

## Original Article

# An *In vitro* Study on the Protective Effect of Melatonin on Human Sperm Parameters Treated by Cadmium

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### ABSTRACT

**Background:** Male infertility account for nearly 50% of infertility cases. Cadmium is regarded as a well-known toxic metal for industrial applications; high amounts of cadmium in the human body can result in chronic toxicity. Melatonin as a free radical scavenger has anti-inflammatory, and even anti-cancer and antiapoptotic functions. **Aim:** In this work, we evaluated the protective effect of melatonin on human sperm parameters treated by cadmium. **Study Setting and Design:** This was an experimental study carried out from May to December 2019. **Materials and Methods:** A total of 41 fresh semen samples were collected from fertile men and were divided into 4 groups: (1) control, (2) sperm +25 Nm cd, (3) sperm +25 nM cd +0.1 mM melatonin, (4) sperm +0.1 mM melatonin treated for 60 min. In all groups, semen analysis was performed for motility, viability and DNA fragmentation index (DFI). **Statistical Analysis:** The groups were compared using the ANOVA test. **Results:** The group treated with cadmium showed a significant decrease in rapid and slow motility, and survival rate compared with the control group ( $P < 0.05$ ). However, the degree of DFI and sperm with non-progressive motility in the group treated with cadmium had a significant increase compared to the control ( $P < 0.05$ ). The use of melatonin significantly improved sperm parameters such as motility, survival rate and decreased sperm DFI with non-progressive motility. **Conclusions:** The use of melatonin reduces the amount of cadmium damage in human sperm *in vitro*.

**KEYWORDS:** Cadmium, human sperm, male infertility, melatonin, semen analysis

## INTRODUCTION

According to the World Health Organisation (WHO), infertility is a reproductive system disorder defined by the failure to reaching clinical pregnancy after 1 year or more of regular, unprotected sex.<sup>[1]</sup> Male partners account for nearly 50% of infertility cases.<sup>[2]</sup> Over the past four decades, decreased male fertility (with 50% worldwide) could be due to low sperm count and declining sperm quality with 1 in 20 men.<sup>[3]</sup> Male infertility can be caused by various factors, such as anatomical aberrations, sexual dysfunction, varicocele and, most importantly, obesity, oligospermia, tobacco smoking and heavy metal toxicity, such as cadmium chloride.<sup>[2,4]</sup> Heavy metal-induced oxidative stress is one

of the causes of abnormal sperm functions and male infertility. It has been demonstrated that elevated heavy metal levels increase lipid peroxidation, and antioxidant depletion and is associated with abnormal sperm functions.<sup>[5]</sup>

Cadmium (Cd) is a toxic element found in the crust of the earth at a low concentration.<sup>[4]</sup> There are different natural sources of heavy metals in the environment; however, anthropogenic activities, such as waste disposal, mining and sludge application, can contribute to their accumulation.<sup>[6]</sup> Humans absorb cadmium (Cd)

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into the body through ingestion or inhalation. Yet, only about 25%–6% of the ingested substance is properly absorbed.<sup>[7]</sup> Cd, a relatively rare substance in nature, was discovered by F. Strohmaier in 1817 and is chiefly applied in the nickel-cadmium (Ni-Cd or NiCad) battery, pigment, painting and plastic production.<sup>[8]</sup> It combines easily with other elements, including carbonate, chloride, oxygen and sulfur, forming cadmium carbonate, cadmium chloride, cadmium oxide and cadmium sulfate, respectively.<sup>[4]</sup>

Cd is regarded as a well-known toxic metal for industrial applications; high amounts of Cd in the human body can result in chronic toxicity. Patients with serum Cd concentrations higher than 30 µg/L have been demonstrated to suffer from chromium toxicity.<sup>[8]</sup> Cd poses a great risk to human health, including steroidogenic defects, semen quality impairment, suppression of oocyte maturation, ovarian failure, defective implantation, spontaneous abortion and congenital disorders.<sup>[9]</sup> Cd can damage enzymes containing sulfhydryl groups and also cause uncoupling of oxidative phosphorylation in mitochondria. Furthermore, competing with other metals (e.g., zinc and selenium (Se), Cd is involved with inclusion into metalloenzymes. Besides, it can influence binding sites on regulatory proteins, such as calmodulin in competition with calcium (Ca).<sup>[7]</sup>

