

Ameliorative Effect of Melatonin Versus the Passage of Time and Lipid Peroxidation on Sperm Motility in Asthenotratospermic Men

Ghadir Sohrabi ¹, Mahdi Malmir ², Aliasghar Ghafarizadeh ^{3*}

1. Tabriz University of Medical Sciences International Branch Aras, Tabriz, Iran

2 Department of Midwifery, Tuyserkhan Branch, Islamic Azad University, Tuyserkhan, Iran

3 Academic Center for Education, Culture and Research, Arak, Iran

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*Correspondence:

Aliasghar Ghafarizadeh,
Academic Center for Education,
Culture and Research, Arak, Iran

Ghafarizade@acecr.ac.ir



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Abstract

Background and Objective: Downscale outcome in assisted reproductive techniques (ART) is associated with lipid peroxidation. Melatonin may have beneficial effects against lipid peroxidation and oxidative stress. This work was aimed to evaluate the ameliorative capacity of melatonin versus the passage of time and lipid peroxidation on sperm parameters from the men with ATS in the ART process.

Material and Methods: Semen samples were collected from 50 asthenoteratozoospermic (ATS) men. Samples were divided into control and melatonin group. The test group were incubated with 6 mM melatonin at 2, 4, 6, and 24 hours. Then total and progressive motility, membrane integrity, and Malondialdehyde levels (MDA) were evaluated. Statistical analysis was carried out by SPSS software (repeated-measures ANOVA).

Results: In the control group, total ($p < 0.01$) and progressive ($P < 0.05$) motility and also, membrane integrity ($P < 0.03$) significantly decreased although, MDA levels of sperm significantly increased ($P < 0.04$). Moreover, in the melatonin group, the mentioned parameters significantly compensated compared to the control group ($P < 0.05$).

Conclusion: In the outcome, melatonin in vitro treatment paves the way for motility and membrane integrity up-regulating in the ATS men.

Keywords: Melatonin, lipid peroxidation, asthenospermia, teratozoospermia, membrane integrity, ART

Highlights

The use of antioxidants, including melatonin, can reduce oxidative stress and lipid peroxidation in human semen.

In the andrology laboratory, melatonin can be added as a supplement to the semen of asthenoteratozoospermic volunteers, which can increase the chances of fertility in them.

Introduction

Infertility can be mentioned as one of the most common problems in men and women nowadays. One of the important factors in male infertility is ATS that in these patients, sperm motility is less than 40% and abnormal sperm morphology is more than 96% (1). Considering that the use of antioxidants in several studies has had a positive effect on the treatment of male infertility (2-5), in the present study, melatonin was used as an antioxidant to overcome induction of oxidative stress and lipid peroxidation in ATS patients. During the ART process, they show a greater reduction in sperm motility because of the waste of time (2) that subsequently increases free radicals in the sperm (3-5) so that about 10-15% of couples are infertile (6). Researches has shown that reactive oxygen species (ROS) levels in men with ATS are higher than fertile men (2).

Aforementioned surplus ROS contributes to a mitochondrial disorder and sperm antioxidants reduction that can be manufactured pending defective spermatogenesis, as well as, originate from the nicotinamide-adenine dinucleotide phosphate oxidase system (NADPH; 1, 6). It seems that fatty acid peroxidation in the plasma membrane is so important and it makes a contribution to hurt to sperm via oxidative stress induction. So It is necessary

to mention the fact that PUFA is a considerable agent in the ROS risk (7). Furthermore, preparing and maintaining of sampling from ATS patients in vitro for ART presses may take several hours (1, 4). Over time, in the ART process manages to generate oxidative stress, which leads to impaired quality of sperm parameters (3, 4). Given the above, make use of antioxidants with the ROS scavenging effect can be beneficial to men fertility (2, 8). Melatonin is an antioxidant that contributes recommended to the sperm preparation process in ATS men (in vitro).

