Sperm dysfunction, a root cause of male infertility, is a commonly allied complication of diabetes mellitus. Our previous studies cogitate that cold stress (15°C) exacerbates the complications and the resultant oxidative stress plays a major role in testicular and epididymis dysfunction in diabetic rats. Despite the strong biologic prospect for this postulation, establishing a direct link between free radicals and specific disease is an in-dire need, and in this context, this study focuses on investigating the sperm quality parameters and their relationship to testicular oxidative stress indices of cold stress diabetes in Wistar rats. The results indicate a cumulative impact by diminishing sperm parameters, viz. sperm density, viability, motility, mortality, and acrosome intactness in cold-stressed diabetic rats. The findings also reveal a strong positive Pearson's correlation between the sperm quality parameters and testicular lipid peroxidation, which reflects the influence of oxidative stress on sperm dysfunction. Together with dual stressor effects, the efficacy of *Moringa oleifera* leaf ethanolic (MOLE) extract is appended to assess its therapeutic role. The apparent effectiveness of MOLE therapy at 250 and 500 mg/kg bw for 60 days aided in suppressing oxidative stress and improved semen quality demonstrating the causative nature of these associations; hence, *Moringa* usage is recommended as a therapeutic agent for male reproductive dysfunctions in population residing in colder climates.

1. INTRODUCTION

The process of spermatogenesis is extremely sensitive to fluctuations in the environment, particularly hormones and temperature [1,2]. A significant reduction in testis weight was reported by Heroux and Campbell [3,4] in mature rats upon exposure to 6°C for 3 months. Similarly, by subjecting immature rats to cold, Johnson et al. [5] confirmed that chronic cold exposure resulted in severe testicular atrophy and histological changes. Using a cold water immersion-induced stress model in rats, Juarez-Rojas et al. [6] reported that stress can potentially activate intrinsic/extrinsic apoptosis pathways in testes resulting in reduced testosterone levels. Recent works of Saeedy et al. [7] indicated that long-term ice cold water drinking induced

testicular damage and altered sperm characteristics in rats, such as reduced sperm count and sperm progressive motility, increased the percentage of non-motile sperm, changed normal morphology of sperm, and destroyed sertoli and leydig cells. Their findings infer that long-term ice cold water drinking ought to be noxious for testis function and structure.

Diabetes mellitus (DM) is closely linked to sexual dysfunction, which leads to infertility [8,9]. Hyperglycemia promoted excessive reactive oxygen species (ROS) shown to interrupt the antioxidant enzyme system, while the clear-cut causes accountable for the spermatogenic dysfunction in diabetes are poorly interpreted. Few studies indicated that DM provokes molecular changes that are vital for sperm quality and function [9,10]. Despite the prevalence of diabetes worldwide, no reports on diabetics residing in temperate and continental zones exist. Majority of the diabetic population residing in temperate and continental zones, where the average temperature is 10°C or less, experience stress reactions in response to sudden cold air outbreaks.

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A cold climate is associated with minimal sweat production and increased metabolic heat production. Studies also indicated altered glycated hemoglobin (HbA1C) levels and poor glycemic management in the diabetic population of these zones [10]. In this context, our preliminary study advocated that cold stress (15°C) exacerbates the complications and the resultant oxidative stress plays a major role in testicular and epididymis dysfunction in diabetic rats, [11]. Due to the paucity of studies on sperm quality parameters in diabetic subjects residing in cold-temperate regions, this continuation study was undertaken with the sole aim to assess the correlates of oxidative stress indices with sperm quality parameters.

