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RESEARCH

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The magnetic cell separation method reveals protective effect of melatonin on human spermatozoa from peroxide-induced apoptosis

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Abstract

Background The selection of non-apoptotic sperm is related to successful fertilization. This study investigated the protective effects of melatonin and the role of the MACS (magnetically activated cell sorting) method to prevent oxidative damage in vitro and improve sperm quality parameters such as viability and DNA integrity.

Materials and methods Semen analysis was performed on 20 different eligible donors participating in the study. Sperm viability and concentration were checked at time of 0 (0 h). In order to conduct more studies after 24 h (24 h), the remaining sperm suspension was divided into a control group and six experimental groups. DNA fragmentation was assessed using the TUNEL assay.

Results The treatment of human spermatozoa with 100 µM hydrogen peroxide for 24 h induced a significant increase in phosphatidylserine externalization and significantly increases apoptotic sperm ($p \leq 0.001$). TUNEL analysis of human sperm pretreated with 100 µM hydrogen peroxide for 24 h showed that the percentage of sperm with fragmented DNA was significantly reduced after sorting by MACS ($P \leq 0.001$). However, pretreated human sperm with 1 µM melatonin for 24 h could effectively maintain sperm motility and progressive motility.

Conclusions Pretreated human spermatozoa with 1 µM melatonin for 24 h could be effective for maintenance of sperm motility and progressive motility. Although 100 µM hydrogen peroxide-treated sperm were used, MACS was used to retain the appropriate sperm and select high-quality sperm.

Keywords Apoptosis, Human spermatozoa, Magnetic cell separation, Melatonin

Introduction

Oxidative stress is the main reason of sperm DNA fragmentation, which is caused by endogenous and exogenous factors. Although sperm function requires proper reduction and oxidative balance, antioxidants play a protective role in reducing oxidative stress [1–3]. Researchers have found that reactive oxygen species (ROS), especially superoxide anion, hydroxyl radicals and hydrogen peroxide, are normal by-products in various metabolic and physiological processes, but excessive production can lead to oxidative stress [4, 5].

In mammals, the pineal gland synthesizes melatonin (*N*-acetyl-5-methoxytryptamine) during the night in response to environmental changes in light levels [6]. In recent years, several reports have appeared on the beneficial effects of melatonin on sperm motility as an antioxidant [7–9]. Although melatonin's role in the modulation of physiological processes is very complex, one of the

