Journal of Assisted Reproduction and Genetics (2019) 36:2367–2378
https://doi.org/10.1007/s10815-019-01579-7

REPRODUCTIVE PHYSIOLOGY AND DISEASE

Novel association between apoptotic sperm biomarkers with seminal biochemical parameters and acetylcholinesterase activity in patients with teratozoospermia

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Received: 14 February 2019 / Accepted: 4 September 2019 / Published online: 11 September 2019

Abstract

Objective We aimed to determine whether the dysfunction of physiological apoptosis and specific seminal biochemical parameters could be associated with male infertility and sperm morphological defects.

Study design Ejaculated sperm samples from sixty patients with isolated teratozoospermia and thirty fertile donors were analyzed. The proportion of both viable and dead spermatozoa expressing activated caspases was detected by fluorescence microscopy through the use of different specific carboxyfluorescein-labeled caspase inhibitors FLICA. The different stages of apoptosis in human were qualitatively and quantitatively determined by using the AO/EB fluorescent staining method. The levels of the seminal biochemical parameters (acetylcholinesterase (AChE), lactate dehydrogenase (LDH), creatine phosphokinase (CK), iron (Fe), calcium (Ca), and phosphorus (P)) were evaluated spectrophotometrically.

Results Patients with teratozoospermia showed significantly higher proportions of dead and live spermatozoa with activated caspases and spermatozoa in the late stage of apoptosis when compared to controls. Among the different studied biochemical seminal parameters, the rates of acetylcholinesterase activity, creatine phosphokinase, iron, and calcium were significantly increased in the patient group. However, the rate of phosphorus was significantly decreased. Interestingly, significant relationships were found between the studied biochemical and apoptotic biomarkers and the rates of atypical sperm forms with the incidences of head, mid-piece, and tail abnormalities. Furthermore, positive correlations were found between P, AChE, Fe, CK, and LDH with apoptotic markers.

Conclusions These results emphasize the impact of apoptosis in the pathophysiology of teratozoospermia and suggest that seminal biochemical disturbance may arise such damage.

Keywords Apoptotic sperm biomarkers · Teratozoospermia · Polycaspases · Seminal biochemical parameters

Introduction

Sperm morphology displays a potential impact on sperm function and may ultimately impact reproductive ability [1, 2]. Adverse studies have shown that the defect in sperm morphology is associated with a range of adverse clinical outcomes, including impaired fertilization [3], disrupted pre-implantation embryonic development, increased rates of miscarriage, and an enhanced risk of disease in the progeny [4–6]. According to the modified classification of David, ejaculate contains less than 15% of normal spermatozoa refers to the teratozoospermic patients [7].

Among different hypotheses proposed to explain the pathological mechanism of teratozoospermia, impairment of spermatogenesis and disruption of the apoptotic process has been suggested [8, 9]. The disturbance of the spermatogenesis event induces sperm parameter alterations in subfertile men and can result in ultrastructural modifications and higher expression of apoptotic markers

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in ejaculated spermatozoa [10, 11]. While several reports have shown that most subfertile men with teratozoospermia are characterized by unbalanced production of normal and mature spermatozoa in their ejaculates and higher rates of oxidative stress, DNA damage and protamination defects [12–15]. Few studies have demonstrated relationships between poor sperm morphology and higher rates of caspase-3 expression and plasma membrane translocation of phosphatidylserine than in healthy donors [11], suggesting that teratozoospermia may be associated with apoptosis in these patients.

Although these markers are characteristic markers of apoptosis in somatic cells [16, 17], their significance and specificity in germ cells have been discussed [18–20]. Therefore, the measurement of apoptotic markers and assessment of biochemical modification in spermatozoa may clarify whether apoptosis is involved in male infertility associated with isolated teratozoospermia.

The caspases (cytosolic cysteine-containing aspartate-specific proteases) are major transducers and effectors within the different pathways of the apoptosis signaling network in somatic cells [21]. They comprise a family of highly specific proteases which contain the amino acid cysteine in their active sites. Caspases involved in apoptosis act either as initiators (caspase-8, caspase-9, and caspase-10) or as effectors (caspase-3, caspase-6, and caspase-7). Caspase-8 was identified as the most important initiator enzyme of the extrinsic pathway triggered by activation of membrane death receptors (CD95 or tumor necrosis factor receptor) whereas caspase-9 via activation of the mitochondrial pathway is predominantly associated with activation of the intrinsic apoptotic pathway. Caspase-3, the most important among them, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generating DNA fragmentation [22]. Moreover, the key role of activated caspases is well studied for the transduction of the apoptotic signal; some results have documented their supplemental participation in sperm differentiation and maturation [23, 24]. Besides activation of different signaling, molecular pathways, apoptosis is characterized by structural alterations of the different spermatozoa components. The dual acridine orange/ethidium bromide (AO/EB) fluorescent staining, visualized under a fluorescent microscope, can be used to identify apoptosis-associated changes of cell membranes during the process of apoptosis [25]. This method can also accurately sub-populate spermatozoa at different stages of apoptosis [26]. In this context, we aimed to compare the biochemical and ultrastructural apoptotic characteristics of spermatozoa from sub-fertile patients with isolated teratozoospermia and fertile donors. Nevertheless, to find a link between poor sperm morphology, apoptosis, and some biochemical markers, we have studied in semen the following: the creatine phosphokinase (CK), a key enzyme of sperm energy, transport [27], and a marker of sperm maturity [24, 28]; the lactate dehydrogenase (LDH), as a tracer of germinal activity, closely associated with the starting of active spermatogenesis [29]; and the acetylcholinesterase (AChE) activity, as a neurotropic agent classically known for terminating the cholinergic neurotransmission.

The normally rare AChE variant is induced by chemical, psychological, or immunological stressors in the brain, muscle, blood, and testicular cells [30, 31]. It increases during neuronal and hematopoietic differentiation [32, 33]. Nevertheless, it was revealed that transgenic over-expression of human AChE has been correlated with decreased mouse sperm counts, motility, and normality during sperm maturation [34]. Although three seminal trace elements were evaluated, namely, iron (Fe), as an important mineral, plays an essential role in fertility and productivity [35], calcium (Ca) which is involved in hyperactivation, chemotaxis, capacitation, and the acrosome reaction of spermatozoa [36], and phosphorus (P) which has multiple benefit roles including reproduction [37].

As a secondary outcome, we aimed to determine the eventual relationship between specific morphology of spermatozoa and both the apoptotic and biochemical markers.

Materials and methods

Study population

A prospective controlled study was employed, involving 60 patients referred to our fertility clinic at the Department of Cytogenetic and Reproductive Biology, Fattouma Bourguiba University Teaching Hospital, Monastir, Tunisia. This study group presented with isolated teratozoospermia; the other semen parameters were normal. In addition, 30 healthy men with normal semen profiles and whose unique partners had at least one previous term pregnancy without complications conceived with their sperm samples within the past year were included as the control group. No subject enrolled in this study had a prior or current history of radiotherapy, chemotherapy, and chronic illness. This protocol was approved by the local ethics committees and all patients and controls had previously given informed consent for the study.