Many believe that herbal treatment is a promising therapy effective for sexual dysfunction and has shown to improve men's infertility [12]. The journey of literature survey in these lines highlighted the efficacy of *Moringa oleifera* (family Moringaceae), an indigenous tree of the Indian sub-continent. As per the available reports, the *Moringa* leaves have a high nutritional value and are also used in traditional Indian dishes. The leaves provide a cure for cancer, diarrhea, nutrition, hypertension, paralysis, diabetes, and neuronal disorders [13]. In our previous study, we have reported the anti-hyperglycemic and antioxidant efficacy of *M. oleifera* leaf ethanolic (MOLE) extract wherein 500 mg/kg bw/days dose is helpful in ameliorating the impaired antioxidant system in cold-stressed diabetic rats and our results inferred that *Moringa* leaf extract acts as a robust agent to quench nitric oxide radicals [11]. Therefore, using the same dosage paradigm of the MOLE extract as reported earlier, the ameliorative efficacy was checked on sperm quality parameters in cold-stressed diabetic rats. More emphasis is given on the correlates of oxidative stress (OS) indices and sperm parameters considering the association of ROS/OS in causing detrimental effects on sperm quality and function.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents used were of analytical grade obtained from Sigma-Aldrich SRL, India Pvt Ltd..

2.2. Plant Sample Collection and Authentication of Plant Material and Extraction

Fresh leaves of *M. oleifera* (Moringaceae) were gathered in the month of November, 2018, from the botanical gardens and surroundings of Jnana Bharathi Campus, Bangalore University Bengaluru, India. The plant materials were authenticated by Dr. T.G. Umesh, Professor and consultant taxonomist. A voucher specimen was deposited with number BUB/DB/PMB/MO/2018. They were dried under the shade for 25–30 days at room temperature and then crushed to powder. Initially, 1 kg of powdered leaves was taken for extraction using Soxhlet apparatus at 78°C in 70% ethanol (solvent). Later, the concentrated extract was obtained using a rotary evaporator. Furthermore, the MOLE extract was subjected to phytochemical screening, and it was found to have potential free radical scavenging ability and significant flavonoid as well as phenolic contents.

2.3. Animals

Male Wistar rats that are 3 months old and weighing 200 ± 10 g were selected. The rats were acclimatized to standard animal

laboratory conditions (12 /12 hours light/dark cycle, temperature $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and humidity $50\% \pm 5\%$) and segregated into groups having six rats in each group [14]. All experimental procedures complied with the set of guidelines (Rules for the Care and Use of Laboratory Animals) laid down by the National Institution of Nutrition, Hyderabad, and the protocol of the study was approved by the Bioethics Committee of the Faculty of Zoology at Bangalore University, Bangalore (Protocol number: DOZ/BUB/2018–19 and 402/CPSCSEA 2009–12 and revival thereon). Care was taken to minimize animal usage and suffering.

2.4. Inducing Diabetes

To induce diabetes, the procedure described by Saumya and Basha [9] was adopted accordingly and the animals were given a single intraperitoneal (i.p.) injection of streptozotocin at 45 mg/kg bw (in 0.1 mol/l of citrate buffer, pH 4.5). Blood glucose levels were monitored using Accu-Check glucometer. After 72 hours, diabetic animals (hyperglycemia <200 mg/dl) [9] were selected for the study, which was considered as the first day of the experiment (day 0).

2.5. Exposure of Cold Stress

Animals were subjected to cold stress in Colton biological oxygen demand (BOD) incubator for 6 hours/days for 60 days [15].

2.6. Experimental Design

All experimental procedures complied with the National Institution of Nutrition, Hyderabad (Guidelines for the Care and Use of Laboratory Animals) and were approved by the Bioethics Committee of the Faculty of Zoology at Bangalore University, Bangalore (Protocol numbers: DOZ/BUB/2018-19 and 402/CPSCSEA 2009-12 and revival thereon). Every effort was made to reduce the number of animals used and their suffering. Control animals served as Group-I and diabetics as Group-II. Animals exposed to cold stress at 15°C were considered Group-III. Diabetic animals exposed to cold stress at 15°C as Group-IV and Groups-V–VII was the prophylactic group supplemented with MOLE using oral gavage. We conducted a pilot trial by supplementing the following grades of MOLE at 100, 250, and 500 mg/kg bw/days dose to assess the feasibility of dose–response and the regimen continued for a period of 1, 7, 14, 30, 45, and 60 days with weekly and fortnight intervals. Consequently, the doses of 250 and 500 mg/kg bw/days offered better protection in the 60-days treatment; thereby, only two doses, i.e., 250 and 500 mg, for the 60-days regimen was continued in the study. This sample size ($n = 6$) was derived by the power analysis method by taking into account the effect size and standard deviation of a particular variable taken from previously published studies [14].