Melatonin (N-acetyl-5-methoxytryptamine) (MT) is an indoleamine neurohormone that is synthesised from an essential exogenous  $\alpha$ -amino acid, i.e., tryptophan secreted by the pineal gland. It can affect circadian and seasonal rhythm adjustment.<sup>[10,11]</sup> It is generally considered a potent antioxidant thanks to its efficacy as a free radical (FR) scavenger, anti-inflammatory, and even anti-cancer and antiapoptotic functions.<sup>[12-14]</sup> It protects against apoptosis via the common pathway, leading to increased expression of Bcl-2 and reduced expression of Bax in C2C12 murine myoblast cells.<sup>[14]</sup> It counteracts the toxic effects of ROS and reactive nitrogen species. MT has been shown to act as a potent protector against oxidative stress for humans, boars, buffalos, mice and mouse sperm.<sup>[12]</sup> Melatonin (MT) is regarded as a more powerful antioxidant than vitamins E or C and 5–15 times greater than glutathione.<sup>[13]</sup> As an intracellular antioxidant, it protects cells against ROS-mediated oxidative damage both *in vitro* and *in vivo*.<sup>[11]</sup> It has a significant mitochondrial affinity, accumulates in the organelles, and reverses mitochondrial disorders by reducing oxidative stress.<sup>[10]</sup> MT molecules bear both hydrophilic and lipophilic affinities, thus dispersed widely in various subcellular compartments, such as membranes, cytoplasm, nucleus and mitochondria.<sup>[14]</sup>

MT is capable of directly yielding sperm characteristics, along with DNA integrity, enhanced sperm membrane, enhanced total motility, modulation of sperm capacitation, progressive motility, reduced membrane lipid peroxidation and viability rates.<sup>[11]</sup> In this work, we evaluated the protective effect of melatonin on human sperm parameters treated by cadmium.

## MATERIALS AND METHODS

This was an experimental study carried out on men referred to the infertility clinic of Al-Zahra Hospital, Rasht, Iran, from May to December 2019.

### Chemicals

All chemicals were purchased from Sigma-Aldrich; Germany. Melatonin was initially dissolved in absolute ethanol and diluted further with phosphate-buffered saline (PBS). CdCl<sub>2</sub> pure powder was dissolved in distilled water (dH<sub>2</sub>O) and diluted further with PBS.

### Semen sample collection and treatment

Semen samples were taken from 41 fertile (without sample size calculation) men with a sperm count of higher than 20 million per mL after 3–4 days of abstaining from sexual intercourse. Participants were asked to provide semen samples by masturbation using sterile plastic containers. Ethical approval for this study was obtained from the Ethics Committee of Guilan University of Medical Sciences (Approval ID: IR. GUMS. REC.1397.169). All sample donors signed a written informed consent before sample collection in adherence to the Declaration of Helsinki. To liquefy clotted semen samples, they were kept in an incubator at 37°C for about 30–60 min. They were then analysed using a computer-assisted sperm analysis system to reliably assess morphometric characteristics, motility parameters and sperm count. The samples were washed by the Sage washing medium and then centrifuged at 5000 g to isolate sperms from seminal plasma. The cell plaque was then washed again with the sperm washing medium. Human sperm samples were assigned to four groups: (1) control (sperm), (2) sperm +25 Nm cd, (3) sperm +25 nM cd +0.1 mM melatonin, (4) sperm +0.1 mM melatonin. The four groups of human sperm were incubated at 37°C for 60 min.

### Sperm motility

Sperm motility was assessed using a phase-contrast microscope with a 40X objective lens. WHO (2010) categorised it into four grades, include (1) Class-A or Grade-IV: Rapid progressive sperm motility (2), Class-B or Grade-III: Slow non-linear progressive sperm motility (3); Class-C or Grade-II: Nonprogressive

rotational sperm motility; and (4) Class-D or Grade-I: Immotile (Grade 1), nonprogressive (Grade 2) and progressive (Grade 3 and 4) sperm motility. No motility or immotile means sperm has no kind of motility, but non-progressive motility means sperm that do not travel in straight lines or that swim in very tight circles.

### Sperm viability

The trypan blue staining technique was used to evaluate sperm viability. Sperm sample and trypan blue were mixed (1:1) and preserved in an incubator for 15 min. A certain amount of sperm suspension was spread carefully on another slide to prepare the smear of human sperm on a clean glass microscope. The slides were then air-dried. Afterwards, the sperm count was measured immediately using an inverted microscope at  $\times 400$ . We assessed 100 sperms in each sample for viability. Viable sperms will not appear stained, but non-viable sperms will take up the stain.