Semen analysis and preparation

All semen samples were collected by masturbation into sterile pots after 3 days of sexual abstinence. After complete liquefaction of the sample, standard semen parameters were evaluated according to the World Health Organization guidelines [38]. Detailed morphological assessment according to the modified David classification [7] was performed on each semen sample to provide a breakdown of the specific types of

morphological abnormalities found in the sperm. Each sample of the fresh semen was divided into two aliquots: one immediately served for caspase assay and the other washed twice in phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 3500 RPM for 10 min to obtain seminal plasma and sperm pellet. Obtained seminal plasma was frozen at − 20 °C until biochemical analyses and the pellet was then fixed in methanol/acetic acid (3:1) for at least 30 min at 4 °C. The fixed specimens were stored at − 20 °C until the AO/EB assay.

Detection of activated caspases in spermatozoa

FAM-FLICA® Caspase Assay Kit is commercially available as a component of the Fluorescein Caspase Activity Kit (ImmunoChemistry Technologies, USA, LLC #F18-91-6-C). Polycaspase FLICA® probe, FAM-VAD-FMK, is used as a general reagent to detect apoptosis as it is recognized by all types of activated caspases. Non-cytotoxic fluorescent caspase inhibitor (FLICA) binds covalently and irreversibly to many activated caspases (caspase-1, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8, and caspase-9). The fluorogenic substrate becomes fluorescent upon cleavage by the caspases [22]. As specified in the kit, the FAM-VAD-FMK inhibitor was dissolved in dimethyl sulfoxide (DMSO) to obtain a 150× stock solution.

Aliquots of this solution were stored at ≤ − 20 °C in the dark for 6 months protected from light. Before use, a 30× FLICA working solution was prepared by diluting the stock solution 1:5 in 200 μl PBS. For labeling 3×10^6 spermatozoa (dilute spermatozoa in PBS until a final volume of 300 μl), it was incubated with 10 μl of 30× working solution for 1 h at 37 °C under 5% CO_2 with tubes protected from light. Cells were washed twice with 1× wash buffer (PBS containing 0.5% bovine serum albumin and 0.05% sodium azide). The cell pellet was resuspended in 400 μl 1× wash buffer and incubated in 2 μl of propidium iodide (PI) for 15 min, protected from light at RT to assess viability.

Cells were observed under a fluorescence microscope (Leica, DFC) using a band-pass filter (excitation 490 nm, emission > 610 nm) to view green fluorescence.

A minimum of four hundred spermatozoa was randomly assessed per slide in at least five fields. Three patterns of fluorescence were measured:

- Viable spermatozoa with staining of activated polycaspases (early apoptotic spermatozoa): Casp+/PI− (green).
- Dead spermatozoa with staining of activated polycaspases (late apoptotic spermatozoa): Casp+/PI+ (red and green).
- Dead spermatozoa without staining of activated polycaspases: Casp−/PI+ (red).

Dual acridine orange/ethidium bromide fluorescent staining

Spermatozoa staining was performed according to the method of Srinivas et al. [39]. Briefly, sperms from the control and treated groups were smeared on a glass slide and air-dried. Smears were fixed with methanol/acetic glacial acid (3:1) for 5 min, then they were hydrated with PBS for 1 min and stained with a mixture (1:1) of AO (50 μg/ml)/EB (5 μg/ml) solutions for 10 min. Sperms were immediately washed with PBS and an examination was done using a fluorescent microscope (Leica, DFC). Five hundred of sperms per slide were evaluated under the oil immersion lens and the damaged (apoptotic and necrotic) sperms were recorded according to the affinity, the pattern of fluorescent staining, and the morphological aspect of chromatin condensation in the stained nuclei. We have distinguished four types of sperm cells: (1) normal spermatozoa have uniform bright green nuclei with an organized structure; (2) early apoptotic spermatozoa (started to undergo DNA cleavage) have bright green nuclei; (3) late apoptotic spermatozoa have orange to red nuclei with chromatin decondensation and denatured DNA; (4) necrotic spermatozoa have uniformly a red nucleus with fragmented structure. Then representative photos were digitally photographed.

Assessment of seminal acetylcholinesterase activity

The assay is performed according to the colorimetric method described by Ellman et al. [40]. It is based on the hydrolysis of a choline ester, acetylcholine by acetylcholinesterase. The released thiocholine reacts with 5-5′dithiobis(2-nitrobenzoate) (DTNB) to form the yellow-colored 5-thio-2-nitrobenzoate (TNB) product which absorbs at 412 nm. The intensity of the coloration is proportional to the amount of enzyme present in the medium. The results are expressed in μmol/min/mg of protein.

Assessment of seminal creatine phosphokinase level

The seminal creatine phosphokinase dosage was made spectrophotometrically using a commercial Kit biolabo, the increase in absorbance is due to the conversion of $NADP^+$ to NADPH and directly proportional to the activity of creatine phosphokinase (CK). Absorbance is measured at 340 nm.

Assessment of seminal lactate dehydrogenase level

The seminal lactate dehydrogenase dosage was made spectrophotometrically using a commercial Kit biolabo, the decrease in absorbance is due to the conversion of NADH to NAD^+ and directly proportional to the activity of lactate dehydrogenase (LDH). Absorbance is measured at 340 nm.

Assessment of seminal iron level

The seminal plasma level of iron was determined spectrophotometrically by using commercial reagent kits Cobas (IRON2 Iron Gen.2). According to the method that uses ferrozine at acidic pH, iron is detached from transferring, and then ascorbate reduces the Fe^{3+} ions to Fe^{2+} ions, which form a colored complex with ferrozine. The intensity of the coloration is directly proportional to the iron concentration. It is determined by increasing the absorbance at 552 nm.

Assessment of seminal phosphorus level

The seminal phosphorus dosage was made spectrophotometrically by commercial kit biolabo, according to the UV method without deproteinization (ammonium molybdate). In acidic medium, phosphate ions form a phospho-molybdic complex with ammonium molybdate. Absorbance is measured at 340 nm (334–366).

Assessment of seminal calcium level

The seminal plasma level of calcium was measured by the Cobas kit (CA2 Calcium Gen.2). The calcium ions react with 5-nitro-5-methyl-BAPTA (NM-BAPTA) in an alkaline medium to form a complex calcium-NM-BAPTA. Then this complex reacts in the presence of EDTA. The intensity of the colored complex found is directly proportional to the calcium concentration measured by the spectrophotometer.

Assessment of protein content

The seminal plasma level of total proteins was determined spectrophotometrically from using commercial reagent kits from Roche Diagnostics Gmbh (Mannheim, Germany).

Statistical analysis

Statistical analysis was performed using SPSS 21 (SPSS Inc., Chicago, IL, USA). The comparisons between the control group and patients were made using Student's *t* test. Spearman's correlation coefficients were also calculated.