2.7. Antioxidant Assays

The following antioxidants were assessed by using the standard methods viz., lipid peroxidation (LPO) assay Niehaus and Samuelsson [16], super oxide dismutase (SOD) assay Misra and Fridovich [17], catalase (CAT) assay Aebi [18], reduced glutathione (GSH) assay Ellman [19], glutathione peroxidase (GPx) Lawrence and Burk [20], glutathione S transferase (GST) assay Habig et al. [21], and total proteins Lowry et al. [22].

2.8. Sperm Analysis

On day 60, all animals were sacrificed by spinal dislocation and the cauda epididymal duct on one side was incised and minced. By using a capillary tube, the semen that oozed out was sucked and transferred to an Eppendorf tube. A 200 times dilution was made using 10 mM phosphate buffer saline (PBS) (i.e., 0.05 μ l of sperm with 99.95 μ l of PBS). After proper mixing, the sperm suspension was used to analyze motility, morphology, and density [23].

2.9. Assessment of Sperm Density and Motility

To analyze the aforementioned indices, a single drop of diluted sperm suspension was placed on a hemocytometer and at least 10 microscopic fields were counted at $\times 400$ magnification using a standard optical microscope [24]. The sperm density was expressed in millions per milliliter as per dilution. To evaluate sperm motility, only sperm suspensions showing active motility were counted and it was expressed as “percent motility.”

2.10. Assessment of Sperm Viability

To determine sperm viability, the standard protocol as given by the World Health Organization [25] was carried out. Accordingly, the sperms were stained with 25% (v/v) eosin in saline solution and analyzed by light microscopy. The percent viability was determined as “the number of sperms that did not incorporate the dye over the total number of sperm cells.”

2.11. Assessment of Sperm Morphology

To analyze the sperm morphology, abnormal heads and tails were assessed by using the standards given by Nahas *et al.* [26], Mori *et al.* [27], and Okamura *et al.* [28]. The standards for head abnormality were no hook, amorphous, pin, and short head. The abnormalities of the tail were indicated as coiled flagellum, bent flagellum, and bent flagellum tip. A total of 2,000 sperms on each slide were analyzed and expressed as percentage of the abnormal head and abnormal tail.

2.12. Assessment of Acrosomal Integrity

Sperms were suspended in 4% p-formaldehyde solution (pH 7.4) and later in 10 mM ammonium acetate (pH 9.0). Sperms were air-dried on a microscopic slide and stained for 2 minutes with 0.22% Coomassie Brilliant Blue solution prepared in 50% methanol and 10% acetic acid. After washing them in distilled water, they are air-dried and observed under $\times 1,000$ magnification. Spermatozoa with intact acrosomes showed a blue stain over both the dorsal (convex) and ventral (concave) surfaces of the head and for acrosome-reacted spermatozoa, only ventral surface showed blue stain [29].

2.13. Statistical Analysis

Data on biochemical parameters were screened by one-way analysis of variance (ANOVA) and Bonferroni *post-hoc* at the significance of $p < 0.05$ was used to compare with control and positive controls by using Statistical Package for the Social Sciences software (version 20.0). Comparisons among MOLE supplemented groups were carried out by one-way ANOVA at $p < 0.01$ with Duncan's Multiple Range Test (DMRT) *post-hoc*. Correlation analysis was carried out based on Pearson's correlation.

