Testicular malondialdehyde level in azoospermic patients

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Objective
This study aimed to evaluate malondialdehyde (MDA) levels in human testicular tissue of azoospermic patients.

Design
This was a cross-sectional study.

Patients and methods
Azoospermic patients with obstructive (OA) and nonobstructive (NOA) were subjected to surgical sperm retrieval with needle aspiration using a 14 G cannula. Assay of MDA level was performed using colorimetric methods in testicular samples. In addition, assessment of the number of retrieved sperm in the samples was performed.

Results
The study included 21 OA (group A), 16 positive NOA (group B with positive sperm retrieval), and 21 negative NOA (group C with negative sperm retrieval). The MDA level was significantly higher in the positive and negative NOA (31.50 ± 15.81 nmol/g) (40.38 ± 14.42 nmol/g) groups than the OA group (21.33 ± 9.61 nmol/g) ($P = 0.043$, $P = 0.000$), respectively. The MDA level correlated negatively with the mean number of retrieved sperm (in groups with positive sperm retrieval A and B) (r=–0.261, $P = 0.048$, r=–0.402, $P = 0.002$), respectively.

Conclusion
Men with NOA seem to have increased basal testicular oxidative stress compared with those with OA as indicated by increased MDA levels in testicular samples. The MDA level correlated negatively with sperm concentration; thus, it may be considered a predictor marker for sperm retrieval in NOA cases.

Keywords:
azoospermia, malondialdehyde, oxidative stress

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Introduction

Oxidative stress is an important factor for the development of male infertility because of the very high rate of cell division and mitochondrial oxygen consumption in testicular tissue as well as comparably higher levels of unsaturated fatty acids in this tissue than in other tissues. Moreover, the level of oxygen pressure is low because of the weakness of the testicular artery; therefore, there is a severe cell competition for oxygen [1].

Researchers believe that the sperm is more susceptible to oxidative stress than other cells because of the limited amount of cytoplasm in a mature sperm and the concentration of antioxidants in the sperm as well as high levels of unsaturated fatty acids in the sperm structure. The free radical (FR)-induced oxidative stress contributes significantly toward producing and increasing abnormal sperm and decreasing sperm count and transformation and fragmenting sperm DNA [2].

Increased production of reactive oxygen species (ROS) induces lipid peroxidation in spermatozoa [2]. Lipid peroxidation can be described generally as a process in which oxidants such as FRs or nonradical species attack lipids containing carbon–carbon double bond (s), especially polyunsaturated fatty acids (PUFAs) [3].

Malondialdehyde (MDA) is an end-product of lipid peroxidation induced by ROS, which can cause cross-linking in lipids, proteins, and nucleic acids, and can be considered a major manifestation of oxidative stress [4]. It is generated by the decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes [5]. Its level in seminal plasma has been reported to correlate negatively with sperm viability, sperm motility, sperm morphology, and sperm concentration, whereas MDA levels have been reported to correlate positively with acrosome anomalies and the presence of residual cytoplasmic droplets [6]. MDA molecules cause an asymmetric distribution of

lipid membrane components by penetrating into the cell membrane structure. It is used as a marker (biomarker) to determine the rate of oxidative damage to lipids. It is noteworthy that currently, the damage caused by lipid peroxidation is the most important factor for testicular dysfunction [2]. Therefore, MDA should be an alarm bell for the diagnosis and treatment of diseases in the acute phase and to possibly prevent infertility [6].

Azoospermia accounts for 10–15% of male infertility cases and generally affects 1% of the male population [7]; it is defined as the absence of spermatozoa in the ejaculate verified in at least two samples, including assessment of the centrifuged pellet [8], and is classified as obstructive (OA) or nonobstructive (NOA) [9].

OA azoospermia occurs as a result of a blockage in the epididymis, vas deferens, or ejaculatory ducts in which spermatogenesis is normal [10], whereas NOA is caused by intrinsic testicular disease (primary testicular failure) or secondary testicular failure (endocrinopathy or other conditions suppressing sperm production) [9].

The advent of intracytoplasmic sperm injection has revolutionized the management of male infertility and made it possible for even an azoospermic man to father a child using sperm retrieved from his epididymis or testis [11].