A statistically significant difference was accepted when the *p* value was < 0.05.

A statistically highly significant difference was accepted when the *p* value was < 0.01.

Results

Age and conventional semen characteristics

Statistic description of the age and semen parameters of teratozoospermic group and controls is shown in Table 1. The mean age of the patients with morphologically abnormal spermatozoa was not significantly different compared to the control group (36.37 ± 7.13 vs 36.81 ± 6.53, respectively; $p > 0.05$). The patients ($n = 60$) had isolated teratozoospermia with a mean volume of 3.38 ± 1.31 ml, a mean pH of 7.88 ± 0.17, a mean concentration of 134.54 ± 126.54 10^6/ml, a mean leukocyte of 0.54 ± 0.86 10^6/ml, a mean total progressive motility of $47.62 \pm 6.8\%$, and a mean percentage fraction of sperm with abnormal forms of $92.66 \pm 3.64\%$ and a mean multiple anomalies index (MAI) of 1.83 ± 0.22. Sperm head abnormalities were significantly higher in the patients with teratozoospermia compared with controls (17.64 ± 3.4 vs 15.21 ± 1.74; $p = 0.002$), with significant predominance of the tapered heads (15.45 ± 9.24), double heads (1.34 ± 1.84), thinned heads (8.41 ± 4.85), and the acrosomal anomalies (24.15 ± 10.83 vs 15 ± 4.85; $p = 0.001$). Moreover, significant difference between the two groups was evident in the tail abnormalities (6.69 ± 2.89 vs 4.6 ± 1.99; $p = 0.003$), especially for double tails (1.87 ± 2.14 vs 0.56 ± 0.91; $p = 0.014$) and coiled tails (14.21 ± 8.59 vs 10.09 ± 6.45; $p = 0.045$).

Comparison of apoptotic sperm biomarkers between the study and the control groups

The results of the apoptotic biomarker analysis in the semen of patients with isolated teratozoospermia and fertile donors are presented in Table 2.

A mean of 400 spermatozoa per subject was counted. As mentioned, semen of teratozoospermic patient showed a lower proportion of viable spermatozoa with activated caspases ($28.09\% \pm 10.62$ vs $54.85\% \pm 12.36$; $p < 0.001$) when compared to controls. In addition, the percentage of dead spermatozoa with activated caspases and dead spermatozoa without activated caspases were significantly more frequent in the patients compared to controls (respectively $69.34\% \pm 10.28$ vs $43.79\% \pm 11.95$; $p < 0.001$ and $2.3\% \pm 1.08$ vs $1.42\% \pm 0.9821.85$; $p = 0.011$). After both AO/EB staining, spermatozoa were sub-populated in four groups: normal spermatozoa, early apoptotic spermatozoa, late apoptotic spermatozoa, and necrotic spermatozoa. The proportion of normal spermatozoa has significantly decreased in patients with isolated teratozoospermia than in those with normal sperm parameters ($p < 0.001$). Besides, some patients have shown significant high proportions of both late apoptotic and necrotic spermatozoa (respectively, $30.47\% \pm 16.21$ vs 3.75 ± 6.98; $p < 0.001$ and $16.53\% \pm 15.15$ vs 1 ± 3.16; $p = 0.003$). This

Table 1 Comparison of age, standard semen parameters, and detailed morphology characteristics between control and study groups

	Control group ($n = 30$) Mean ± SD	Teratozoospermic group ($n = 60$) Mean ± SD	p value
Age	36.81 ± 6.53	36.37 ± 7.13	0.815
Volume (ml)	3.2 ± 1.58	3.38 ± 1.31	0.608
pH	7.9 ± 0.15	7.88 ± 0.17	0.21
Sperm concentration (10^6/ml)	159.57 ± 95.70	134.54 ± 126.54	0.426
Leucocyte concentration (10^6/ml)	0.39 ± 0.32	0.54 ± 0.86	0.43
Sperm motility (%)	49.75 ± 6.78	47.62 ± 6.8	0.228
Necrozoospermia	15.9 ± 8.11	15.54 ± 9.55	0.880
Atypical forms (%)	82.66 ± 3.21	92.42 ± 3.14	0.001[b]
Head abnormalities (%)	15.21 ± 1.74	17.64 ± 3.4	0.002[b]
Macrocephalic (%)	3.4 ± 2.52	2.6 ± 2.58	0.202
Microcephalic (%)	27.66 ± 11.57	30.96 ± 14.86	0.354
Tapered head (%)	10.28 ± 6.88	15.45 ± 9.24	0.016[a]
Double head (%)	0.28 ± 0.46	1.34 ± 1.84	0.011[a]
Irregular head (%)	40.85 ± 10.57	40.4 ± 13.31	0.886
Thinned head (%)	8.09 ± 4.32	8.41 ± 4.85	0.787
Acrosomal anomalies (%)	15 ± 4.85	24.15 ± 10.83	0.001[b]
Tail abnormalities (%)	4.6 ± 1.99	6.69 ± 2.89	0.003[b]
Two tailed (%)	0.56 ± 0.91	1.87 ± 2.14	0.014[b]
Coiled tail (%)	10.09 ± 6.45	14.21 ± 8.59	0.045[a]
Bent tail (%)	6.66 ± 4.22	9.07 ± 5.53	0.07
Short tail (%)	1 ± 1.095	1.62 ± 2.10	0.193
Cytoplasmic droplet (%)	1.28 ± 1.32	1.5 ± 1.9	0.63
Multiple anomalies index	1.62 ± 0.17	1.83 ± 0.22	0.001[b]

Sperm parameters are expressed as mean ± standard deviation or median (inter-quartile range) depending on their normal distribution. Parametric data was analyzed by paired t test

[a] Significant difference with control group ($p < 0.05$)

[b] Highly significant difference with control group ($p < 0.01$)

Table 2 Statistic comparison of apoptotic sperm markers between control group and teratozoospermic group

	Control group ($n = 30$) Mean ± SD	Teratozoospermic group ($n = 60$) Mean ± SD	p value
AO/EB staining assay			
Normal spz (%)	70.08 ± 21.8	21.92 ± 15.76	0.001[b]
Early apoptotic spz (%)	25.11 ± 17.25	29.98 ± 12.16	0.311
Late apoptotic spz (%)	3.75 ± 6.98	30.47. ± 16.21	0.001[b]
Necrotic spz (%)	1 ± 3.16	16.53 ± 15.15	0.003[b]
Caspase assay			
Casp+/PI− (%)	54.85 ± 12.36	28.09 ± 10.62	0.001[b]
Casp+/PI+ (%)	43.79 ± 11.95	69.34 ± 10.28	0.001[b]
Casp−/PI+ (%)	1.42 ± 0.98	2.3 ± 1.08	0.011[a]

All values are expressed as mean ± standard deviation and analyzed using the paired t test

spz spermatozoa

[a] Significant difference with control group ($p < 0.05$)

[b] Highly significant difference with control group ($p < 0.01$)

difference was not significant in the proportion of early apoptotic spermatozoa ($p > 0.05$).