Melatonin with the chemical formula N-acetyl-5-methoxy tryptamine is a hormone. This hormone is produced in the pineal gland in the brain and helps regulate the sleep-wake cycle in the body (9). The secretion level of this hormone reaches a maximum at night and decreases around noon (9, 10). Melatonin is synthesized from the essential amino acid tryptophan to serotonin (9). Researches have shown that melatonin increases antioxidant enzyme activity and cellular mRNA levels for these enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (11, 12). Treatment of different concentrations of melatonin in studies had obviously illustrated that this antioxidant improves motility, velocity, viability, total antioxidant capacity (TAC), and acrosome health (13, 14, 15). Also, a reduction in the rate of sperm lipid peroxidation membranes in infertile men had shown by 6 mM melatonin (16). According to a scrutinizes, melatonin was used in this study because of its antioxidant impacts.

As mentioned, studies have shown that ROS levels are higher in ATS volunteer than in normal men. On the other hand, in the sperm preparation process in ART, the sample stays in the laboratory for several hours, which increases oxidative stress and lipid

peroxidation. In this study, melatonin was used as an antioxidant to improve total and progressive motility, membrane integrity, and MDA of sperm in men with ATS.

Materials and Methods

Materials

L-carnitine (Merck, Germany), TPTZ reagent (Sigma Aldrich, St. Louis, MO, USA), Feso₄ (Merck KGaA, Darmstadt, Germany), hypoosmotic fluid and mOsm (Merck KGaA, Darmstadt, Germany), and Trichloroacetic acid, thiobarbituric acid, and Hydrochloric acid (15% w/v TCA, 0.375% w/v TBA and 0.25N HCl; Merck KGaA).

Patient selection and collection samples

The inclusion criteria were the volunteers who had asthenozoospermia and teratozoospermia simultaneously. Also, they had orderly intercourse (sexual) two or three times a week, but in no way were they able to fertilize. These patients had gone to the Omid Royan Infertility Center, Arak, Iran for treatment (July and December 2016). In no circumstance did the men expend any medicine including alcohol, tobacco, and vitamin supplements, as well as, they did not have prostatitis, azoospermia, and varicocele (exclusion criteria). After careful examination, 50 men between 20 and 40 years old were selected for this study. Next, 3-4 days after the last ejaculation, samples were taken into non-toxic containers. Finally, they were analyzed based on the World Health Organization (WHO) criteria.

Patient selection and collection samples

Based to WHO 2010 criteria, after sampling, for semen liquefaction, they were incubated (37°C for 30 min; 3). Then, each sample was divided into control groups and test group (L-carnitine) group. The next, after removing the

upper layer via centrifugation (10 min in 1000 g), samples were mixed by media (Ham's F-10), then they were embrocated twice. Then, for melatonin dose selection that was 6 mM according to past research (in vitro) under the impact of melatonin on sperm quality and quantity (16, 17). Ultimately, samples were incubated at 2, 4, and 6 hours.

Semen total antioxidant capacity by FRAP assay

Based on Benzie and Strain's method, the total antioxidants capacity of semen was measured by Ferric Reducing Antioxidant Power (FRAP). This method is according to the ability of plasma to reduce Fe³⁺ ion and convert to Fe²⁺ ion at acidic pH in the presence of 2,4,6-Tris (2-pyridyl)-s-triazine (Sigma Aldrich, St. Louis, MO, USA) reagent. The amount of plasma regenerative power is proportional to the concentration of this complex. At low pH, a blue complex is formed in the presence of TPTZ, which can be measured at a wavelength of 593 nm. Standard solution of ferrous sulfate (Feso₄, 7H₂O, Merck KGaA, Darmstadt, Germany) with 1mM concentration and various dilutions were prepared. Then the standard curve of ferrous sulfate was drawn in the FRAP experiment. The regression formula was obtained from the standard curve ($R^2 = 0.9975$, $y = 0.6333x + 0.1013$), which Y is equal to conservation. The regression formula from the standard curve was used to measure the FRAP of the ATS Men seminal plasma (18).

