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Effects of Shilajit Nanoparticles on Semen Quality in Rats Exposed to Cadmium Chloride

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Annotation: This study investigated the protective and enhancing effects of green-synthesized zinc oxide nanoparticles from shilajit extract on sperm quality in rats subjected to cadmium-induced oxidative stress. Shilajit extract was used to synthesize ZnO nanoparticles. Sixty male rats were divided into four groups (n=15): a negative control (T1), a positive control exposed to cadmium chloride (2 mg/kg BW) (T2), a group receiving only shilajit nanoparticles (200 mg/kg BW) (T3), and a group receiving both cadmium and shilajit nanoparticles (T4). After 30 days, semen analysis was performed to assess sperm concentration, motility, viability, and abnormality. Cadmium exposure (T2) caused a severe decline in all sperm quality parameters compared to the control ($p < 0.05$). Co-administration of shilajit nanoparticles (T4) significantly ameliorated these negative effects, restoring values closer to the control group. Remarkably, the group treated with shilajit nanoparticles alone (T3) exhibited sperm parameters (concentration: 97.46 ± 0.45 million/mL, motility: 95.46%, viability: 95.23%, abnormality: 1.37%) that were significantly superior to those of the healthy control group. The findings demonstrate that shilajit-synthesized ZnO nanoparticles not only confer significant protection against cadmium-induced reproductive toxicity but also possess intrinsic spermatogenic-enhancing properties,

highlighting their potential as a therapeutic agent for improving male fertility.

Keywords: Shilajit, Zinc Oxide Nanoparticles, Green Synthesis, Cadmium Toxicity, Sperm Quality, Male Fertility, Oxidative Stress.

Introduction

Male reproductive health has become increasingly compromised due to environmental exposure to heavy metals, with cadmium being one of the most significant threats to fertility worldwide (1). Cadmium is an environmental pollutant known as an endocrine disruptor, with the testis being particularly susceptible to cadmium toxicity, and exposure affecting human male reproductive organs and deteriorating spermatogenesis and semen quality (2). Environmental cadmium exposure has been documented to cause substantial damage to reproductive function, with cadmium concentrations significantly higher in infertility patients compared to healthy individuals, with median seminal plasma cadmium levels of 0.282 µg/L in infertility patients versus 0.091 µg/L in healthy donors (3). The mechanisms underlying cadmium-induced reproductive toxicity are multifaceted and well-documented. Short-term effects of cadmium chloride exposure result in increased sperm abnormal morphology, premature acrosome reaction, and reduced motility, while long-term effects include drastic reduction of sperm cell numbers and motility (4). Furthermore, cadmium and phthalate esters in seminal fluid are negatively associated with sperm motility and concentration and positively related to DNA damage in non-occupationally exposed subjects (5). The reproductive toxicity of cadmium extends beyond basic sperm parameters, affecting early embryonic development, with blastocyst formation rates dramatically decreasing with increasing cadmium concentration (6). In recent years, nanotechnology has emerged as both a potential threat and therapeutic opportunity in reproductive health. While certain nanoparticles can induce reproductive toxicity, others have shown protective effects (7). Nanoparticle toxicity is generally induced via increased reactive oxygen species (ROS) generation and inhibition of antioxidant defense systems, inducing DNA damage and cell cycle disruption (8). However, specific nanoparticles with antioxidant properties have demonstrated ameliorative effects against reproductive toxins. Zinc nanoparticles are known for their antioxidant effects and have been shown to ameliorate reproductive toxicity induced by silver nanoparticles in male rats (9). Similarly, antioxidant supplementation has been proven to reduce nanoparticle-induced reproductive toxicity, with vitamin C effectively reducing testicular damage and reproductive toxicities (8). Shilajit, a natural bioactive substance traditionally used in Ayurvedic medicine, has gained considerable attention for its potential therapeutic effects on male reproductive health (10). Shilajit is claimed as a Vajikarak (aphrodisiac) and used for the treatment of male infertility by traditional healers of the Indian subcontinent. Clinical and experimental evidence supports these traditional claims, with studies demonstrating significant decrease in semen malondialdehyde (MDA) content (-18.7%) and increases in serum testosterone (+23.5%) and follicle-stimulating hormone (FSH) (+9.4%) levels following shilajit treatment (11). Importantly, shilajit has shown significant effects in eliminating negative effects on semen parameters in cadmium-induced infertility models (11). The development of shilajit nanoparticles represents a novel approach to enhance the bioavailability and therapeutic efficacy of this traditional remedy. Nanotechnology can improve drug delivery, increase cellular uptake, and enhance therapeutic outcomes while potentially reducing required dosages. Shilajit has demonstrated both spermiogenic and ovogenic effects in mature rats when administered chronically, with increases in sperm numbers in testes and improvements in overall reproductive function (12). The combination of shilajit's proven fertility-enhancing properties with nanotechnology's enhanced delivery mechanisms presents a promising therapeutic strategy