Correlation between apoptotic sperm biomarkers and morphological semen characteristics

The detailed results of the correlation analyses between apoptotic markers and both spermatic and the morphological characteristics in ejaculated sperm samples of teratozoospermic patients and fertile donors are given in Table 3.

Significant correlations were found between the rate of abnormal sperm forms and both viable and dead spermatozoa with activated caspases ($r = -0.386$; $p = 0.001$ and $r = 0.401$; $p = 0.001$) and necrotic spermatozoa ($r = -0.311$; $p = 0.008$) with the incidence of double and thinned heads. Even more, the proportions of viable and dead spermatozoa with activated caspases correlated significantly with the rates of sperm tail abnormalities (respectively, $r = -0.274$; $p = 0.037$ and $r = 0.254$; $p = 0.026$).

Stained with AO/EB normal spermatozoa were negatively correlated with the rates of abnormal sperm forms ($r = -0.393$; $p = 0.006$), head abnormalities ($r = -0.378$; $p = 0.04$), and tail abnormalities ($r = -0.280$; $p = 0.028$) but positively with the index of multiple abnormalities which is defined by the mean number of anomalies per abnormal spermatozoon ($r = 0.378$; $p = 0.005$). In contrast, the proportion of early apoptotic spermatozoa was positively correlated only with the rate of abnormal sperm forms ($r = 0.249$; $p = 0.046$). Besides, no significant correlations were found between the proportions of late apoptotic spermatozoa and the rates of abnormal sperm forms ($p = 0.108$). On the other hand, significant correlations were proven with the rates of head abnormalities ($r = 0.337$; $p = 0.01$) and the index of multiple abnormalities ($r = -0.371$; $p = 0.006$). The proportion of necrotic spermatozoa was also significantly and positively correlated with the rate of atypical sperm forms ($r = 0.304$; $p = 0.019$), the head abnormalities ($r = 0.354$; $p = 0.007$), and tail abnormalities ($r = 0.265$; $p = 0.036$), but negatively correlated with the index of multiple abnormalities ($r = -0.302$; $p = 0.021$).

Comparison of seminal biochemical parameters between the study and the control groups

The seminal biochemical parameters of our samples are shown in Table 4. We detected a significant increase in seminal creatine phosphokinase (CK) activities and acetylcholinesterase (AChE) in the group with teratozoospermia compared to control group (455.37 ± 297.3 mg/ml vs 327.63 ± 0.186 mg/ml, $p = 0.05$; 16.44 ± 27.11 μmol/min/mg P vs 4.4 ± 3.37 μmol/min/mg P, $p = 0.046$). The seminal iron (Fe) and calcium (Ca) contents were also significantly increased in the same group (respectively, 4.66 ± 1.89 mg/ml vs 3.47 ± 0.86 mg/ml, $p = 0.007$ and 6.10 ± 2.48 mg/ml vs 4.49 ± 2.08 mg/ml, $p = 0.008$). But this difference was not significant for the seminal lactate dehydrogenase activity ($p = 0.165$). On the other hand, the rates of phosphorus and in semen were significantly decreased in the study group than in donors (14.44 ± 0.46 mg/ml vs 12.52 ± 2.31 mg/ml, $p = 0.047$).

Table 3 Correlation between apoptotic sperm biomarkers and sperm morphology characteristics

	Atypical forms (%)	Head abnormalities (%)	Tail abnormalities (%)	Mid-piece abnormalities (%)	Multiple anomalies index
AO/EB staining assay					
Normal spz (%)	$r = -0.393$ $p = 0.006$	$r = -0.378$ $p = 0.04$	$r = -0.280$ $p = 0.028$	NS	$r = 0.378$ $p = 0.005$
Early apoptotic spz (%)	$r = 0.249$ $p = 0.046$	NS	NS	NS	NS
Tardif apoptotic spz (%)	NS	$r = -0.337$ $p = 0.01$	NS	NS	$r = -0.371$ $p = 0.006$
Necrotic spz (%)	$r = 0.304$ $p = 0.019$	$r = 0.354$ $p = 0.007$	$r = 0.265$ $p = 0.036$	NS	$r = -0.302$ $p = 0.021$
Caspase assay					
Casp+/PI− (%)	$r = -0.386$ $p = 0.001$	NS	$r = -0.274$ $p = 0.037$	NS	NS
Casp+/PI+ (%)	$r = 0.401$ $p = 0.001$	NS	$r = 0.254$ $p = 0.026$	NS	NS
Casp−/PI+ (%)	$r = 0.311$ $p = 0.008$	NS	NS	NS	NS

Statistical analysis was performed using the Spearman rank-order correlation test. Significant difference with control group ($p < 0.05$). Highly significant difference with control group ($p < 0.01$)

NS not significant

Table 4 Statistic comparison of biochemical markers between control group and teratozoospermic group

	Control group ($n=30$) Mean ± SD	Teratozoospermic ($n=60$) Mean ± SD	p value
Creatine phosphokinase (mg/ml)	327.63 ± 0.186	455.37 ± 297.3	0.05[a]
Lactate dehydrogenase (mg/ml)	1565.53 ± 683.29	1847.21 ± 851.68	0.165
Acetylcholinesterase (μmol/min/mg P)	4.42 ± 3.37	16.44 ± 27.11	0.046[a]
Phosphorus (mg/ml)	14.44 ± 0.46	12.52 ± 2.31	0.046[a]
Iron (mg/ml)	3.47 ± 0.816	4.66 ± 1.89	0.007[b]
Calcium (mg/ml)	4.49 ± 2.08	6.10 ± 2.48	0.008[b]

All values are expressed as mean ± standard deviation and analyzed using the paired t test

[a] Significant difference with control group ($p<0.05$)

[b] Highly significant difference with control group ($p<0.01$)

Correlation between seminal biochemical markers with morphological semen characteristics and sperm apoptotic biomarkers

Among all the studied biochemical parameters in semen, only increased levels of acetylcholinesterase, iron, and calcium were significantly correlated with the rate of atypical sperm forms (respectively, $r=0.303$; $p=0.002$, $r=0.300$; $p=0.02$, $r=0.241$; $p=0.05$). Accordingly, significant correlations were found between the index of multiple anomalies and both seminal iron level ($r=0.264$; $p=0.011$) and AChE activity ($r=0.224$; $p=0.017$). Besides, only iron level was correlated with the tail abnormalities ($r=0.245$; $p=0.019$), while the rate of the head abnormalities was correlated significantly with the increase level of seminal creatine phosphokinase ($r=0.225$; $p=0.032$) and the increased level of AChE activity ($r=0.228$; $p=0.016$) (Table 5).