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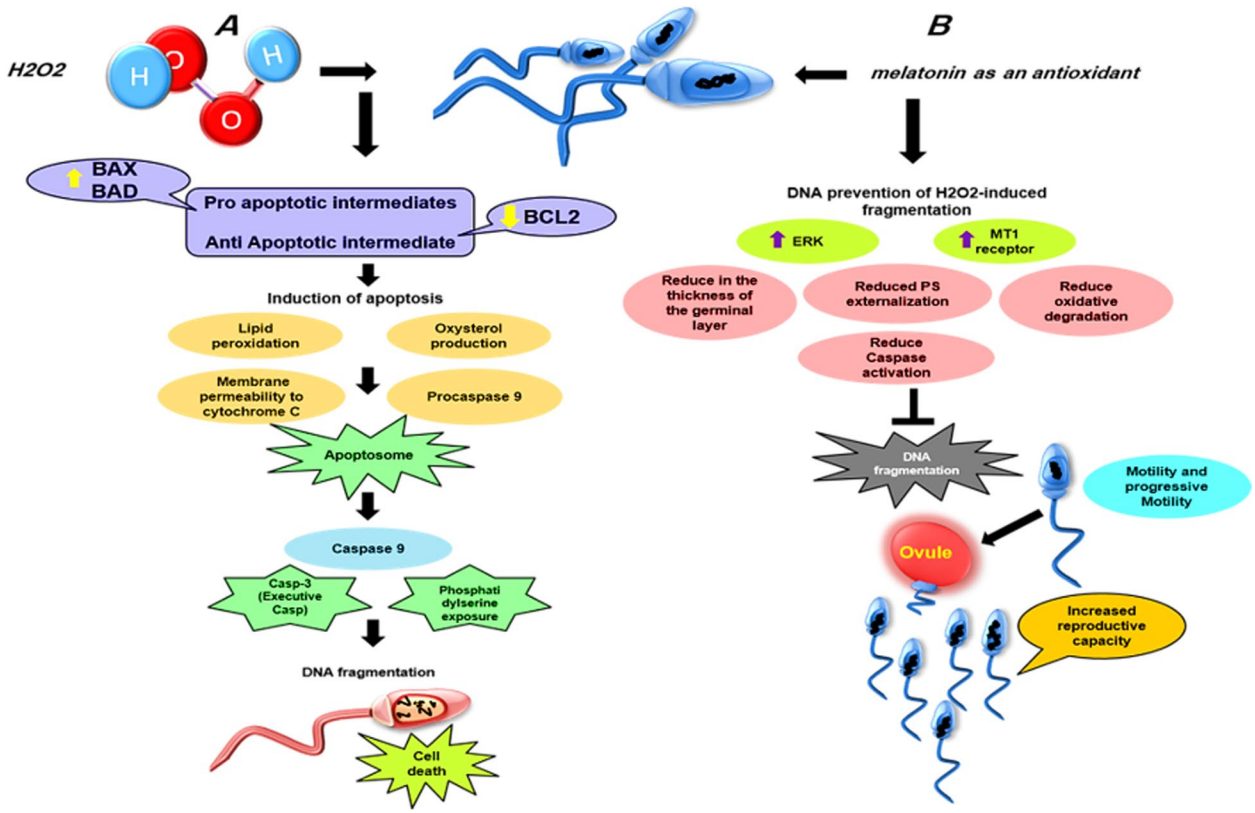

Fig. 1 **A** In the presence of H_2O_2 as a ROS in the environment, increasing pre-apoptotic factors and decreasing anti-apoptotic factors will increase the production of oxysterol production. Thus increasing the membrane's permeability to cytochrome C and producing procaspase 9, one of these factors leads to the production of apoptotic bodies, and ultimately the production of caspase 3 will be carried out as a caspase and DNA fragmentation and ultimately destroy the sperm nucleus. **B** In the presence of melatonin as an antioxidant in the environment, by increasing ERK and MT1 receptor, activity of caspases and oxidative degradation reduced and it leads to reduction of the germinal layer and then blocking the DNA fragmentation pathway, along with increasing sperm fertility capacity, such as improvement morphology and movement, it will increase the chance of fertilization

metabolites of melatonin is the cyclic 3-hydroxymelatonin that results from the reaction between OH and melatonin [10].

The intrinsic pathway of apoptosis starts with mitochondrial alterations [11]. Cells react to apoptotic stimuli and several proteins are released from the mitochondria into the cytoplasm [11] and this activates the caspase-9 pathway [12]. Activation of caspase-9 triggers a cascade of caspase activation, including caspase 3, which promotes cellular apoptosis.

Hydrogen peroxide (H_2O_2) has been implemented in literature and may induce a significant increase in caspase-9 and caspase-3. Pretreatment of ejaculated human spermatozoa with melatonin in a dose-dependent way has been shown to reduce caspase activation [13]. However, antiapoptotic effects of melatonin in ejaculated human sperm may involve membrane melatonin receptor MT1 by survival-promoting pathway of extracellular signal–regulated kinase (ERK). Moreover, melatonin has a role in the protective actions in ejaculated human spermatozoa by prevention of H_2O_2-induced DNA fragmentation [14] (Fig. 1). In year 2011, Researchers demonstrated that melatonin has the anti-apoptotic actions in human spermatozoa clearly dose dependent. In this study, melatonin with high dose (1 mM) had the most of effect for prevent apoptotic events [15]. In recent years, the ART Centers have evaluated methods for identifying apoptotic sperm using magnetic cell sorting (MACS) as a reproductive technology, which can be helpful in orienting patient treatment plans. Magnetic cell sorting (MACS) using annexin V-conjugated microbeads eliminates apoptotic spermatozoa with annexin V-positive. MACS tools are based on the externalization of phosphatidylserine residues and also displayed the lowest percentage of capsase 3 activation as well as the highest mitochondrial membrane integrity with high motility in Annexin V-negative [16, 17].