3. RESULTS AND DISCUSSION

3.1. Cumulative Impact of Cold Stress on Sperm Quality Indices in Diabetes

Infertility is a major concern of the current population as sexual dysfunction is intricately linked to social and biological relationships. Additionally, diabetes has a detrimental impact on male and female reproductive function(s) [9,10]. Diabetic subjects often face disruption in sexuality where they lose their desire and become impotent and/or infertile [30], characterized by reduced sperm motility, elevated abnormal sperm morphology [9,31], higher sperm DNA damage, and deletion of mitochondrial DNA [32]. Besides, we hypothesized in the present study that diabetics exposed to cold stress may have to tackle sexual dysfunction and male infertility. Reports of Oliva *et al.* [33] also indicated that male infertility and sexual dysfunction are strongly associated with environmental, physiological, and genetic factors. In this study, when diabetic animals were subjected to cold stress at 15°C for 60 days (6 hours/days), the sperm density and viability decreased significantly ($p < 0.05$), while the sperm-mortality rate increased (Fig. 1a–c). Contrarily, non-diabetic rats at 15°C exhibited an insignificant ($p < 0.05$) decrease in viable sperms. Likewise, the rate of motility in sperms (both progressive and non-progressive) was altered significantly in non-diabetic rats at 15°C when compared to control (Fig. 1d–f). Explicitly, the diabetic group exhibited an exacerbated reduction in the rate of progressive motile sperms, while a magnified increase in non-progressive sperms was witnessed in the co-exposure group (D+CS 15°C). The percentage (%) of intact acrosomes had diminished significantly ($p < 0.05$) in the co-exposure groups (Fig. 2a), while cold stress at 15°C resulted in a moderate reduction. The representative microphotographs of sperm morphology are shown in Figure 3a–i wherein the percentage of sperms with normal tails was significantly decreased ($p < 0.05$) in all experimental groups. Visibly, the witnessed common head defects include detached head and sperm with an abnormal head number (Fig. 3b–f) and equally the sperms with knob-twisted flagellum /broken tails (Fig. 3g–i). These observations, strongly advocating the impact of diabetes in causing reproductive dysfunction and cold stress, brought about an exacerbated change in sperm morphology and diminished acrosome integrity (Fig. 4). The obscure factor behind the cumulative impact could be attributed to oxidative stress. Although ROS mediate vital cellular mechanisms such as capacitation and sperm maturation, excess ROS overwhelm the enzymatic and non-enzymatic antioxidant system, leading to oxidative injury. Sperm are essentially vulnerable to OS as their membranes are rich in Polyunsaturated fatty acid (PUFA) and their limited cytoplasm lacks an efficient enzymatic antioxidant system. Besides, our previous studies cogitate that cold stress (15°C) exacerbates the complications and the resultant oxidative stress plays a major role in testicular and epididymis dysfunction in diabetic rats (supplementary data). Hyperglycemia in diabetics could have enhanced the production of advanced glycation end products (AGEs), which in turn resulted in ROS-induced cellular damage and reproductive dysfunctions [9,34,35]. Our prior study and substantial reports of Kenny *et al.* [10] suggested that, aside