Sperm motility assay

Based on the WHO criteria, sperm motility evaluation was performed (1). Firstly, 10 ml of suspension (medium plus sperm) were laid on the lamel to the sperm mobility measurement under a light microscope (via 40X lens). Next, 10 scope (Includes not less

than 150 spermatozoa) were seen for every slide. Finally, the percentage of mobility was measured by recording of progressive, non-progressive, and immotile sperm.

Sperm membrane integrity by Hypo-osmotic swelling (HOS) test assay

According to Check's method, the HOS test for membrane integrity was performed (fig 1). The sperm are swollen with a normal plasma membrane by exposing to a hypoosmotic fluid through penetration of liquid into.

firstly, 30 μ l of the sperm suspension was gently co-incubated and then 300 μ l of 150 mOsm/kg hypoosmotic solution were simultaneously added. Then they were placed on a slide for one hour (at 37° C). Finally, 200 sperm were approximately counted in 5 fields of view via a contrast phase microscope. The percentage of the swollen sperms with warping tail (normal) was recognized and they were recorded as the response to a hypoosmotic solution (19).

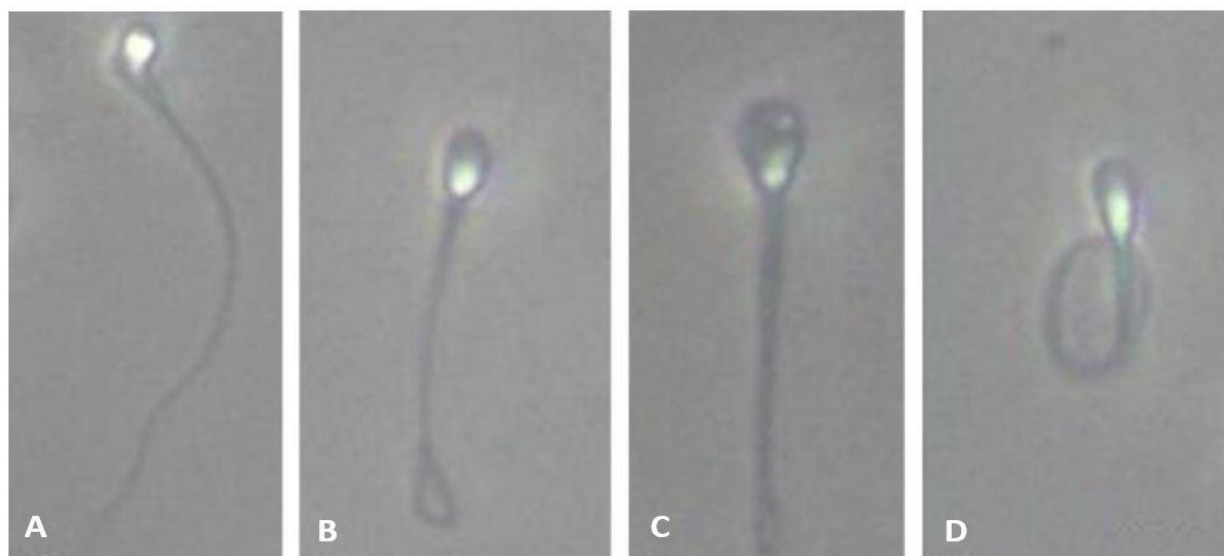


Figure 1. Sperm membrane integrity (SMI) via HOS staining at 2, 4, and 6 hours of incubation with the 6 mM melatonin, which shows different degrees of swelling of the human sperm tail (A-D). Sperm with a normal plasma membrane by the tail bent (B-D), and sperm with a damaged plasma membrane by the tail without swell (A, $\times 1000$ magnification).