Sperm DNA integrity is very important for transmission of genetic material to the oocyte. Strand breaks of sperm DNA have been investigated by several techniques

such as; terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL), sperm chromatin structure assay (SCSA), acridine orange staining (AOT) and commet assay [18]. TUNEL method can detect single and double strand breaks [19]. Therefore, the selection of nonapoptotic spermatozoa is one of the correlatives for achieving optimal conception rates following ART. This study will further investigate the ability of melatonin with MACS, to protect against peroxidative damage in vitro and increase sperm quality parameters such as motility and DNA integrity.

Materials and methods

Patient selection

The procedure of this study was approved by the gametogenesis research center in Kashan. Signed informed consent for study participation was obtained from all participants. The basic semen analysis procedure in this study was performed according to the WHO guidelines [20, 21].

Semen sample collection and preparation

A total of 20 semen samples with age 28–36 years were obtained from healthy male partners for analysis according to the World Health Organization criteria (WHO, 1999) [15, 22, 23]. Following a period of 3–5 days of sexual abstinence, fresh semen samples were collected by masturbation into sterile plastic jars on the day of the analysis [23]. After liquefaction at room temperature (25 °C) within 1 h of ejaculation, all samples were analyzed by a computer-assisted sperm analysis (CASA) system to quantify sperm count, motility parameters, and morphometric features. The ejaculated spermatozoa with a suitable concentration (20 million per mL after 3–4 days of sexual abstinence) was checked for motility and concentration and prepared by density gradient centrifugation ($300 \times g$ at 20 min) using 40/80 gradient (Cook medical company) [24] and the pellet resuspended with 4 ml Potassium Simplex Optimized Medium (KSOM-H; Merck, MR-121-D) handling medium in a new conical tube [25]. One aliquot (0.5 ml) of the sperm suspension was subjected to MACS. Motility and concentration was checked, and a sample taken for tunnel before and after MACS. The remaining sperm suspension was divided into 6 tubes [2 × control, 2 × peroxide (addition of 100 µM peroxide), 2 × peroxide/melatonin (addition of 100 µM peroxide and 1 µM melatonin)].

TUNEL assay (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate-biotin nick-end labeling assay)

DNA fragmentation was evaluated using the TUNEL assay [26] with some modifications. Briefly, 1 million of spermatozoa from sample was added to 1 ml phosphate-buffered saline (PBS) followed by centrifuged at $300 \times g$ for 5 min and the pellet was resuspended with 1 ml of 3.5% formaldehyde in PBS and incubated for 1 h, washing through centrifugation (300 G/1800 rpm) for 5 min. Resuspend pellet with 300–400 µl of PBS allows slides to reach room temperature gradually. Slides are stored at 4 °C in darkness.

TUNEL staining

The spermatozoa was treated with a solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 15 min at 4 °C. Sample was washed twice with PBS for 3 min each time and then dried area surrounding it. Sperm was treated with a solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 15 min at 4 °C. The sample was then washed twice with PBS for 3 min and then the area surrounding the sample was dried.

A 35-µl TUNEL mixture consisting of terminal deoxynucleotidyl transferase (TdT) and fluorescein dUTP was added to the same volume of each sample for 5 min in to 4 °C. The samples were incubated for 60 min at 37 °C in a moist chamber in darkness, washed two times with PBS 3 min each time. The area surrounding the sample was dried and stained with 1 µg/ml propidium iodium (PI) in PBS for 3 min in darkness, and washed two times in PBS (3 min in each wash) and the area surrounding the sample was dried. The slides were mounted with Vecta shield and sealed with nail polish and then analyzed using fluorescence microscopy (Nikon Reading Using Olympus Cellsens dp70) with blue wavelength (460–490 nm). At least 400 cells were counted. The presence of green fluorescent signals was regarded as positive (PI stained all sperm red).