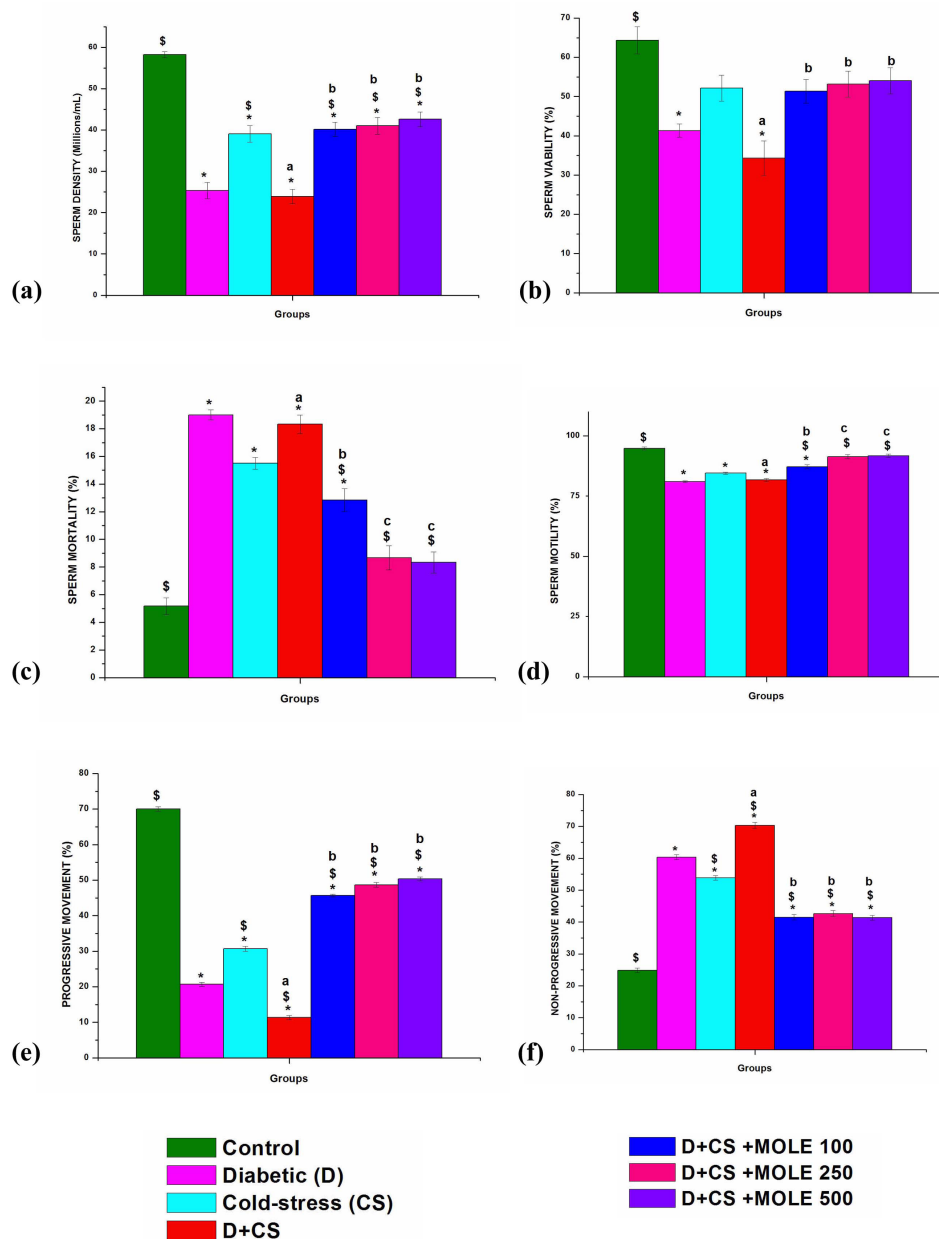


Figure 1: Dose-dependent effect of MOLE extract on sperm quality indices in cold stress exposed diabetic rats. (a) Changes in sperm density; (b) changes in sperm viability; (c) changes in sperm mortality; (d) changes in sperm motility; (e) changes in sperm progressive movement; (f) changes in sperm non-progressive movement. * $p < 0.05$ significantly different from control; \$ $p < 0.05$ significantly different from diabetic control (D) by using Bonferroni post hoc. Different superscripts (a, b and c) indicate significant ($p < 0.01$) differences among antioxidant treatments compared to positive control (D+CS15°C) using DMRT post hoc.