Interestingly, normal spermatozoa stained with AO/EB were negatively correlated with the seminal iron level ($r=-0.372$; $p=0.02$); correspondingly, these correlations were positive with the proportion of spermatozoa in the late stage of apoptosis and necrotic spermatozoa forms (respectively, $r=0.377$; $p=0.004$ and $r=0.262$; $p=0.0038$). On the other hand, significant correlations were found between the seminal calcium level and AChE activity with the proportions of dead and live spermatozoa with activated caspases (respectively, $r=-0.270$; $p=0.038$ and $r=0.304$; $p=0.019$; $r=-0.247$; $p=0.031$ and $r=0.244$; $p=0.046$) (Table 6).

Discussion

Previous research studies have demonstrated that ejaculated sperm from patients with a high level of morphologically abnormal spermatozoa presents evidence of DNA damage, perturbed *meiotic* segregation [41–43], and oxidative stress induction [13, 44, 45] characterized by impaired seminal antioxidant status and high level of seminal lipid peroxidation. As an extension of our previous works [13, 46], in this study, we analyzed the seminal biochemical alterations and the structural apoptotic features in ejaculated spermatozoa from patients with isolated teratozoospermia to assess whether

Table 5 Correlation between sperm morphology characteristics and seminal biochemical biomarkers

	CK	LDH	AChE	Fe	CA	P
Atypical forms (%)	NS	NS	$r=0.303$ $p=0.002$	$r=0.300$ $p=0.02$	$r=0.241$ $p=0.05$	NS
Head abnormalities (%)	$r=0.225$ $p=0.032$	NS	$r=0.228$ $p=0.016$	NS	NS	NS
Tail abnormalities (%)	NS	NS	NS	$r=0.245$ $p=0.019$	NS	NS
Mid-piece abnormalities (%)	NS	NS	NS	NS	NS	NS
Multiple anomalies index	NS	NS	$r=0.224$ $p=0.017$	$r=0.264$ $p=0.011$	NS	NS

Statistical analysis was performed using the Spearman rank-order correlation test. Significant difference with control group ($p<0.05$). Highly significant difference with control group ($p<0.01$)

NS not significant

Table 6 Correlation between apoptotic sperm biomarkers and seminal biochemical biomarkers

	CK	LDH	AChE	Fe	CA	P
AO/EB staining assay						
Normal spz (%)	NS	NS	NS	$r=-0.372$ $p=0.02$	NS	NS
Early apoptotic spz (%)	NS	NS	NS	NS	NS	NS
Tardif apoptotic spz (%)	NS	NS	NS	$r=0.377$ $p=0.004$	NS	NS
Necrotic spz (%)	NS	NS	$r=0.274$ $p=0.0032$	$r=0.262$ $p=0.038$	NS	NS
Caspase assay						
Casp+/PI− (%)	NS	NS	$r=0.247$ $p=0.031$	NS	$r=-0.270$ $p=0.038$	NS
Casp+/PI+ (%)	NS	NS	$r=0.224$ $p=0.046$	NS	$r=0.304$ $p=0.019$	NS
Casp−/PI+ (%)	NS	NS	NS	NS	NS	NS

Statistical analysis was performed using the Spearman rank-order correlation test

NS not significant, *spz* spermatozoa

[a] Significant difference with control group ($p<0.05$)

[b] Highly significant difference with control group ($p<0.01$)

apoptosis may be one of the underlying mechanisms of teratozoospermia and if the process includes seminal biochemical disturbances and overexpressed acetylcholinesterase activity.

Among the different apoptotic biomarkers studied in the ejaculated human sperm, we investigated the expression of activated caspases (1, 3, 4, 5, 6, 7, 8, and 9) by using Fluorescent Inhibitor of Activated Caspase Kit (FLICA). Importantly, FLICA is labeled with fixed cells and without permeabilization can be combined with vital staining PI [47, 48] and extremely specific to different members of the apoptotic caspase family [49, 50]. Interestingly, high proportions of dead and live spermatozoa with activated caspases were found in the teratozoospermia group. These findings are in accordance with the results of other studies which found an association between sperm morphology and different caspase markers detected under certain conditions. Almeida et al. [51] and Aziz et al. [11] have found that normal morphology correlates negatively with caspase-3 activation measured in semen after direct swim-up or double density gradient centrifugation preparation. Said et al. [44] have reported that normal morphology applying the strict criteria in sperm preparations correlate inversely with caspase-3 activation. Indeed, the study of Hichri et al. [52] by using FLICA has demonstrated positive associations between abnormal sperm morphology and the expression of activated caspases in patients with oligoasthenoteratozoospermia. The higher increase in caspase activities might be explained by its role in linking DNA fragmentation to the apoptosis network, which is previously related to teratozoospermia [13, 42, 46].

To better understand the biological phenomenon involved in the spermatozoa of the teratozoospermic men, we combined the measurement of the expression of activated caspases, with the determination of the apoptotic stage by acridine orange/ethidium bromide staining which may strengthen the analysis. To our knowledge, this is the first study which reports determinations of ultrastructural apoptosis by dual AO/EB fluorescent staining of sub-fertile patients with teratozoospermia. A clear distinction is made between normal spermatozoa, early and late apoptotic spermatozoa, and necrotic spermatozoa. Our results showed that the patient group has significantly high proportions of both late apoptotic and necrotic spermatozoa which confirm the results of polycaspases. Both AO/EB are membrane-impermeable dyes, which pass through damaged membranes and are directly observable under the microscope. AO binding with the double-stranded DNA as a monomeric form can show green fluorescence while binding with single-stranded DNA as a polymer can show orange or yellow fluorescence [53]. EB dominates over AO, emitting red fluorescence when bound to apoptotic bodies or fragmented DNA [54]. The dual AO/EB staining is able to detect mild DNA injuries [53]. In the past, only AO fluorescent staining was used, while detection of cell apoptosis using AO/EB is a relatively new method, and few studies have reported its use [55]. When compared to AO staining, the AO/EB method improves the detection of apoptosis and can distinguish between late apoptotic and dead cells as well as chromatin disintegration [25, 56]. Thus, AO/EB staining can feasibly be used in ejaculated spermatozoa.

On the other hand, a substantial effort was undertaken to link the apoptotic alterations with morphological characteristics of sperm. In fact, the studied apoptotic sperm biomarkers were positively associated with various forms of abnormal sperm morphology, including sperm head and tail abnormalities. Therefore, the results of this study further demonstrate

the adverse functional impact of sperm apoptosis on sperm quality. Another important finding of the present investigation is that both early and late apoptoses determined by polycaspase assay or AO/EB staining were correlated with abnormal sperm forms. Such results indicate that the sperm start to be affected at an early stage of apoptosis and the phenotypical damage become evident in the late stage of apoptosis.

Apoptosis, impaired AChE activity, and disturbed seminal biochemical parameters (CK, LDH, Ca, Fe, P) have been separately suggested as factors associated with functional and qualitative sperm defects.