MACS procedure

Spin samples followed by centrifuged for $400 \times g$ during 6 min, and resuspend pellet in 0.5 ml MACS buffer (miltenyi biotec, bergisch gladbach, Germany), and spermatozoa was incubated with 100 µl MACS microbeads (Miltenyi Biotec) per 10 millions sperm annexin V-conjugated microbeads and put in mixer shaking at 34 °C for 20 min. After washing the MS column (MiniMACS) with 500 µl of MACS buffer, the column was placed in the MiniMACS magnetic separation unit with a sample collection tube placed below, 500 µl buffer and 500 µl sperm/bead suspension was added. Drop wise simultaneously and the collected sample was then centrifuged at $400 \times g$ for 6 min, and the pellet re-suspend with 500 µl KSOM handling media. Sperm motility was evaluated.

Statistical analysis

Data were analyzed using GraphPad InStat (Version 3.10) differences between treated group and control groups at 0 h were compared by the independent-samples t-test. Collected data were presented as percent and $P<0.05$ was considered to be significant.

Results

The treatment of human spermatozoa with 100 µM hydrogen peroxide for 24 h induced a significant increase in PS externalization and significantly increases apoptotic sperm ($p\leq0.001$). Results of the TUNEL assay in pretreatments of human spermatozoa with 100 µM hydrogen peroxide for 24 h revealed that the percentage of sperm with fragmented DNA was significantly lower in the sperm after sorting MACS ($P\leq0.001$ versus control) (Table 1).

The pretreatment of human spermatozoa with peroxide/melatonin (addition of 100 µM peroxide and 1 µM melatonin) or 1 µM melatonin for 24 h could reduce DNA damage, which indicates that melatonin is able to avoid DNA damage in ejaculated human spermatozoa (Fig. 2).

MACS separation method results in removal of spermatozoa with caspase activated. The treatments of human spermatozoa with 100 µM hydrogen peroxide for 24 h with MACS were removed apoptotic sperm (sperm DNA fragmentation were removed). Following the pretreated human spermatozoa with hydrogen peroxide for 24 h, percentage mean of sperm motility and progressive motility were significantly reduced ($P\leq0.001$ versus control) (Table 2).

Following the pretreated human spermatozoa with hydrogen peroxide for 24 h, percentage mean of sperm motility and progressive motility were significantly reduced ($P\leq0.001$ versus control). Although the treatments of human spermatozoa (except in hydrogen peroxide treated group), for 24 h affected by apoptosis, there is no significantly difference between them. But, sperm motility and progressive motility had significantly differences between each group in comparison with fresh sample ($p\leq0.0001$).

Also, pretreated human spermatozoa with 1 µM melatonin for 24 h could be effective for maintenance of sperm motility and progressive motility. Despite the sperm was treated with 100 µM hydrogen peroxide sorting using MACS retain appropriate spermatozoa and select sperm with good quality. Our findings showed that motility and progressive motility characteristics of the sperm samples were not affected after 24 h if pretreated with melatonin.

Table 1 The percentage mean of spermatozoa in 0 h and 24 h within the whole sample and the annexin V MACS–generated subpopulations in samples

TUNEL assay	Control	Hydrogen peroxide	Hydrogen peroxide with MACS	Melatonin	Melatonin with MACS	Hydrogen peroxide and melatonin	Hydrogen peroxide and melatonin with MACS
0 h	2 ± 0.1						
24 h	2 ± 0.1	26 ± 0.15**	1 ± 0.13	2 ± 0.12	2 ± 0.14	2.5 ± 0.1	2 ± 0.2
P value		0.001					

All values are given as mean ± SD. *P* values < 0.05 were considered statistically significant