from hyperglycemia, cold temperature could also elevate the risk of free radical formation from raised metabolic rate and elevated levels of blood glucose. Increased oxidative stress has a major role in disrupting the hypothalamic–pituitary–adrenal axis hypothalamic pituitary–gonad axis and sperm functionality [36]. In this context, investigating the relationship between the testicular antioxidant system and sperm quality parameters is an in-dire need. In the present study, the results indicate a significant decline in sperm motility and morphology in rats upon exposure to individual and combined stressors. Furthermore, normal sperm morphology was significantly

affected by the increased percentage of sperm with a detached head and increased abnormal sperm tail morphology. To strengthen the drawn inferences, the relative coefficients (r) were assessed to interpret the relationship between the aforesaid indices and oxidative stress parameters (Table 1) and the data showed a high negative correlation ($R = -0.803$; -0.817) of sperm quality parameters (viz. sperm density, sperm viability, and sperm motility) to testicular lipid peroxidation [$LPO = 0.803$; -0.752 ; -0.904 ; -0.734 ; -0.862 , respectively]. Likewise, a negative correlation was observed between GSH and sperm motility/morphology aspects [$GSH = \text{sperm motility}$,

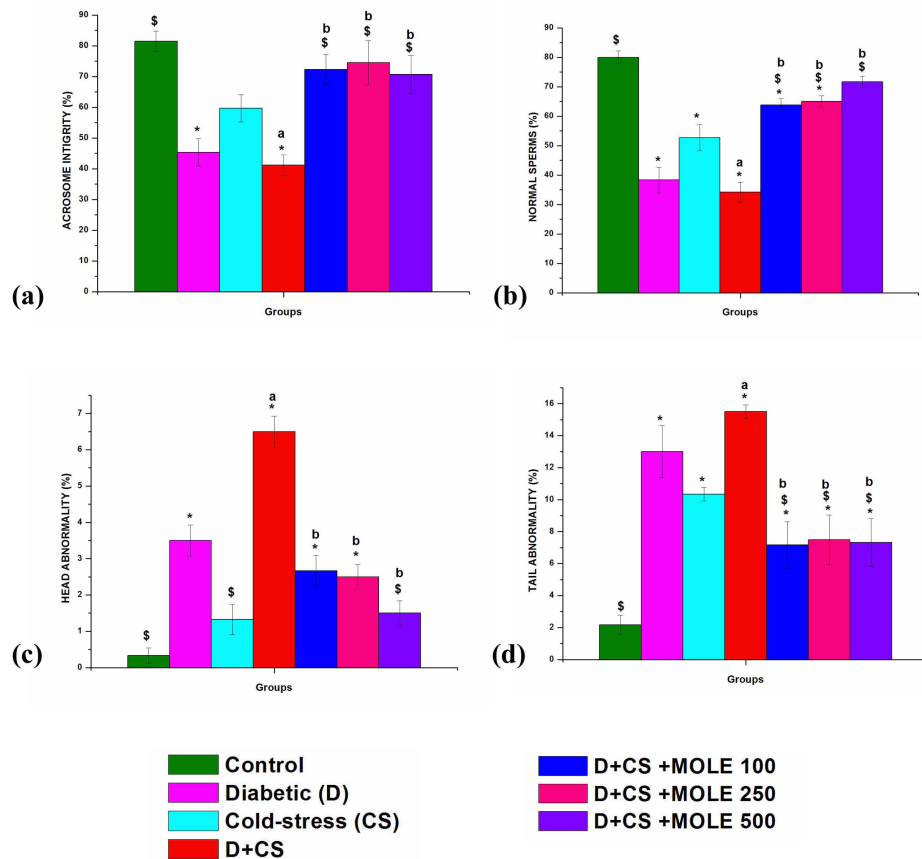


Figure 2: Dose-dependent effect of MOLE extract on sperm quality indices in cold stress exposed diabetic rats. (a) Changes in acrosome integrity; (b) changes in normal sperm; (c) changes in abnormal sperm; (d) changes in abnormal tails of sperm. * $p < 0.05$ significantly different from control; \$ $p < 0.05$ significantly different from diabetic control (D) by using Bonferroni post hoc. Different superscripts (a and b) indicate significant ($p < 0.01$) differences among antioxidant treatments compared to positive control (D+CS 15°C) using DMRT post hoc.