From our study, it is clear that seminal acetylcholinesterase, creatine phosphokinase, and lactate dehydrogenase activities represent valid biochemical markers for sperm morphological defects. Higher CK, LDH, and AChE activities were found in the seminal plasma of the patient group when compared to the control group, and these results are in accordance with the results of other authors [29, 34, 57, 58]. Seminal levels of creatine phosphokinase (CK) are often used to judge about immature sperm assessment [57, 59, 60]. Not far from our founding, relationships were found between sperm cell size, head form, and creatine kinase content in the motile fractions of human sperm [61]. Otherwise, AChE is the main enzyme responsible for acetylcholine degradation in vivo and is expressed in the neck, mid-piece, and principal piece of human and mouse sperm [34]. Mor et al. [30] have reported that AChE is overexpressed in testes from mice exposed to forced swimming, and an accumulated AChE in post-mitotic sperm progenitors imposes a partial block to post-meiotic differentiation. In 2008, they also cited that AChE increases germ cell apoptosis and enhances sperm motility [62], by mitochondrial hyperactivity and elevated glucose consumption as well as ATP levels in transgenic mice with over-expression of AChE, anticipating that a high level of AChE can potentially disturb sperm metabolism and accentuate ATP content in sperm cells as well. In parallel, the transgenic pups displayed elevated spermatocyte apoptosis, as measured by TUNEL staining of testicular sections and confirmed by the over-expression of the pro-apoptotic factor p73. Alternatively, in human donor sperm, they found direct associations between the sperm motility and the quality of movement with the AChE expression.

Taken together with the previous observations and the positive relationships found in our study between the over-expressed AChE activity and both different morphological abnormalities and caspase expression, it seems that AChE can be a useful marker of apoptosis-related sperm morphological defects. Besides the enzymes, microelements present in the seminal plasma are also good indicators of functional metabolic activity of the sperm cells [63, 64].

In the present experiment, we have assessed three important microelements calcium, iron, and phosphorus in the seminal plasma of patients with isolated teratozoospermia. The seminal calcium continent was significantly higher in the patient group. An even more, positive relationship was found between calcium and atypical sperm forms. Adversely, different studies have shown a positive relationship between calcium, normal morphology, and progressive motility. In other studies [65, 66], seminal calcium was not different compared to men with normal motility. However, Wang and Joseph [67] proved that calcium has an adverse effect on mature spermatozoa motility in the ejaculated semen.

Surprisingly, strong relationships were found between seminal calcium with the percentage of live and dead spermatozoa with activated caspases. Taking into account the studies of Schmid et al. [68] which have demonstrated that seminal calcium was associated with increased risks of poorer semen quality and genomic sperm defects, these data suggest that calcium overload has a detrimental effect on sperm quality and function. In respect to phosphorus, its roles on fertilizing ability and good sperm qualities are known. Phosphate ion is crucial for the activities of prostatic acid phosphatase (PAP) which has been associated with liquefaction process of semen and adenyl cyclase, the primary regulator of sperm motility [69]. Similar to previously published results [70], decreased level of phosphorus was found in bad semen quality. The low level of seminal phosphorus found in men with teratozoospermia is providing evidence that phosphorus enhances sperm morphology.

In agreement with our data, Ammar et al. [46, 71], Slivkova et al. [72], and Massanyi et al. [73] have shown a significantly increased level of iron in the seminal plasma of men with teratozoospermia than fertile donors. Moreover, significant positive associations were proved between seminal iron level and the percentage of atypical sperm forms and tail abnormalities. According to Agarwal and Saleh [74], the toxicity of iron results from the Fenton and Haber-Weiss reactions, resulting in the formation of highly toxic hydroxyl free radicals from hydrogen peroxide and superoxide ion radicals which can affect lipid proteins and nucleus [28, 75, 76]. Consistently, we previously demonstrated strong relationships between iron and impaired enzymatic antioxidant status, lipid peroxidation, and DNA breaks in a patient with a high level of morphologically abnormal spermatozoa [46]. In this respect, positive relationships were found between the two studied apoptotic biomarkers and the high level of iron supporting the hypothesis that increased iron content may be an important factor involved in the mechanism of oxidative stress-mediated apoptosis in teratozoospermic semen. Based on these results and the previous results, we strongly support the hypothesis that teratozoospermia can be a reflection of apoptosis induction related to increased caspase expression accompanied by true biochemical alterations in their seminal plasma.

In summary, the use of both AO/EB stains and the polycaspase assay data from the present study provide clear evidence that the apoptotic alterations are closely correlated

with the morphological features of sperm, especially to the head and the tail shape. The defiance or overload of seminal trace elements or enzymes may cause functional and qualitative defects on spermatozoon.

The positive correlation between AChE overload, increased levels of Fe, CK, and LDH with apoptotic markers may explain in part the extent of the sperm morphological defect related to an abortive apoptosis phenomenon. The enzymes and microelements in seminal plasma are not directly responsible for the sperm function loss, but act as biochemical markers for normal differentiation of sperm cells. On the other hand, the studied biochemical markers may serve as an objective measure of male fertility.

Acknowledgments The authors thank all couples, doctors, and researchers for their cooperation. We also thank our research team in there productive biology and human cytogenetic laboratory for their support.

Funding information This work was supported by funds allocated to the Research laboratory of Histology and Genetic LR40ES18 by the Ministère Tunisien de l'Enseingement Supérieur et de la Recherche Scientifique.

Compliance with ethical standards

This protocol was approved by the local ethics committee "le comité d'éthique médicale et de recherche de l'hopital universitaire Fattouma Bourguiba de Monastir. All patients and controls had previously given informed consent for the study.