### Sperm DNA fragmentation with non progressive motility and DNA fragmentation index

An aliquot of the sperm sample was diluted to 10 million/ml in PBS. The resulting suspensions were mixed with 1% low-melting-temperature aqueous agarose. Afterwards, 50  $\mu$ L mixture aliquots were pipetted onto a glass microscope slide precoated by 0.65% standard agarose. They were then left for solidification for 4 min at 4°C using a 24–60 mm coverslip. Coverslips were then completely removed, and slides were immediately immersed horizontally in a tray containing newly prepared acid-denatured solution (0.08N HCl) at 22°C for 7 min in darkness. They were then soaked horizontally in 25 ml lysing solution containing 0.05M Ethylenediaminetetraacetic acid, methanol, 2M NaCl, sodium dodecyl sulfate and 0.4M Tris-Hcl at pH 7.5 for 25 min. The slides were then completely washed with dH<sub>2</sub>O for 5 min, and were dehydrated in ethanol (70%, 90% and 100%) for 2 min each and finally air-dried. They were covered with a Wright's stain solution-PBS (1:1) mixture for 5-10 min. They were then washed under tap water and left to dry. This study scored at least 200 spermatozoa per sample using a 100X objective lens. Four SCD patterns are defined: (1) big halo-sized sperm cells whose halo width is equal to or more than the minor core diameter, (2) medium halo-sized sperm cells whose halo size ranging from big to very small, (2) very small halo-sized sperm whose halo width is equal to or smaller than 1/3 of the minor core diameter, and (2) halo-free sperm cells. In measuring DNA fragmentation index (DFI), big-to-medium halo-sized nuclei were determined to be sperms with a non-fragmented DNA, while small halo-sized, halo-free and degraded sperm nuclei were

determined to be sperms with fragmented DNA. DFI was estimated by dividing the number of spermatozoa with small or no halo by the total number of analysed spermatozoa multiplied by 100.

### Statistical analysis

The SPSS (Statistical Package for the Social Sciences) version 21 (Chicago, USA) was used to perform statistical analysis. All data derived from the above experiments were expressed in the form of mean  $\pm$  Standard deviation. The Kolmogorov–Smirnov test has been used to test the normality of the data. The groups were compared using the ANOVA test. The Tukey's Ben-Froeny test was conducted in case of statistical significance ( $P < 0.05$ ).

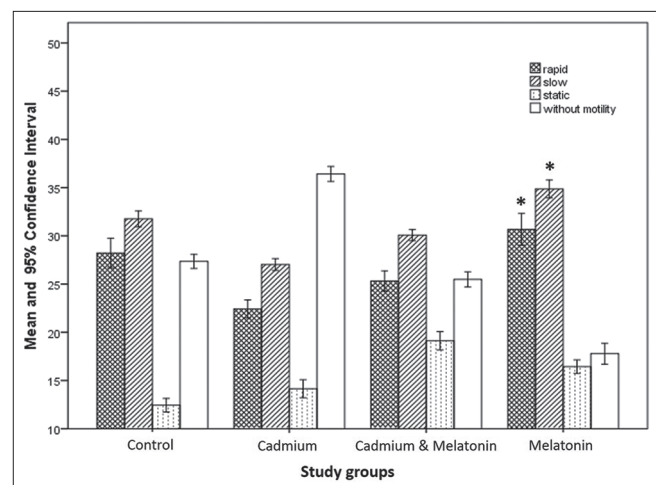
## RESULTS

### Effects of MT and cadmium exposure on total motile sperm *in vitro*

Figure 1 indicates a significant increase in the percentage of spermatozoa with rapid and slow motility in the MT-treated group when compared with the Cd-treated, Cd-MT-treated and control groups ( $P < 0.05$ ). However, the percentage of sperm with rotational or non-progressive motility and immotile sperm in MT-treated groups decreased with respect to the Cd-treated, Cd-MT-treated and control groups ( $P < 0.05$ ).