non-progressive sperm motility, Abnormal heads, abnormal tail; -0.817 ; -0.876 ; -0.795 ; -0.680 , respectively]. Contrarily, the antioxidant enzymes clearly exhibited a high positive correlation (r) [SOD = $+0.743$; $+0.855$; $+0.849$; $+0.753$]; [GST = $+0.770$; $+0.857$; $+0.834$; $+0.754$] [GPx = $+0.752$; $+0.825$; $+0.826$; $+0.735$]; [CAT = $+0.643$; $+0.795$; $+0.837$; $+0.706$] with the sperm quality parameters, viz., sperm motility, and head and tail abnormalities, respectively. Thus, the analysis cogitated the influence of oxidative stress, from hyperglycemia and cold stress, and is responsible for the impaired sperm quality parameters in cold-stressed diabetic animals. Furthermore, the results are corroborating with studies of El-Taieb et al. [37] and Dutta et al. [38], wherein the correlation analysis substantiated testicular oxidative stress as the main culprit of male infertility.

3.2. Ameliorative Role of MOLE Extracts

Although herbal medicine is widely used to treat many ailments, especially reproductive dysfunctions, identifying an effective therapeutic agent as well as easily available medicine in the best dosage is a meticulous task. *M. oleifera* is known for the nutritional value of its parts like bark, leaves, flower as well as fruits and its biological benefits on consumption.

They are a rich source of potassium, calcium, phosphorous, iron, and vitamins [39]. The preponderance of studies reported the use of *M. oleifera* for traditional diabetes and infertility treatments [40,41]. Several studies proved the protective role of *M. oleifera* against oxidative damage in discrete organs of diabetic models [41–43]. We have reported previously that MOLE extract at doses of 250 and 500 mg/kg bw had substantially offered hypoglycemic effects in cold-stressed diabetic rats, and its efficacy due to the ample presence of alkaloids, phenols, and quercetin in the extract (paper in review). In the present study, the supplementation of MOLE extracts for 60 days resulted in significantly ($p < 0.01$) high sperm density and viability (Fig. 1a and b) than positive control (D+CS 15°C). Besides, MOLE extract at doses of 250 and 500 mg/kg bw/days showed significant recovery in ($p < 0.01$) sperm mortality where 100 mg/kg bw/days supplemented group displayed mild significant recovery in the percentage of viable sperm. MOLE extract at 500 mg/kg bw/days showed the highest significant ($p < 0.01$) recovery in progressive, non-progressive motility and sperm abnormalities. The ameliorative effect of MOLE extracts could be attributed to its potential antioxidant efficacy. In cold-exposed diabetic rats,

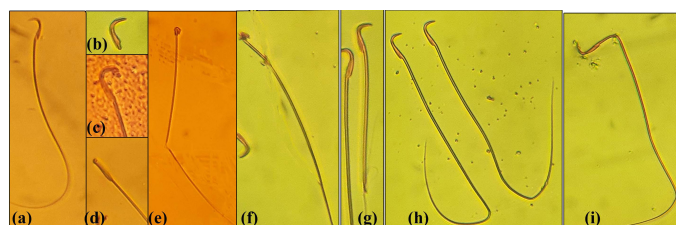


Figure 3: Different types of sperms observed in study. (a) normal sperm; (b) detached head; (c–f) – abnormal sperm heads, (g) broken tail; (h & i) – twisted tail in sperms.