MDA levels assay

According to Buege and Aust's method with some modifications, the HOS test was performed to measuring MDA levels (20). In this method, MDA reacts with thiobarbituric acid (TBA) and they produce a combination of orange color that set the stage for observing rays with a wavelength of about 532-535 nm. The first, TCA (Trichloroacetic acid)-TBA-HCL (Hydrochloric acid) reagent (15% w/v TCA, 0.375% w/v TBA and 0.25N

HCl; Merck KGaA) was blended (2:1) with sperm suspension. Then, the samples were placed in a boiling water bath for 15 min. Then, after taking the samples from the bath and cooling, they were centrifuged for 10 min. Next, after the supernatant removal, their adsorption was read via spectrophotometry in the presence of Blank. Finally, MDA concentration was calculated by the extraction coefficient that is $1.56 \times 10^5 \text{ M}^{-1} \text{ Cm}^{-1}$ (18, 19).

Ethical consideration

Current study was carried out in agreement with ethical laws under No: IRANKMU.REC.1394.37 of the medical ethics committee of Arak University of medical sciences. It should be noted that oral consent was received from all volunteers.

Statistical analysis

Statistically, measurements of data were carried out via SPSS software (Chicago, IL, USA; SPSS 16). Moreover, for the comparison between time intervals, repeated-measures ANOVA was used, also for

comparison between the control and L-carnitine groups, an independent t-test was used (data were significantly considered with means \pm SE and $P < .05$; 2).

Result

Total sperm motility assay

In the control group, the total sperm motility significantly decreased after 2 hours. Although the comparison of the total sperm motility in the melatonin group at different incubation times showed a significant increase at 6 hours compared to the control group ([fig 2](#)).

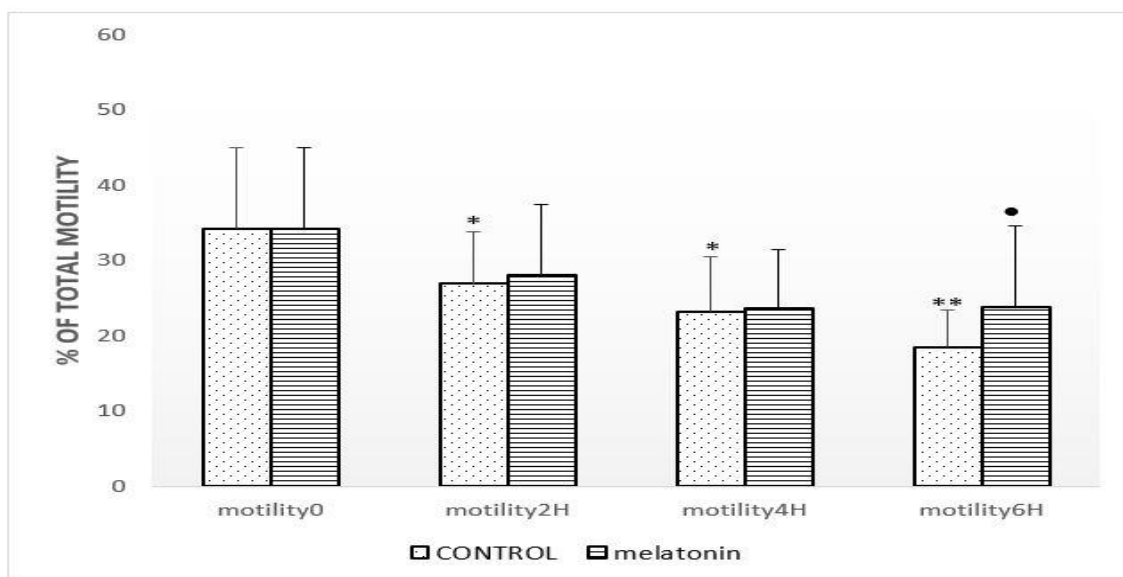


Figure 2. The effects of melatonin (6 mM) on the total sperm motility at 2, 4, and 6 hours of incubation. Mean \pm SD, $P < 0.05$. * $P < 0.048$ and ** $p < 0.001$, a significant difference in the control group at different time. ● $p < 0.001$, significant difference in the test group versus the control group at different time.

Progressive sperm motility assay

As illustrated in [fig 3](#), in the control group, the progressive sperm motility significantly decreased after 2 hours. However, the

comparison of the progressive sperm motility in the melatonin group at different incubation times showed a significant increase at 6 hours compared to the control group.