The success rate of sperm retrieval depends on the type of azoospermia and the retrieval technique used. The sperm-retrieval rate is high in OA cases compared with NOA. In men with OA, sperm-retrieval rates are close to 100% even after repeated procedures [12]; the greater challenge is sperm retrieval in men with NOA. Only some of these men will have a few sperm in the testes, and the distribution of these scanty sperm may be multifocal or very localized, necessitating different sperm-retrieval techniques [13].

Aim

The aim of our study is to evaluate the MDA level in human testicular tissue of azoospermic patients.

Patients and methods

This is a cross-sectional study that was carried out at the Andrology Unit, Dermatology, Venereology and Andrology Department, Assiut University Hospitals, in collaboration with the Biochemistry Department, Assiut Faculty of Medicine, in the period from October 2016 to July 2018.

This study was approved by the Ethical Committee at Assiut Faculty of Medicine. Privacy and confidentiality of all data were assured.

Patients were selected from among infertile men with repeated azoospermia undergoing preparation for surgical sperm retrieval (SSR) before intracytoplasmic sperm injection. Before inclusion in the study, the participants underwent thorough assessment of history, clinical examination, semen analysis, hormonal assay (follicle-stimulating hormone and luteinizing hormone), and sonographic imaging (scrotal and transrectal). Patients with pyospermia, systemic diseases, cryptorchidism history, smoking, and antioxidant intake within 3 months were excluded.

Testicular surgical sperm retrieval

Testicular SSR was performed by fine-needle aspiration using a 14-G cannula (INPHARVEN; Farcomake for Advanced Medical Industries, New Burg El Arab, Egypt) that was passed directly into the testis under spermatic cord infiltration anesthesia using xylocaine. Once the needle was in the testicular substance, a strong negative pressure was exerted using a 10-ml attached syringe (B. Braun Melsungen AG, Melsungen, Germany). The cannula was moved within the substance of the testis until small aliquots of aspirated testicular tissue could be observed to appear within the microtubing set. The needle was then withdrawn slowly from the testis through the scrotal skin and a core of attached testicular tissue was cut off, on withdrawal, from the skin surface.

Sample preparation and microscopic examination of testicular aspirates

The testicular tissue was collected in a falcon 35-mm Petri dish containing 2 ml of culture media (Ferticult Flushing Medium containing bicarbonate, physiologic salts, glucose, lactate, and human serum albumin). Preparation was carried out in another dish containing 2 ml of the same medium by milking the tissue with two needles attached to a 1-ml syringe until good mixing yielded a suspension. Then, a drop from the suspension was examined on the glass slide under a light microscope with magnification power × 400 for the detection of sperm in 20 HPF.

Measurement of the malondialdehyde level

An MDA level assay was used for the assessment of positive and negative sperm samples; a colorimetric method was used. A chemical kit was used, manufactured by Biodiagnostics diagnostics and research reagents (catalog no. MD 25 29; Dokki, Giza, Egypt), based on the reaction between thiobarbituric

acid with MDA in acidic medium at a temperature of 95°C for 30 min, forming a thiobarbituric acid-reactive product. The absorbance of the resultant pink product was measured at 534 nm. The color intensity of the final product is directly proportional to the concentration of MDA in the initial reaction. The concentrations of MDA in different samples were calculated using the absorbance of the MDA standard, whose concentration was 10 nmol/ml using the following formula [13,14]:

A sample / A standard × 10 nmol/ml.

MDA concentrations were quantified in reference to protein concentrations in a ground sample by dividing the MDA concentration of each sample by the protein concentration of that sample, and the final MDA concentration in the sample is referred to as nmol/g protein. The protein concentration was measured by a colorimetric method using a chemical kit manufactured by Spectrum Diagnostics, an Egyptian company for Biotechnology (Ref 001,310; Obour City, Cairo, Egypt), based on the Biuret reaction. In an alkaline medium, the copper reacts with the peptide bonds of proteins to form the characteristic pink to purple biuret complex. The color intensity is directly proportional to the protein concentration. It is determined by measuring the increase in the absorbance at 546 nm. The concentrations of protein in different samples were finally calculated using the absorbance of the protein standard, whose concentration was 6 g/dl using the following formula [15]:

A sample / A standard × 6 g/dl.