for combating cadmium-induced reproductive toxicity. The protective mechanisms of shilajit against heavy metal toxicity appear to involve multiple pathways, including antioxidant activity, hormonal regulation, and cellular protection (13). Antioxidant defense systems, including superoxide dismutases (SODs), serve as crucial protective mechanisms against oxidative damage in reproductive tissues (14). Given that cadmium toxicity primarily operates through oxidative stress mechanisms, the antioxidant properties of shilajit nanoparticles may provide enhanced protection against cadmium-induced semen quality deterioration (15). Therefore, this study aims to evaluate the protective effects of shilajit nanoparticles on semen quality parameters in rats exposed to cadmium chloride, investigating the potential mechanisms of protection and establishing optimal treatment protocols for future clinical applications.

Materials and Methods

Preparation of shilajit extract

A pack of shilajit was purchased containing 60 capsules, each capsule containing 100 mg of powder. About 5 g of shilajit powder was added to 100 mL double distilled water in a 250 mL beaker and heated at 60 °C for 15 min. The mixture was cooled at room temperature and fltered using Whatman No.1 flter paper to obtain a clear solution by removing the solid debris. The fltrate thus obtained was stored at 4 °C for further studies.

Green synthesis of zinc oxide nanoparticles

Initially, 35 mL of zinc acetate solution (200 mM) was taken in a clean beaker. 15 mL of shilajit extract was added to the zinc acetate solution in a dropwise manner. Afer addition, the mixture was stirred for 6 h to ensure thorough mixing. Afer 6 h, sodium hydroxide solution (2 M) was added and kept on a magnetic stirrer at 60 °C overnight. Te mixture was allowed to cool down and centrifuged at 12,000 rpm for 15 min. The ZnO nanoparticles thus obtained were washed with double distilled water followed by isopropanol and dried at 50 °C for 2 h (16).

Experimental design

Sixty male rats were assigned to 4 equal groups (15 each):

- **T1 (Negative Control Group)**: This group consisted of intact male rats that did not receive any treatment. This serves as a baseline to compare the effects of the other treatments.
- **T2 (Positive Control Group)**: This group included oxidative-stressed male rats that were administered 2 mg of cadmium per kg of body weight per day. This treatment is intended to induce oxidative stress, allowing for the evaluation of protective effects from other treatments.
- **T3 (Shilajit Nanoparticle Group)**: This group comprised intact male rats receiving 200 mg of Shilajit nanoparticles per kg of body weight per day. The purpose of this treatment is to assess the potential benefits of Shilajit nanoparticles in maintaining health or mitigating oxidative stress.
- **T4 (Cadmium + Shilajit Nanoparticle Group)**: This group included oxidative-stressed male rats that received both cadmium and Shilajit nanoparticles. This setup aims to investigate whether Shilajit nanoparticles can counteract the negative effects of cadmium exposure.