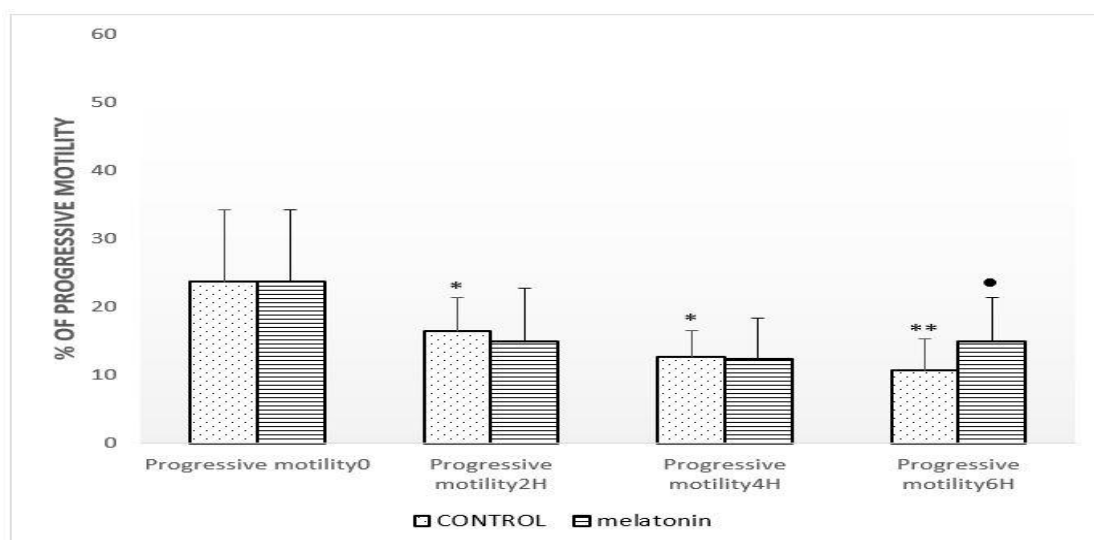


Figure 3. The effects of melatonin (6 mM) on the progressive sperm motility at 2, 4, and 6 hours of incubation. Mean \pm SD, $P < 0.05$. * $P < 0.046$ and ** $p < 0.029$, a significant difference in the control group at different time. • $p < 0.003$, significant difference in the test group versus the control group at different time.

Sperm membrane integrity (SMI) assay

The SMI significantly decreased after 4 hours in the control group. Although the

comparison of the SMI in the melatonin group at different incubation times showed a significant increase after 4 hours compared to the control group ([fig.4](#)).