** $p\leq0.001$ (Comparison with the control group in the 24-h group)

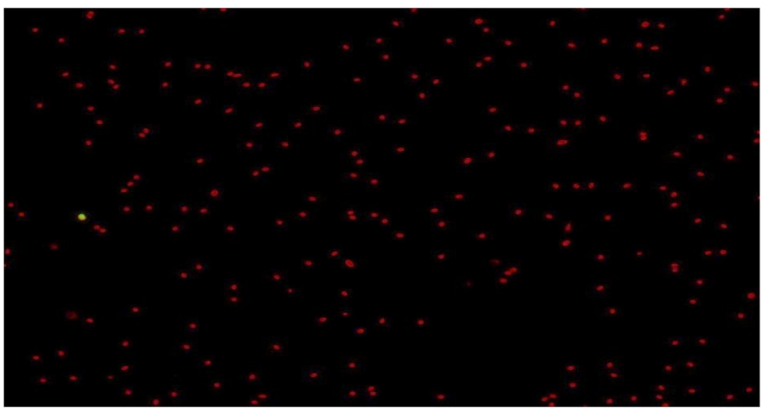

Fig. 2 The picture indicates DNA damage (green) and normal (red) human spermatozoa

Table 2 The percentages mean of sperm motility and progressive motility in 0 h and 24 h within the whole sample with or without MACS

Motility and progressive motility		Control	Hydrogen peroxide	Hydrogen peroxide with MACS	Melatonin	Melatonin with MACS	Hydrogen peroxide and melatonin	Hydrogen peroxide and melatonin with MACS
0 h	Motility	90 ± 1.2						
	Progressive motility	85 ± 1.5						
24 h	Motility	56.5 ± 2	27.5 ± 1.5**	33 ± 0.8**	48 ± 2.1	90 ± 2.8	51 ± 2.2	90 ± 2.6
	Progressive motility	37.5 ± 2.2	12.3 ± 1.2**	13 ± 1.1**	33 ± 1.95	85 ± 1.67	12 ± 2.1	80 ± 1.7
P value			0.001	0.001				

Values are expressed as mean ± SD. *P* values < .05 was considered statistically significant

** $p \leq 0.001$ (Comparison with the control group in the 24-h group)

Pretreatments of human spermatozoa for 24 h revealed that the percentage mean of sperm motility and progressive motility were significantly reduced by 100 µM hydrogen peroxide and MACS with hydrogen peroxide, versus zero hour in control group ($P < 0.001$). However, with both melatonin and MACS did not affect sperm motility. MACS separation method results in removal of apoptotic spermatozoa did not show significantly difference between peroxide/melatonin and MACS in comparison with control group. Storage time significantly reduced sperm motility and progressive motility. The use of MACS will select only sperm with intact which results in high percentage motility and progressive motility.

Discussion

Our results showed protective effect of melatonin in MACS method, which produced high-quality sperm compared to control group. Following MACS separation, as mentioned before in the annexin V-negative fraction, apoptotic sperm will be retained and only non-apoptotic spermatozoa could pass through the sorting. Said et al. (2005) and Berteli et al. (2017) demonstrated that that sperm motility can improve by MACS, which is in agreement with present study [27, 28]. In the current study, the incidence of DNA fragmentation in non-apoptotic sperm was lowest after the MACS method. However, this fragmentation did not match with the percentage of motile spermatozoa or other markers of apoptosis. This may be participated to the fact that DNA integrity is an independent factor of sperm quality that is not correlated with other sperm parameters [29]. Moreover, markers of apoptosis do not always appear simultaneously [30, 31].

The selection of nonapoptotic spermatozoa is one of the correlatives for achieving optimal conception rates following ART. Several studies expressed the beneficial effects of melatonin on spermatozoa as an antioxidant [32–34]. Although the density gradient centrifugation is not sufficient for identification of apoptotic markers in spermatozoa [35, 36], novel tools such as electrophoretic separation and MACS have demonstrated commendation results for the separation of spermatozoa with good quality [27, 37].