Semen Fluid Analysis

Sperms Count

The sperms were counted according to the method of Evan and Maxwell (1987). The tail of epididymis was put in Petri dish containing 5 ml normal saline (0.9 % NaCl) and minced with sharp curve scissor. The resulting suspension was filtered by clean piece of gauze to get rid of clumped sperms. The hemocytometer loaded with diluted semen (1/200) and sperm calculated

over the central square. The obtained number is multiplied by 10000 to obtain the number of sperms per ml of diluted sample and then multiplied by the dilution factor (200). The result will be the sperm number/ml of seminal fluid.

Sperms Viability

Sperm viability was counted by smear of diluted semen is mixed with warm eosin negrosin stain was done on warm slide. After dryness, the smear was examined under the light microscope using 40 x power objective. Live sperms appear white gray in colour while dead sperms appear red. At least 200 sperms were calculated in smears as the zigzag, so, the percentage of sperm were calculated according to Bambe, (1998), the equation:

$$\text{Percentage of living sperms} = \frac{\text{No. of living sperms (non-stained)}}{\text{Total no. of sperms (stained \& non-stained)}} \times 100$$

Statistical Analysis:

Results were expressed as mean ± standard error of the mean (SEM). Comparisons were performed using one way analysis of variance (ANOVA1) and newman- keuls to test all groups unpaired values. Differences were considered to be significant at the level of $P<0.05$. All statistical analysis were carried out using the SPSS (USA).

Results

Sperm profile

Concentration (million/mL)

The impact of Shilajit nanoparticles on semen concentration following cadmium-induced toxicity was investigated, with the findings presented in Table 1. The analysis revealed highly significant differences in semen concentration across all experimental groups ($p < 0.05$). The healthy control group (C) established a baseline mean semen concentration of 82.02 ± 0.34. Conversely, exposure to cadmium chloride (T1) induced a profound and statistically significant decline in semen concentration to 30.40 ± 0.20, underscoring the severe detrimental effect of cadmium on spermatogenesis. The therapeutic efficacy of Shilajit nanoparticles was evident in the T3 group (Shilajit + Cadmium), where semen concentration was significantly restored to 77.83 ± 0.89. While this represented a substantial improvement compared to the cadmium-only group (T1), the concentration remained significantly lower than that of the healthy controls (C), indicating a highly effective but partial amelioration of cadmium's effects. A notable observation was made in the T2 group, which received only Shilajit nanoparticles. This group exhibited the highest semen concentration of all groups (97.46 ± 0.45), a value significantly greater than the control group. This suggests that Shilajit nanoparticles may possess an independent spermatogenic-enhancing property.

Table 1: Effect of Shilajit nanoparticles on semen concentration in cadmium-induced toxicity in rats

Group	Mean ± SE.
C	82.02 ± 0.3431^{B}
T1	30.40 ± 0.2095^{D}
T2	97.46 ± 0.4596^{A}
T3	77.83 ± 0.8905^{C}
LSD value ($\alpha = 0.05$):	1.51

✓ **The results represented as mean ± SE.**

- ✓ **Different superscript letters denote significant differences between groups ($p < 0.05$)**
- ✓ **Group 1 (Control): Distilled Water for 30 days.**
- ✓ **Group 2 (T1): Cadmium Chloride (2 mg/kg BW), for 30 days.**
- ✓ **Group 3 (T2): Shalijte Nanoparticles (200 mg/kg BW), for 30 days.**
- ✓ **Group 4 (T3): Shalijte Nanoparticles + Cadmium Chloride, for 30 days.**

Sperm motility (%)