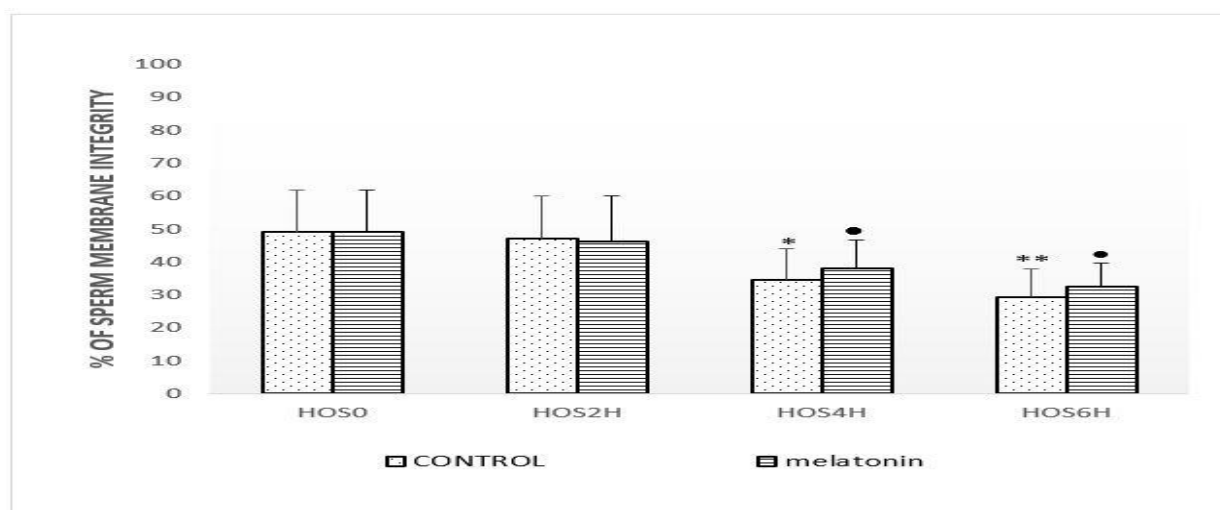


Figure 4. The effects of melatonin (6 mM) on the sperm membrane integrity (SMI) at 2, 4, and 6 hours of incubation. Mean \pm SD, $P < 0.05$. * $P < 0.028$ and * $P < 0.029$, a significant difference in the control group at different time. • $p < 0.045$, significant difference in the test group versus the control group at different time.

Sperm lipid peroxidation assay

As illustrated in [fig 5](#), in the control group, the MDA levels significantly increased at 6 hours. Although the comparison of the MDA

levels in the melatonin group at different incubation times showed a significant decrease after 4 hours compared to the control group.

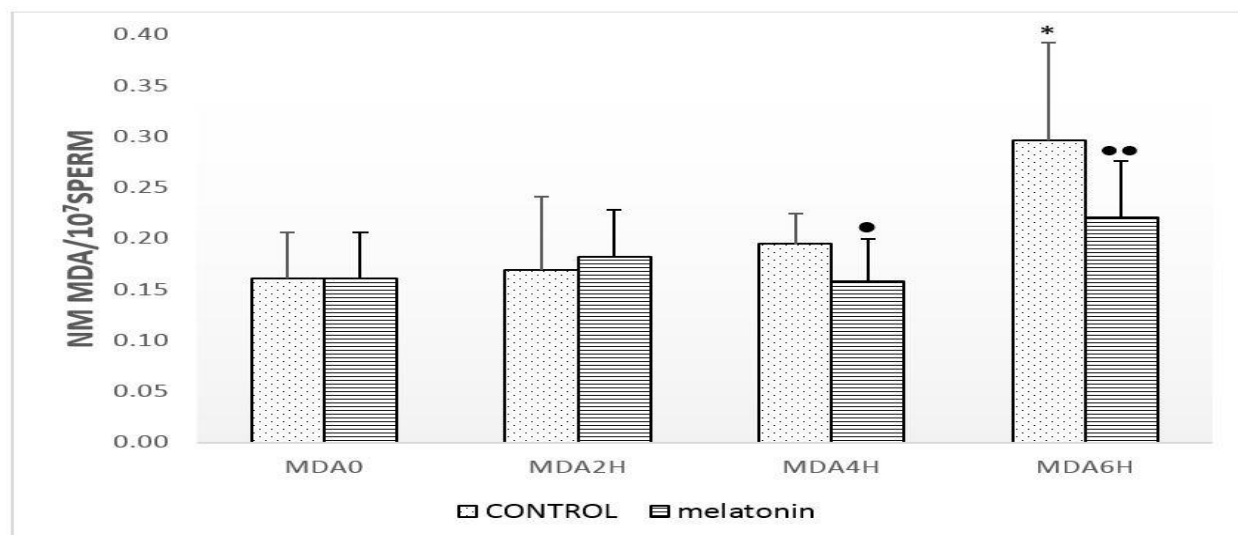


Figure 5. The effects of Melatonin (6 mM) on the sperm lipid peroxidation via MDA levels assay at 2, 4, and 6 hours of incubation. Mean \pm SD, $P < 0.05$. * $P < 0.036$, a significant difference in the control group at different time. • $p < 0.001$ and •• $p < 0.001$, significant difference in the test group versus the control group at different time.