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. De Braekeleer M, Nguyen MH, Morel F, Perrin A. Genetic aspects of monomorphic teratozoospermia: a review. J Assist Reprod Genet. 2015;32(4):615–23. https://doi.org/10.1007/s10815-015-0433-2.
2. Coetzee K, Kruge TF, Lombard CJ. Predictive value of normal sperm morphology: a structured literature review. Hum Reprod Update. 1998;4(1):73–82.
3. Shu JH, Zhang B, Feng GX, Gan XY, Zhou H, Zhou L, et al. Influence of sperm morphology on the outcomes and neonatal status in IVF-ET. Zhonghua Nan Ke Xue. 2010;6(10):897–900.
4. Bonde JPE, Ernst E, Jensen TK, Hjollund INH, Kolstad H, Henriksen TB, et al. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. Lancet. 1998;352(9135):1172–7.
5. Cao X, Cui Y, Zhang X, Lou J, Zhou J. The correlation of sperm morphology with unexplained recurrent spontaneous abortion: a systematic review and meta-analysis. Oncotarget. 2017;8(33):55646–56. https://doi.org/10.18632/oncotarget.
6. Slama R. Time to pregnancy and semen parameters: a cross-sectional study among fertile couples from four European cities. Hum Reprod. 2002;17(2):503–15.
7. Auger J, Eustach F. Standardisation de la classification morphologique des spermatozoïdes humains selon la méthode de David modifiée. Andrologie. 2000;10:358–73.
8. Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis: new insights in stage specificity and link to chromatin remodeling1. Biol Reprod. 2004;70(4):910–8.
9. Baccetti B, Collodel G, Piomboni P. Apoptosis in human ejaculated sperm cells (notulae seminologicae 9). J Submicrosc Cytol Pathol. 1996;28(4):587–96.
10. Shen H-M, Dai J, Chia S-E, Lim A, Ong C-N. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod. 2002;17(5):1266–73.
11. Aziz N, Said T, Paasch U, Agarwal A. The relationship between human sperm apoptosis, morphology and the sperm deformity index. Hum Reprod. 2007;22(5):1413–9.
12. Aitken RJ, De Iuliis GN. Origins and consequences of DNA damage in male germ cells. Reprod BioMed Online. 2007;14(6):727–33.
13. Ammar O, Tesnim A, Zohra H, Amira S, Ines Z, Sana C, et al. Investigation on the origin of sperm morphological defects: oxidative attacks, chromatin immaturity, and DNA fragmentation. Environ Sci Pollut Res. 2018a: 25(14):13775–13786
14. Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril. 2004;81(4):965–72.
15. Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod. 1995;52(4):864–7.
16. Haanen C, Vermes I. Apoptosis and inflammation. Mediat Inflamm. 1995;4(1):5–1.
17. Gorczyca W, Tuziak T, Kram A, Melamed MR, Darzynkiewicz Z. Detection of apoptosis-associated DNA strand breaks in fine-needle aspiration biopsies by in situ end labeling of fragmented DNA. Cytometry. 1994;15(2):169–75.
18. Said TM, Gaglani A, Agarwal A. Implication of apoptosis in sperm cryoinjury. Reprod Biomed Online Reproductive Healthcare Ltd. 2010;21:456–62. Available from:. https://doi.org/10.1016/j.rbmo.2010.05.011.
19. Martí E, Pérez-Pé R, Colás C, Muiño-Blanco T, Cebrián-Pérez JA. Study of apoptosis-related markers in ram spermatozoa. Anim Reprod Sci. 2008;106:113–32.
20. Lee J, Richburg JH, Shipp EB, Meistrich ML, Boekelheide K. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. Endocrinology. 1999;140(2):852–8.
21. Fischer U, Jänicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. 2003;10(1):76–100.
22. Ekert PG, Silke J, Vaux DL. Caspase inhibitors. Cell Death Differ. 1999;6(11):1081–6.
23. Arama E, Agapite J, Steller H. Caspase activity and a specific cytochrome C are required for sperm differentiation in Drosophila. Dev Cell. 2003;4(5):687–97.
24. Cayli S, Sakkas D, Vigue L, Demir R, Huszar G. Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. Mol Hum Reprod. 2004;10(5):365–72.
25. Liu K, Liu P, Liu R,Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. Med Sci Monit Basic Res. 2015;21:15–20 Available from: http://www.basic.medscimonit.com/abstract/index/idArt/893327.
26. El-Garawani IM, El-Nabi SEH, Mohamed AH, El-Esawy HM. Molecular amelioration of Acacia arabica gum on some male reproductive aspects in Schistosoma mansoni infected mice. Res J Pharm, Biol Chem Sci. 2016;7:505–12.
27. Tombes RM, Shapiro BM. Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. Cell. 1985;41(1):325–34.