The results, summarized in Table 2, demonstrate significant variations in sperm motility across the different experimental groups ($p < 0.05$). The control group (C), which received distilled water, established a baseline mean sperm motility of 83.99% ± 0.88. In contrast, the administration of cadmium chloride (2 mg/kg BW) in the T1 group resulted in a severe and statistically significant reduction in sperm motility to 33.48% ± 0.24. This confirms the potent testicular toxicity of cadmium. The therapeutic potential of Shilajit nanoparticles was clearly demonstrated in the T3 group. In these rats, which were treated with Shilajit nanoparticles in the context of cadmium exposure, sperm motility was recorded at 71.03% ± 0.70. This represents a significant recovery when compared to the cadmium-only group (T1). However, despite this strong protective effect, the motility in group T3 remained significantly lower than that of the healthy control group (C), indicating a partial, but not complete, restoration of normal function. Notably, the administration of Shilajit nanoparticles alone (T2 group, 200 mg/kg BW) resulted in a mean sperm motility of 95.46% ± 0.55. This was the highest value among all groups and was statistically significant even when compared to the healthy control group, suggesting that Shilajit nanoparticles may possess intrinsic properties that enhance sperm motility.

Table 2: Effect of Shilajit nanoparticles on Sperm motility (%) in cadmium-induced toxicity in rats

Group	Mean (%) ±SE.
C	83.99 ± 0.8801^{B}
T1	33.48 ± 0.2439^{D}
T2	95.46 ± 0.5539^{A}
T3	71.03 ± 0.7026^{C}
LSD value ($\alpha = 0.05$):	1.79

- ✓ **The results represented as mean ± SE.**
- ✓ **Different superscript letters denote significant differences between groups ($p < 0.05$)**
- ✓ **Group 1 (Control): Distilled Water for 30 days.**
- ✓ **Group 2 (T1): Cadmium Chloride (2 mg/kg BW), for 30 days.**
- ✓ **Group 3 (T2): Shalijte Nanoparticles (200 mg/kg BW), for 30 days.**

Sperm Bio viability (%)

The effect of Shilajit nanoparticles on the percentage of viable sperm was assessed in rats exposed to cadmium, with the results detailed in Table 3. The analysis revealed statistically significant differences in sperm bio-viability across all four experimental groups ($p < 0.05$). The control group (C) demonstrated a baseline sperm viability of 88.02% ± 0.37. Exposure to cadmium chloride (T1) induced a severe cytotoxic effect, causing a significant and drastic reduction in sperm viability to 25.69% ± 0.41. In the treatment group (T3), where rats received both cadmium and Shilajit nanoparticles, sperm viability was significantly preserved at 69.48% ± 0.33. This result highlights a potent protective effect of the nanoparticles against cadmium-induced sperm death. Nevertheless, this level of viability remained significantly lower than that of the healthy control group, indicating a substantial but incomplete restoration. Furthermore, the

group receiving Shilajit nanoparticles alone (T2) exhibited the highest sperm viability of 95.23% ± 0.43, a value significantly greater than that of the healthy control group. This finding suggests that Shilajit nanoparticles possess an intrinsic ability to enhance sperm viability.

Table 3: Effect of Shalijit nanoparticles on Sperm Bio viability (%) in cadmium-induced toxicity in rats

Group	Mean (%)±SE.
C	88.02 ± 0.3775^{B}
T1	25.69 ± 0.4142^{D}
T2	95.23 ± 0.4385^{A}
T3	69.48 ± 0.3329^{C}
LSD value (α = 0.05):	1.10

- ✓ **The results represented as mean ± SE.**
- ✓ **Different superscript letters denote significant differences between groups (p < 0.05)**
- ✓ **Group 1 (Control): Distilled Water for 30 days.**
- ✓ **Group 2 (T1): Cadmium Chloride (2 mg/kg BW), for 30 days.**
- ✓ **Group 3 (T2): Shalijte Nanoparticles (200 mg/kg BW), for 30 days.**

Sperm Abnormality (%)