Espino et al. (2011) reported that human spermatozoa is protected from apoptosis via melatonin receptor and extracellular signal–regulated kinase-mediated pathways. They found the stimulation with melatonin triggers a set of events culminating in cell death prevention in ejaculated human spermatozoa. The results of these researchers were disagreement with our data because their study did not work MACS method [15]. MACS using annexin V-conjugated microbeads eliminate apoptotic spermatozoa with annexin V-positive. MACS tools are based on the externalization of phosphatidylserine (PS) residues and also displayed the lowest percentage of capsase 3 activation as well as the highest mitochondrial membrane integrity with high motility in annexin V-negative [38]. Therefore, the selection of nonapoptotic spermatozoa is one of the correlatives for achieving optimal conception rates following ART. MACS paired with annexin V microbeads have been shown to effectively isolate spermatozoa with activated caspases (apoptotic spermatozoa) [39, 40]. In this matter, MACS may suppose that as a specific molecular preparation technique that supplements conventional sperm preparation.

Externalization of phospholipid is the first process of apoptosis. Depending on Ca2+, PS has an excessive affinity for annexin V, which is 35–36 kDa phospholipid binding protein showing excessively selective binding to PS. Annexin-V cannot pass the intact sperm membrane [41]. Therefore, annexin V is able to recognize cells with membrane abnormality at the beginning stage [42]. In this subject, annexin binding and finally magnetic separation 2 fractions are obtained: annexin-negative (unlabeled-intact membrane; nonapoptotic) and annexin positive (labeled-changed membrane; apoptotic). There

is closely adherence detected between PS externalization, caspase 3 activation, and MMP [43].

Increased sperm chromatin decondensation in selected nonapoptotic spermatozoa of patients with male infertility was evaluated by Grunewald et al. (2009). These researchers determined that sperm motility and progressive motility after MACS are reduced compared to sperm after DGC (density gradient centrifugation) due to the increased centrifugation steps. Moreover, the sperm chromatin decondensation rate (a substitute marker for fertilization capability) after MACS still increase [44]. They indicated sperm chromatin decondensation that is inconsistent with our findings. The efficiency of MACS for subgroups of infertile couples needs further investigation and elucidation. Sperm motility, but not sperm morphology or concentration, is the sole basic sperm parameter correlated with early apoptosis markers (AV, PI2) and DNA fragmentation (TUNEL) [16].

Conclusion

The results of the current study suggest that determining spermatozoa with apoptotic markers may facilitate developing the optimal treatment plan for patients with UI (unexplained infertility) who had failed IUI on two attempts. Furthermore, elimination of spermatozoa with apoptotic markers may improve the fertilization potential of sperm and possibly the outcome of further ART cycles. Therefore, the pretreatment of human spermatozoa with peroxide/melatonin or melatonin for 24 h could reduce DNA damage and increase sperm motility, which indicates that melatonin will able to avoid DNA damage and improve motility in ejaculated human spermatozoa. It is suggested that MAPK pathway be investigated in future studies. This pathway induces apoptotic signal and leading to DNA strand breaks, and also evaluates the direct effect of oxidative stress on spermatozoa that results in Sperm DNA fragmentation (SDF).

Abbreviations

ART	Assisted reproductive technology
MACS	Magnetic-activated cell sorting
ROS	Reactive oxygen species
ERK	Extracellular signal–regulated kinase
H_2O_2	Hydrogen peroxide
PS	Phosphatidylserine
SCSA	Sperm chromatin structure assay
TUNEL	Terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling
AOT	Acridine orange staining
PBS	Phosphate-buffered saline
PI	Propidium iodium
KSOM	Potassium simplex optimized medium
UI	Unexplained infertility
IUI	Intrauterine insemination

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Authors' contributions

HHB and HHK provided direction and guidance throughout the preparation of this manuscript. HHB and ASS conducted the literature and drafted the manuscript. Other authors reviewed the manuscript and made significant revisions on the drafts. All authors read and approved the final version.

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Availability of data and materials

The dataset used in this study is available with the authors and can be made available upon request.

Declarations

Ethics approval and consent to participate

All procedures performed in the study involving human were in accordance with the 1964 Helsinki Declaration and ethical standards of the institutional and national research committee of Kashan University of Medical Sciences. The protocol was approved by the research committee of Kashan University of Medical Sciences, Kashan, Iran.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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