Statistical analysis

Data entry and data analysis were carried out using SPSS, version 19 (Statistical Package for the Social Science) IBM Corp., Armonk, New York. Data were presented as number, percentage, mean, median (interquartile range), and SD. The χ^2 test and Fisher exact test were used to compare between qualitative variables. The Mann–Whitney test was used to compare between two quantitative variables in case of nonparametric data. Spearman's correlation was carried out to measure the correlation between quantitative variables. Medcalc was used to calculate the sensitivity, specificity, positive, and negative predictive values, and receiver operating characteristic (ROC) curves. *P* value was considered statistically significant when less than 0.

Results

Fifty-eight infertile azoospermic men were included in the study. Their age ranged from 18 to 45 years. On the basis of clinical data and previous investigations, they were divided into OA azoospermia patients (group A) (21 participants) and NOA azoospermia patients (37 participants), who were subdivided according to the SSR outcome into a positive NOA group B (16 participants with positive sperm retrieval) and a negative NOA group C (21 participants with negative sperm retrieval). Descriptive data of the patients are shown in Table 1.

Comparison between the mean MDA level in the testicular samples among the groups studied showed that the mean MDA level was very significantly higher in the positive and negative NOA groups than the OA group (P = 0.053, 0.000), respectively, with no significant difference between both NOA groups as shown in Table 2.

The correlation between the mean MDA level and the mean sperm concentration per 20 HPF showed a very highly significant negative correlation between the mean MDA level and the mean sperm concentration/20 HPF (r=–0.402)) Fig. 1).

Table 1 Descriptive data of the participants

	Group A (*n*=21)	Group B (*n*=16)	Group C (*n*=21)	*P*[a]	*P*[b]	*P*[c]
Age (years)						
Median (IQR)	32 (26.0-35.0	30 (25.5-38.5)	30 (27.0-32.0)	0.667	0.240	0.854
Residence [*n* (%)]						
Rural	13 (61.9)	10 (62.5)	14 (66.7)	0.970	0.747	0.793
Urban	8 (38.1)	6 (37.5)	7 (33.3)			
Marital status						
Single	4 (19.0)	3 (18.8)	5 (23.8)	1.000	1.000	1.000
Married	17 (81.0)	13 (81.3)	16 (76.2)			
Duration of infertility (years)						
Median (IQR)	3 (1.0-4.0)	4 (1.0-4.0)	4 (1.0-5.0)	0.758	0.801	0.461
Sperm concentration per 20 HPF						
Mean±SD	23.57±6.15	14.00±6.18	0.00±0.00	0.000*	0.000*	0.000*
Median (range)	20 (20-40)	13 (1-25)	0 (0-0)			

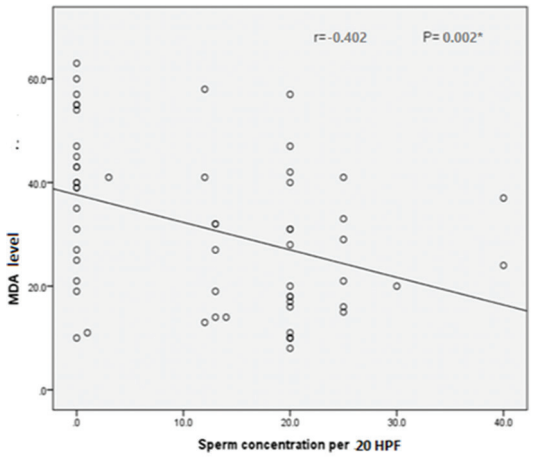
[a]Comparison between groups A and B. [b]Comparison between groups B and C. [c]Comparison between groups A and C. IQR, interquartile range. *Significant *P* value less than 0.05.