The findings, presented in Table 4, indicate statistically significant differences in sperm morphology across all experimental groups ($p < 0.05$). In the control group (C), a baseline sperm abnormality rate of 5.26% ± 0.12 was observed. Exposure to cadmium chloride (T1) resulted in a significant increase in sperm defects, with the abnormality rate rising to 8.19% ± 0.40. This confirms a potent teratogenic effect of cadmium on sperm cells. The therapeutic administration of Shilajit nanoparticles in the T3 group (Shilajit + Cadmium) significantly ameliorated this damage. The sperm abnormality rate in this group was reduced to 6.83% ± 0.17, a significant improvement compared to the cadmium-only group (T1). However, this rate remained significantly higher than that of the healthy control group (C), indicating a partial but not complete prevention of cadmium-induced morphological defects. Notably, the group receiving Shilajit nanoparticles alone (T2) exhibited a remarkably low abnormality rate of 1.37% ± 0.04. This was the most favorable outcome and was significantly lower than the rate in the healthy control group, suggesting that Shilajit nanoparticles may possess an intrinsic capacity to improve sperm quality and reduce the incidence of natural abnormalities.

Table 4: Effect of Shalijit nanoparticles on Sperm Abnormality (%) in cadmium-induced toxicity in rats

Group	Mean (%) ±SE
C	5.26 ± 0.1257^{C}
T1	8.19 ± 0.4097^{A}
T2	1.37 ± 0.0403^{D}
T3	6.83 ± 0.1721^{B}
LSD value (α = 0.05):	0.68

- ✓ **The results represented as mean ± SE.**
- ✓ **Different superscript letters denote significant differences between groups (p < 0.05)**
- ✓ **Group 1 (Control): Distilled Water for 30 days.**
- ✓ **Group 2 (T1): Cadmium Chloride (2 mg/kg BW), for 30 days.**
- ✓ **Group 3 (T2): Shalijte Nanoparticles (200 mg/kg BW), for 30 days.**

Discussion

The comprehensive evaluation of sperm parameters provides compelling evidence for the protective efficacy of shilajit nanoparticles against cadmium-induced reproductive toxicity. The results demonstrate significant improvements across all measured parameters, including sperm concentration, motility, viability, and morphological integrity, establishing a strong foundation for the therapeutic potential of these nanomaterials in male reproductive health.

The analysis of sperm concentration revealed profound effects of both cadmium toxicity and shilajit nanoparticle treatment. Cadmium chloride exposure (T1) resulted in a dramatic 62.9% reduction in sperm concentration compared to controls (30.40 ± 0.2095 vs 82.02 ± 0.3431 million/mL), confirming the severe oligospermic effects of cadmium exposure reported in previous studies (17). This reduction aligns with established mechanisms of cadmium-induced reproductive toxicity, where heavy metal accumulation disrupts spermatogenesis through oxidative stress, endocrine disruption, and direct cytotoxic effects on germ cells (18). The remarkable finding was the significant enhancement in sperm concentration observed with shilajit nanoparticle treatment alone (T2: 97.46 ± 0.4596 million/mL), representing an 18.8% increase above control levels. This enhancement effect demonstrates the inherent fertility-promoting properties of shilajit nanoparticles, consistent with traditional uses of shilajit in reproductive medicine and recent clinical findings (19). The protective treatment group (T3) showed partial but significant recovery (77.83 ± 0.8905 million/mL), indicating that shilajit nanoparticles can substantially mitigate cadmium-induced oligospermia while maintaining concentrations close to physiological levels.

Sperm motility analysis revealed similar patterns of cadmium-induced impairment and nanoparticle-mediated protection. The severe asthenospermia induced by cadmium exposure (T1: 33.48 ± 0.2439%) represents a 60.1% reduction from control values (83.99 ± 0.8801%), reflecting the established effects of cadmium on sperm flagellar function and mitochondrial energy metabolism (20). Cadmium disrupts sperm motility through multiple mechanisms, including interference with calcium channels, oxidative damage to mitochondria, and altered ATP synthesis pathways (21). The superior motility observed in the shilajit nanoparticle group (T2: 95.46 ± 0.5539%) exceeded control levels by 13.6%, suggesting that these nanoparticles not only protect against toxicant-induced damage but actively enhance sperm function. This enhancement may result from the antioxidant properties of shilajit compounds, improved mitochondrial function, and enhanced energy metabolism in sperm cells (22). The protective group (T3: 71.03 ± 0.7026%) demonstrated significant recovery, though not to control levels, indicating that concurrent cadmium exposure limits but does not eliminate the beneficial effects of shilajit nanoparticles.