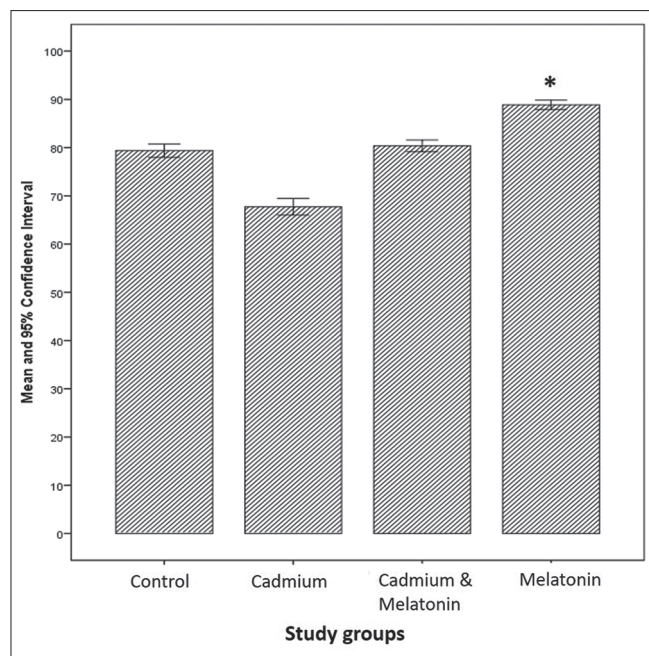
### Effects of MT and cadmium exposure on sperm viability *in vitro*

Figure 2 illustrated the percentage of viable spermatozoa in various sperm groups. Viability in the MT-treated group significantly increased compared with the Cd-treated, Cd-MT-treated and control groups.



**Figure 1:** The effect of MT and Cadmium on sperm motility in study groups. A significant increase in the percentage of spermatozoa with rapid and slow motility has been observed in the MT-treated group compared with the Cadmium-treated, Cadmium-MT-treated, and control groups ( $P < 0.05$ )





**Figure 2:** The effect of MT and Cadmium on sperm viability. Viability in the MT-treated group was higher than in other groups

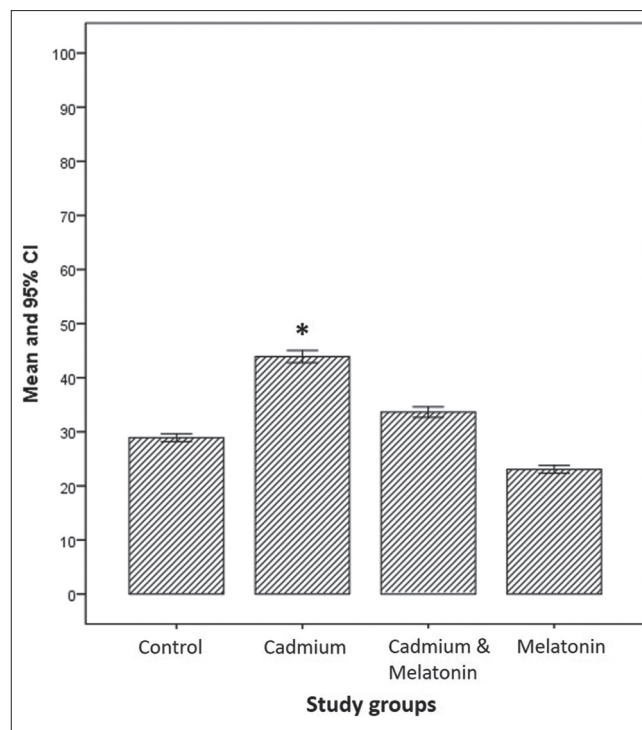
### Effects of MT and cadmium exposure on sperm DNA fragmentation index *in vitro*

The results indicated that the degree of DFI in the Cd-treated group was higher than in the MT-treated, Cd-MT-treated and control groups [Figure 3].

## DISCUSSION

This study showed that Cd has a negative effect on human sperm parameters. Briefly, it decreases sperm motility and survival and increases SDF. Besides, the results of our experiments revealed that the use of MT substantially decreased Cd-induced damage. Cd is also detrimental to the human body due to its long half-life. It impairs the functionality of the liver and kidneys, and respiratory, nervous and testicular systems, but the testes are more sensitive than others. It has been proven that Cd damage is mainly caused by cellular oxidative stress.<sup>[15]</sup> Besides, it could result in cell death by changing the cellular antioxidant system. There are various procedures to curb oxidative stress and decrease the damage caused by oxygen FRs, including the damage to the antioxidant system.<sup>[16]</sup>

MT counteracts or eradicates FRs. Due to its small size and lipophilic characteristics, MT smoothly passes through the cell membrane and spread across the cell, and protects DNA against damaging factors. It exerts antioxidant defense at the cell membrane, mitochondria and cell nuclei, both *in vitro* and *in vivo*. Our study indicated that Grade-3 and Grade-4 sperm motility decreased in the Cd-treated group compared to the control