Table 2 Comparison between the mean malondialdehyde level in testicular samples among the groups studied

MDA level (nmol/g)	Group A (*n*=21) (mean±SD)	Group B (*n*=16) (mean±SD)	Group C (*n*=21) (mean±SD)	P^a	P^b	P^c
Testicular samples						
Mean±SD	21.33±9.61	31.50±15.81	40.38±14.42	0.043*	0.125	0.000*
Median (range)	20 (8-41)	32 (11-58)	40 (10-63)			

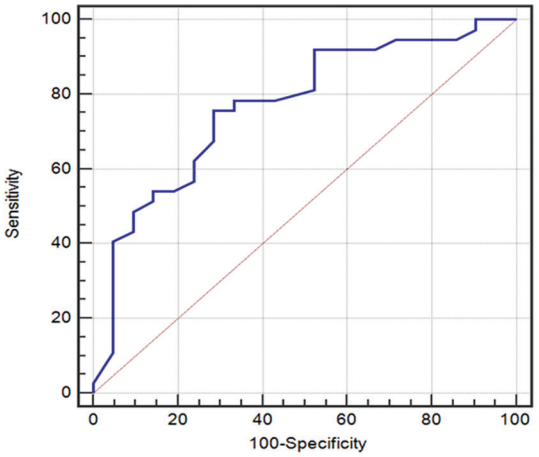
[a]Comparison between groups A and B. [b]Comparison between groups B and C. [c]Comparison between groups A and C. MDA, malondialdehyde. *Significant *P* value less than 0.05.

Figure 1



Correlation between the mean MDA level and the mean sperm concentration per 20 HPF. MDA, malondialdehyde.

Figure 2



ROC curve between the MDA level and sperm retrieval. MDA, malondialdehyde; ROC, receiver operating characteristic.

It was found by ROC analysis that when the MDA level is less than or equal to 33 nmol/g protein, there will be a 75.68% (sensitivity) chance of the presence of one sperm or more in the testicular samples and when the MDA level is more than 33 nmol/g protein, we can expect a 75.68% (specificity) chance of the absence of sperm (Table 3, Fig. 2).

Discussion

Increased production of ROS induces lipid peroxidation in spermatozoa, which has two important effects: (a) reduction of sperm combination with oocyte and (b) increase in spermatozoa ability to bind to the transparent area (zona placida). Also, lipid peroxidation causes abnormality in the middle section of sperm and loss of acrosome capacity of fertilization. MDA molecules induce an asymmetric distribution of lipid membrane components by penetrating into the cell membrane structure. It is used as a marker (biomarker) to determine the rate of oxidative damage to lipids. It is noteworthy that currently, the damage caused by lipid peroxidation is the most important factor for testicular dysfunction [2].

In our study, there was a statistically significant increase in the mean MDA level in both the positive NOA and the negative NOA group than the OA group ($P = 0.053$, 0.000), respectively, with no significant difference between the positive and negative NOA groups. In other studies that support our result, there was increased lipid peroxidation (MDA) in the plasma, liver, lung, kidneys, and testis of rats after cigarette smoke exposure [16–18].

The authors attributed the finding to the fact that testicular tissue is a highly vascular tissue, and because of the rich blood supply, cigarette smoke may disrupt the balance between oxidant and antioxidant enzyme systems. As a result, FRs are generated, which cause testicular tissue damage [18]. Also, in cases of NOA, when FRs and ROS production exceeds the ability of the defense system to scavenge these species, oxidative stress occurs and FRs attack PUFAs found widely in cell membranes. Lipid peroxidation develops in cell membranes, which causes the production of membrane destruction products, such as MDA [18].

In our study, we found a very highly significant negative correlation between the mean MDA level and the mean sperm concentration/20 HPF; this finding is in agreement with the results reported in semen [19–22]. This can be explained by the fact that MDA can lead to the activation of the caspase cascade and externalization of phosphatidylserine, leading to apoptosis of sperm [23], which in turn leads to a reduction in the sperm count because of an increase in its level.

ROC analysis showed a good predictive value of testicular MDA level as a marker for the presence or

Table 3 Cut-off value of the malondialdehyde level and sperm retrieval

Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC
≤33	75.68	71.43	82.4	62.5	74.14	0.768

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

absence of sperm. Further studies are needed to evaluate whether there is a correlation between testicular tissue and seminal fluid in terms of the MDA level, and if found, the MDA level in seminal fluid could be used as a predictive value for the presence of sperm in testicular tissue of NOA cases.

Conclusion

In conclusion, the MDA level in testicular tissues is higher in NOA than in OA cases; the MDA level correlates negatively with the sperm concentration. Therefore, it may be considered a predictor marker for sperm retrieval in NOA cases. Further research on a large scale is needed.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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