Discussion

After melatonin incubation, control group with time crossing, total and progressive motility, and membrane integrity of sperm significantly increased, versus MDA levels significantly decreased.

There is no denying the fact that over time after sperm sampling can increase ROS and decrease antioxidant enzymes. Sperm of humans have low levels of antioxidant enzymes and cannot resist oxidative stress alone, as well as, the high concentration of ROS contributes to reduction in TAC levels, total and progressive motility, and membrane integrity of sperm (21, 22). The sperm membrane has a lot of fatty acids and can be stuffed of lipid peroxidation (22). A study has indicated of relevance between a decline in the viability of sperm and increase ROS levels (23). Beside, MDA causes damage to the sperm membrane, so that the transfer of ionic, as well as, ionic concentration gradient on both sides of the membrane is interrupted (24, 25). In summary, increasing oxidative stress and lipid peroxidation by wasting time after

semen sampling can cause lessen fluidity and flexibility of membranes (22, 24), which they can a contribution to reducing motility and increase membrane integrity of sperm. There is majority truth in the argument that melatonin has several antioxidant effects (13, 14), with this in impact for ATS men were carried out.

According to results, total and progressive motility, membrane integrity, and MDA levels of sperm were significantly compensated in the melatonin group compared to the control group at 6 hours of incubation.

Various results had reported on the effect of melatonin on sperm parameters. Ozgur et al had investigated the protective effect of melatonin (0.01, 0.1, and 1 mM) on the toxicity of titanium dioxide nanoparticles on the fish sperm that they had reported a significant increase in all sperm velocity indexes and antioxidant capacity, versus a decrease MDA (13). As well as, in another study, melatonin treatment (20 mg/kg) versus bisphenol were declared a significant increase

in motility, viability, and normal morphology (14). Hence our results in motility are consistent with previous studies. In the membranes of human sperm study, in the presence of melatonin (6 mM) were shown a significant reduction in lipid peroxidation versus a significant upgrade in acrosome health, TAC, SOD, and GPx regeneration (15, 16). It can be said that this antioxidant has reduced lipid peroxidation by increasing TAC as well as boosting the antioxidant system in sperm. Melatonin has 3 types of receptors including type 1 receptor (MT1), type 2 receptor (MT2), and type 3 receptor (MT3), which exerts its antioxidant effects through MT1 and MT2 receptors (26). Likewise, Melatonin has binding sites within the nucleus and also has amphiphilic properties that allow it to pass freely through cell membranes (27). Accordingly, Espino et al. had reported that the protective effect of melatonin on sperm quality and quantity by H₂O₂ is dependent on the MT1 receptor (26, 28). Oxidative stress can impair membrane integrity (24, 25) and melatonin with its antioxidant effect (26) can protect membranes, so an increase in HOS can be justified. The study of Ghafarizadeh, which was performed using similar materials and methods, confirm the data of the present study (29). Also, other experimental researches have shown that antioxidants therapy in asthenotratospermic men (in vitro) can affect sperm quality (30-33). Moreover, the limitation of this study was the lack of follow-up of most patients for in vitro fertilization. In that case, the fertilization rate in these patients can also be examined.

Conclusion

In conclusion, according present results it seems that melatonin make a contribution to boosts progressive motility, membrane

integrity of sperm in ATS men. Melatonin with its antioxidant role paves the way for defeating lipid peroxidation and oxidative stress.

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Abbreviations:

ART: assisted reproductive techniques

ATS: asthenoteratozoospermia

MDA: Malondialdehyde levels

ROS: reactive oxygen species

NADPH: Nicotinamide adenine dinucleotide phosphate

PUFA: polyunsaturated fatty acids

SOD: superoxide dismutase

GPx: glutathione peroxidase

CAT: catalase

TAC: total antioxidant capacity

WHO: World Health Organization

FRAP: Ferric Reducing Antioxidant Power

HOS: Hypo-osmotic swelling

SMI: Sperm membrane integrity

TBA: thiobarbituric acid

TCA: Trichloroacetic acid