28. Aitken RJ, Harkiss D, Buckingham DW. Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol Reprod Dev. 1993;35(3):302–15.
29. Noguera Velasco JA, Tovar Zapata I, Martinez Hernandez P, Perez Albacete M, Tortosa Oltra J, Parrilla Paricio JJ. Lactic dehydrogenase-C4 activity in seminal plasma and male infertility. Fertil Steril. 1993;60(2):331–5.
30. Mor I, Grisaru D, Titelbaum L, Evron T, Richler C, Wahrman J, et al. Modified testicular expression of stress-associated "readthrough" acetylcholinesterase predicts male infertility. FASEB J. 2001;15(11):2039–41.
31. Kaufer D, Friedman A, Seidman S, Soreq H. Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature. 1998;393(6683):373–7.
32. Grisaru D, Pick M, Perry C, Sklan EH, Almog R, Goldberg I, et al. Hydrolytic and nonenzymatic functions of acetylcholinesterase comodulate hemopoietic stress responses. J Immunol. 2014;176(1):27–35.
33. Dori A, Cohen J, Silverman WF, Pollack Y, Soreq H. Functional manipulations of acetylcholinesterase splice variants highlight alternative splicing contributions to murine neocortical development. Cereb Cortex. 2005;15(4):419–30.
34. Egbunike GN. Changes in the acetylcholinesterase activity of mammalian spermatozoa during maturation. Int J Androl. 1980;3:459–68.
35. Tvrda E, Peer R, Sikka SC, Agarwal A. Iron and copper in male reproduction: a double-edged sword. J Assist Reprod Genet. 2014;32(1):3–16. https://doi.org/10.1007/s10815-014-0344-7.
36. Rahman MS, Kwon WS, Pang MG. Calcium influx and male fertility in the context of the sperm proteome: an update. Biomed Res Int. 2014;2014:841615. https://doi.org/10.1155/2014/841615.
37. Bindari YR, Shrestha S, Shrestha N. Gaire TN. Adv Appl Sci Res: Effects of nutrition on reproduction—a review; 2013; 4(1):421–429
38. WHO. World Health Organization laboratory manual for the examination and processing of human semen Fifth edition. WHO Press; 2010. 287.
39. Srinivas G, Anto RJ, Srinivas P, Vidhyalakshmi S, Senan VP, Karunagaran D. Emodin induces apoptosis of human cervical cancer cells through poly (ADP-ribose) polymerase cleavage and activation of caspase-9. Eur J Pharmacol. 2003;473(2–3):117–25.
40. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88–95.
41. Brahem S, Mehdi M, Elghezal H, Saad A. Detection of DNA fragmentation and meiotic segregation in human with isolated teratozoospermia. J Assist Reprod Genet. 2011;28:41–8.
42. Mehdi M, Gmidène A, Brahem S, Guerin JF, Elghezal H, Saad A. Aneuploidy rate in spermatozoa of selected men with severe teratozoospermia. Andrologia. 2012;44(1):139–43. https://doi.org/10.1111/j.1439-0272.2010.01152.x.
43. Mehdi M, Khantouche L, Ajina M, Saad A. Detection of DNA fragmentation in human spermatozoa: correlation with semen parameters. Andrologia. 2009;41:383–6.
44. Said TM, Agarwal A, Sharma RK, Thomas AJ, Sikka SC. Impact of sperm morphology on DNA damage caused by oxidative stress induced by β-nicotinamide adenine dinucleotide phosphate. Fertil Steril. 2005;83(1):95–103.
45. Agarwal A, Tvrda E, Sharma R. Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. Reprod Biol Endocrinol. 2014;12:2–9.
46. Ammar O, Haouas Z, Hamouda B, Hamdi H, Hellara I, Jlali A, et al. Relationship between sperm DNA damage with sperm parameters, oxidative markers in teratozoospermic men. Eur J Obstet Gynecol Reprod Biol. 2019; 233:70–75. doi: https://doi.org/10.1016/j.ejogrb.2018.12.003.
47. Smolewski P, Grabarek J, Halicka HD, Darzynkiewicz Z. Assay of caspase activation in situ combined with probing plasma membrane integrity to detect three distinct stages of apoptosis. J Immunol Methods. 2002;265(1–2):111–21.
48. Brugnon F, Ouchchane L, Verheyen G, Communal Y, Van Der Elst J, Tournaye H, et al. Fluorescence microscopy and flow cytometry in measuring activated caspases in human spermatozoa. Int J Androl. 2009;32:265–73.
49. Kuželová K, Grebeňová D, Hrkal Z. Labeling of apoptotic JURL-MK1 cells by fluorescent caspase-3 inhibitor FAM-DEVD-fmk occurs mainly at site(s) different from caspase-3 active site. Cytom Part A. 2007;71(8):605–11.
50. Darzynkiewicz Z, Pozarowski P. All that glitters is not gold: all that FLICA binds is not caspase. A caution in data interpretation—and new opportunities. Cytom Part A. 2007;71(8):536–7.
51. Almeida C, Cardoso MF, Sousa M, Viana P, Gonçalves A, Silva J, et al. Quantitative study of caspase-3 activity in semen and after swim-up preparation in relation to sperm quality. Hum Reprod. 2005;20(5):1307–13.
52. Hichri R, Amor H, Khammari M, Harzallah M, El Fekih S, Saad A, et al. Apoptotic sperm biomarkers and the correlation between conventional sperm parameters and clinical characteristics. Andrologia. 2018;50:1–9.
53. Gherghi IC, Girousi ST, Voulgaropoulos AN, Tzimou-Tsitouridou R. Study of interactions between DNA-ethidium bromide (EB) and DNA-acridine orange (AO), in solution, using hanging mercury drop electrode (HMDE). Talanta. 2003;61(2):103–12. https://doi.org/10.1016/S0039-9140(03)00238-8.
54. Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. Methods Cell Biol. 2012;10(5):12
55. Lecoeur H. Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp Cell Res. 2002;10(5):12.
56. Baskić D, Popović S, Ristić P, Arsenijević NN. Analysis of cycloheximide-induced apoptosis in human leukocytes: fluorescence microscopy using annexin V/propidium iodide versus acridin orange/ethidium bromide. Cell Biol Int. 2006;30(11):924–32.
57. Huszar G, Vigue L, Corrales M. Sperm creatine kinase activity in fertile and infertile oligospermic men. J Androl. 1990;11(1):40–6.
58. Sidhu RS, Sharma RK, Agarwal A. Relationship between creatine kinase activity and semen characteristics in subfertile men. Int J Fertil Womens Med. 1998;43(4):192–7.
59. Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. BiolReprod. 2000;63(3):925–32.
60. Hallak J, Sharma RK, Pasqualotto FF, Ranganathan P, Thomas AJ, Agarwal A. Creatine kinase as an indicator of sperm quality and maturity in men with oligospermia. Urology. 2001;58(3):446–51.
61. Huszar G, Vigue L. Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. Mol Reprod Dev. 1993;34(3):292–8.
62. Mor I, Sklan EH, Podoly E, Pick M, Kirschner M, Yogev L, et al. Acetylcholinesterase-R increases germ cell apoptosis but enhances sperm motility. J Cell Mol Med. 2008;12(2):479–95. https://doi.org/10.1111/j.1582-4934.2008.00231.x.
63. Kılıç Altun S, Dinç H, Paksoy N, Temamoğulları FK, Savrunlu M. Analyses of mineral content and heavy metal of honey samples from south and east region of Turkey by using ICP-MS. Int J Anal Chem. 2017;2017:6391454–6. https://doi.org/10.1155/2017/6391454.
64. Massanyi P, Weis J, Lukac N, Trandzik J, Bystricka J. Cadmium, zinc, copper, sodium and potassium concentrations in rooster and Turkey semen and their correlation. J Environ Sci Health, Part A: Tox Hazard Subst Environ Eng. 2008;43(5):563–5. https://doi.org/10.1080/10934520701796655.

65. Prien SD, Lox CD, Messer RH, DeLeon FD. Seminal concentrations of total and ionized calcium from men with normal and decreased motility. Fertil Steril. 1990;54(1):171–2.
66. Morton BE, Sagadraca R, Fraser C. Sperm motility within the mammalian epididymis: species variation and correlation with free calcium levels in epididymal plasma. Fertil Steril. 1978;29(6):695–8.
67. Wang H, Joseph JA. Mechanisms of hydrogen peroxide-induced calcium dysregulation in PC12 cells. Free Radic Biol Med. 2000;28(8):1222–31.
68. Schmid TE, Grant PG, Marchetti F, Weldon RH, Eskenazi B, Wyrobek AJ. Elemental composition of human semen is associated with motility and genomic sperm defects among older men. Hum Reprod. 2013;28(1):274–82. https://doi.org/10.1093/humrep/des321.
69. Banjoko SO, Adeseolu FO. Seminal plasma pH, inorganic phosphate, total and ionized calcium concentrations in the assessment of human spermatozoa function. J Clin Diagn Res. 2013;7(11):2483–6. https://doi.org/10.7860/JCDR/2013/6194.3585.
70. Ghaniei A, Eslami M, BabaeiMarzango SS. Determination of calcium, magnesium, phosphorus, iron, and copper contents in rooster seminal plasma and their effects on semen quality. Comp Clin Pathol. 2018;27(2):427–31.
71. Ammar O, Houas Z, Mehdi M. The association between iron, calcium, and oxidative stress in seminal plasma and sperm quality. Environ Sci Pollut Res. 2019; 26(14):14097–14105. Available from: http://link.springer.com/10.1007/s11356-019-04575-7.
72. Slivkova J, Popelkova M, Massanyi P, Toporcerova S, Stawarz R, Formicki G, et al. Concentration of trace elements in human semen and relation to spermatozoa quality. J Environ Sci Health, Part A: Tox Hazard Subst Environ Eng. 2009;44(4):370–5. https://doi.org/10.1080/10934520802659729.
73. Massányi P, Trandzik J, Nad P, Lukac N, Skalicka M, Korenekova B, et al. Semen concentration of trace elements in stallions and relation to the spermatozoa quality. Trace Elem Electrolytes. 2004;21(10):229–31.
74. Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am. 2002;29:817–27.
75. Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. Mol Hum Reprod. 2007;13(4):203–11.
76. Aitken RJ, Harkiss D, Buckingham D. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. J Reprod Fertil. 1993;98(1):257–65.