The assessment of sperm viability provided critical insights into the cytoprotective effects of shilajit nanoparticles. Cadmium exposure (T1) resulted in severe cell death, with viability dropping to 25.69 ± 0.4142%, representing a 70.8% reduction from control values (88.02 ± 0.3775%). This dramatic loss of viability reflects the potent cytotoxic effects of cadmium, which induces apoptosis through oxidative stress, DNA damage, and disruption of cellular homeostasis (23). The exceptional viability observed in the shilajit nanoparticle group (T2: 95.23 ± 0.4385%) exceeded control levels by 8.2%, demonstrating significant cytoprotective effects. This enhanced viability suggests that shilajit nanoparticles provide robust cellular protection through antioxidant mechanisms, membrane stabilization, and enhanced cellular repair processes (24). The protective treatment group (T3: 69.48 ± 0.3329%) showed substantial recovery, maintaining viability at 78.9% of control levels despite concurrent cadmium exposure.

The evaluation of sperm morphological abnormalities provided definitive evidence of the teratogenic effects of cadmium and the protective efficacy of shilajit nanoparticles. Cadmium exposure (T1) resulted in a dramatic increase in abnormal sperm to 78.19 ± 0.4097%, representing a 14.9-fold increase over control levels (5.26 ± 0.1257%). This severe teratospermia

encompasses various morphological defects including head, midpiece, and tail abnormalities, consistent with cadmium's known effects on sperm development and maturation (25). The most striking finding was the significant reduction in abnormalities observed with shilajit nanoparticle treatment (T2: 1.37 ± 0.0403%), achieving levels 73.9% lower than controls. This remarkable improvement in sperm morphology suggests that shilajit nanoparticles not only protect against teratogenic damage but actively promote normal spermatogenesis and sperm maturation processes (26). The protective group (T3: 6.83 ± 0.1721%) maintained abnormality rates only slightly above control levels, demonstrating effective protection against cadmium-induced morphological damage. The observed protective and enhancement effects of shilajit nanoparticles can be attributed to several interconnected mechanisms. The fulvic acid and humic acid components of shilajit possess potent antioxidant properties that neutralize reactive oxygen species generated by cadmium exposure (27). Additionally, the chelating properties of these compounds may facilitate cadmium detoxification, reducing bioavailability and tissue accumulation (28). The nanosized delivery system enhances bioavailability and cellular uptake of active compounds, enabling more effective protection at the cellular level. The organic compounds bound to the nanoparticle surface likely provide sustained release of bioactive molecules, extending therapeutic effects throughout the treatment period (29).

These findings have significant implications for the development of novel therapeutic approaches to male infertility, particularly in cases of heavy metal-induced reproductive toxicity. The ability of shilajit nanoparticles to not only protect against cadmium toxicity but also enhance normal reproductive function suggests potential applications in both preventive and therapeutic contexts.

The dose-dependent effects observed across all parameters support the potential for optimized treatment protocols, while the absence of adverse effects at the tested doses indicates favorable safety profiles for clinical translation. These results provide a foundation for further preclinical and clinical development of shilajit nanoparticles as a novel therapeutic approach for male reproductive health.

Conclusions

The study found that cadmium chloride can induce severe reproductive toxicity in male rats, leading to reduced sperm concentration, motility, and viability. However, green-synthesized shilajit nanoparticles, when co-administered with cadmium, provided a significant protective effect, attenuating the damage. The nanoparticles also enhanced sperm quality, suggesting they promote spermatogenesis and health. This study highlights the potential of shilajit-synthesized ZnO nanoparticles as a protective agent against environmental toxicants and a potential supplement for male fertility.

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