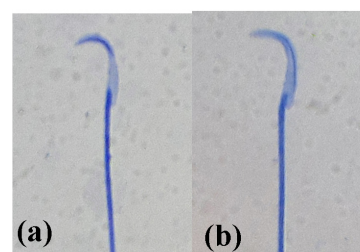


Figure 4: Changes observed in sperms during acrosome integrity analysis. (a) Acrosome intact (b) sperm with reduced acrosome intactness.

Table 1: Pearson correlation coefficients between the testicular oxidative stress indices and sperm quality parameters.

	LPO	SOD	GSH	GST	GPx	CAT
Sperm density	−0.803*	−0.742*	+0.805*	−0.772*	−0.758*	−0.689*
Sperm viability	−0.752*	−0.742*	+0.685*	−0.738*	−0.733*	−0.717*
Sperm mortality	+0.828*	+0.743*	−0.817*	+0.770*	+0.752*	+0.643*
Sperm motility	−0.828*	−0.743*	+0.817*	−0.770*	−0.752*	−0.643*
Progressive sperm motility	−0.904*	−0.849*	+0.886*	−0.859*	−0.830*	−0.776*
Non-progressive sperm motility	+0.896*	+0.855*	−0.876*	+0.857*	+0.825*	+0.795*
Acrosome integrity	−0.734*	−0.726*	+0.726*	−0.727*	−0.711*	−0.584*
Sperm morphology	−0.862*	−0.841*	+0.866*	−0.841*	−0.826*	−0.685*
Abnormal heads	+0.858*	+0.849*	−0.795*	+0.834*	+0.826*	+0.837*
Abnormal tail	+0.768*	+0.753*	−0.680*	+0.754*	+0.735*	+0.706*

*Correlation is significant at $p < 0.01$.

the free radical scavenging ability of MOLE extract might have quenched ROS formed due to hyperglycemia and cold stress, and consequently helped in recuperating the sexual dysfunction. Flavonoids and triterpenoids of *M. oleifera* are known for free radical scavenging and inhibition of protein oxidation [44]. Previous studies on *Moringa* also revealed its efficacy in suppressing testicular apoptosis by downregulating Bax expression and elevating testosterone, follicle-stimulating hormone, as well as luteinizing hormone in rats [45]. Quercetin, chlorogenic acid, and kaempferol as reported in *Moringa* showed to confer its anti-hyperglycemic properties [46]. The suppression of α -glucosidase, pancreatic α -amylase, and intestinal sucrose activities by *Moringa* extract has shown to diminish AGEs which help to regulate ROS generation [47]. In brief, the pharmacological actions of *M. oleifera* extract modulated the oxidative stress disturbance and improved the alterations in spermatogenesis, sperm count, and sperm abnormal morphology in cold stress-exposed diabetic rats.

4. CONCLUSION

Concurrent exposure to cold stress in diabetics poses health concerns like hyperglycemia and reproductive dysfunctions. The findings in this study advocate the exacerbatory actions brought about in sperm quality parameters of diabetic subjects upon their exposure to the cold climate and their close relationship to hyperglycemia-induced oxidative stress. The free radical scavenging efficacy of *M. oleifera* at 250 and 500 mg/kg bw for 60 days aided in improving the diminished sperm quality in cold stress-exposed diabetic rats and hence, *Moringa* usage is recommended as a therapeutic agent

for male reproductive dysfunctions. However, future studies are warranted in formulating the dose regimen suitable for the human population.

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. FUNDING

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8. ETHICAL APPROVAL

Study protocol was approved by the Bioethics Committee of the Faculty of Zoology at Bangalore University, Bangalore (Protocol number: DOZ/BUB/2018–19 and 402/CPSCSEA 2009–12 and revival thereon).

9. AUTHORS' CONTRIBUTIONS

Basha PM designed the research studies and wrote the manuscript. Rakesh H conducted the experiments and acquired data. Saumya SM analyzed data and edited the manuscript.

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