**Figure 3:** The effect of MT and Cadmium on sperm DNA fragmentation index. The Cadmium -treated group had a higher DNA fragmentation index compared to other groups ( $P < 0.05$ )

group. Nevertheless, Grade-1 and Grade-2 sperm motility dramatically increased in the Cd-treated group compared to the control group. On the other hand, the addition of MT significantly increased Grade-3 and Grade-4 sperm motility and decreased Grade-1 and Grade-2 sperm motility in comparison with the control and Cd-treated groups. According to Da Costa *et al.*, Cd reduces sperm motility via sperm ATPase depletion, which affects the biochemical mechanism of sperm.<sup>[17]</sup> Wang *et al.* also confirmed that Cd reduces sperm motility by tyrosine phosphorylation since it functions as an engine for regulating sperm motility.<sup>[18]</sup> Li *et al.* noticed that Cd reduces sperm motility, whereas MT increases it. It has been shown that MT increases sperm motility.<sup>[19]</sup>

In this study, the sperm survival rate reduced in the Cd-treated group compared to the control group. However, following the addition of MT, the sperm survival rate increased in comparison with the control and Cd-treated groups. Cd lowers ATP production and enhances ROS production. Large amounts of ROS cause damage to mitochondria that may trigger apoptosis.<sup>[20]</sup> MT reduces mitochondrial ROS production, and as an antioxidant and antiapoptotic agent, reduces caspase-3 and-9 in human sperm. Thus, it can partially reduce these detrimental effects.

It has been indicated that Cd decreases sperm survival.<sup>[7,21,22]</sup> Wang *et al.* suggested that Cd causes

infertility in mice *in vitro* by reducing sperm survival.<sup>[23]</sup> Nevertheless, Karimfar *et al.* suggested an increase in sperm survival by adding MT to human sperm samples during the freezing process.<sup>[24]</sup> Accordingly, Najafi *et al.* have recently reported that MT significantly increased sperm survival after freezing and thawing.<sup>[25]</sup> du Plessis *et al.* demonstrated that MT reduces dead human sperm *in vitro*.<sup>[26]</sup>

Cd remarkably decreases spermatozoa with large and medium halos and, at the same time, increases spermatozoa with small and no halo, as well as DFI, compared to the control group. However, MT increases spermatozoa with large and medium halos and decreases spermatozoa with small and no halo, as well as DFI. Several factors contribute to DNA fragmentation, including increased intracellular Ca<sup>2+</sup>. Previous studies revealed that Cd might replace Ca<sup>2+</sup> at high levels. Increased Ca<sup>2+</sup> concentrations cause abnormal functions of cellular organs, including mitochondria. Excessive accumulation of Ca in these organelles (or excessive intracellular Ca accumulation) alters the voltage of the mitochondrial membrane.<sup>[20]</sup> It makes the pores of this organ open, which, in turn, leads to the release of apoptotic proteins. During apoptosis, apoptosis inducers and endonuclease G (endoG) from the cytosol enters the nucleus and causes DNA fragmentation.<sup>[27]</sup> However, MT protects spermatozoa against DNA fragmentation and apoptosis by increasing BCL-2 and reducing cytochrome c (Cyt c).<sup>[28,29]</sup> It has been demonstrated that Cd increases DNA fragmentation.<sup>[30]</sup> Sharbatoghli *et al.* demonstrated a positive correlation between DNA fragmentation and MT amounts present in semen. One of the major causes of DNA fragmentation is ROS production. Studies have suggested that MT reduces ROS production.<sup>[24,25,31]</sup>

DNA fragmentation and sperm parameters are significantly correlated so that the percentage of motile sperm and sperm with normal morphology decreases by increasing the DNA fragmentation rate. Factors affecting DNA fragmentation can also affect the structure and function of the sperm.<sup>[32]</sup> This study had some limitations. First, it was not possible to evaluate the direct effects of CD and MT on human testis and spermatogenesis. Second, the exact mechanisms of CD and MT effects have not been assessed.

## CONCLUSIONS

Overall, it may be concluded that Cd, as heavy metal, can harm sperm and the male reproductive system by affecting sperm motility, morphology, survival and DNA fragmentation in sperm. The findings of our study showed that the use of melatonin reduces the amount

of cadmium damage in human sperm *in vitro*. Hence, it may be possible to reduce heavy metal effects on male infertility by antioxidants like melatonin. However, further studies are needed to confirm the findings of this study.

## Data availability and sharing statement

Data supporting the results presented in this paper is available from the corresponding author upon reasonable request.

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This study has been supported by Guilan university of medical sciences.

## Conflicts of interest

There are no conflicts of interest.

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