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New perspectives on male fertility evaluation: Innovative approach for the qualitative analysis of spermatozoa

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Abstract

The identification of idiopathic infertility cases, actually, is impossible. Among new functional tests, developed to improve the male fertility diagnosis, the evaluation of spermatic myo-inositol (MI) level, known as Andrositol® test (AT), is one of the most interesting, considering its weak economic burden and ease of use. The aim of this study was to evaluate the predictive power of AT and its potential use for a preliminary evaluation of semen samples. To evaluate the predictive power of AT, 87 sperm samples were analysed in comparison with spermiogram and sperm chromatin dispersion (SCD) Test, the gold standard analyses for male fertility evaluation. The application of AT resulted very useful for a preliminary sample evaluation, predicting the absence of DNA fragmentation in case of Low Responder samples precisely, and the presence of DNA fragmentation in case of medium or High Responder samples with abnormal morphology, predicting SCD results with a probability of 80% for Medium Responder sample and of 96.7% for High Responder sample. Considering the predictive power of this method, we could imagine, as preliminary qualitative analysis, its application before SCD test, deepening sperm analysis, improving the daily activities of laboratory operators and maintaining a good reliability of sperm evaluation.

KEYWORDS
Andrositol® test, idiopathic infertility, male fertility, myo-inositol, sperm quality

1 | INTRODUCTION

In the last decades, the significative reduction in sperm quality is increasingly evident in the population, determining the heavy worsening of male fertility. The main result of this process is the underestimation of sub-fertile population, not easily identifiable (Carlsen, Giwercman, Keiding, & Skakkebaek, 1992; Merzenich, Zeeb, & Blettner, 2010). Indeed, actually, a real and complete assess of male infertility is impossible considering the analytic instruments at our disposal. Nowadays, the evaluation of sperm quality is based on the parameters reported in the WHO guidelines for semen analysis (semen volume, total sperm number, sperm concentration, total motility, progressive motility, vitality and sperm morphology; Gianaroli et al., 2012; WHO, 2010b). In particular, three of these parameters are mostly considered to define the male fertility: total sperm number, morphology and total motility (WHO, 2010b). However, a reliable evaluation of male fertility does not seem possible. The threshold values used refer to statistical distribution of male population and not to the objective quality of semen. Being based on statistical data, this method is strongly conditioned by distributional fluctuations of male population resulting uncertain and needy of a constant threshold updating (the last one is dating from 2010; Menkveld, Holleboom, & Rhemrev, 2011). Moreover, several factors can impair the fertility evaluation outcomes such as fever, emotional distress, alcohol consumption and abuse of drugs. For this reason, a single evaluation, now, is not considered enough (De Rose et al., 2018). Additionally, some pathologies can impair the reproductive capacity transiently or definitively (cryptorchidism, genital

infections, varicocele, testicular torsion, surgical procedures, endocrine disorders, pharmacological therapies, genetic pathologies). Generally, anything that leads to an increase in the oxidative stress is considered as a factor able to impair the sperm quality reducing male fertility. Oxidative stress is a raise of the reactive oxygen species (ROS) which could exceed the total antioxidant capacity of the sperm cell (Gosalvez, Tvrda, & Agarwal, 2017). Physiologically, spermatozoa have a reduced antioxidant power, especially in case of a reduced intake of vitamin E, vitamin C, folic acid, zinc, selenium, inositol, carnitine and carotenoids. These substances, indeed, carry out their protective function acting as scavengers of free radicals. Their shortage is frequently associated with alterations of spermatozoa such as peroxidation of lipid membrane, nemaspermic DNA alteration or fragmentation (Lombardo et al., 2011), worsening of sperm motility or damages of cytoplasmatic and membrane proteins. Most of the data available in literature report levels of radical oxygen species (ROS) significantly higher in the seminal plasma of patients with idiopathic infertility in comparison with fertile males (Benedetti et al., 2012).

Considering the wide number of variables involved and already mentioned, the lack of uniqueness of current methods for male fertility evaluation is not unusual, as confirmed by the increased number of idiopathic infertility diagnosis. In the light of this evidence, the identification of new instruments of evaluation has become increasingly important and the integration of a quantitative approach with a qualitative evaluation of the sperm sample appears fundamental. Among the currently available tests able to evaluate the quality of sperm samples, the main one is undoubtedly the Sperm DNA Fragmentation (SCD—Sperm chromatin dispersion). This test allows to highlight breaks and lesions inside the sperm DNA strands. As reported in literature, sperm samples with a DNA fragmentation over 30% hardly can induce an oocyte fertilisation or a regular pregnancy. The fragmentation threshold defines the accepted limit of breaks inside DNA, a value beyond which pregnancy and embryo development might be impaired (Rex, Aagaard, & Fedder, 2017; Simon, Emery, & Carrell, 2017). This test is considered a valid complement to the standard evaluation of semen according WHO because it allows to analyse the fertilising capacity of spermatozoa suggesting a more accurate therapeutic programme. Unfortunately, spread of SCD was strong limited because of its complexity. Currently, several new tests have been developed and one of the most interesting appears to us the Andrositol® test (AT) based on the evaluation of spermatic myo-inositol (MI) levels. MI, belonging to the family of inositol stereoisomers, represents the most important precursor of the phosphatidylinositol phosphate and is a fundamental second messenger for several cellular pathways regulating functions such as sperm motility, capacitation and the maintenance of physiological intracytoplasmic calcium level. The MI concentration is not equal from the efferent ducts to the ductus deferens but follows a concentration gradient driving the sperm maturation (Beemster, Groenen, & Steegers-Theunissen, 2002; Condorelli et al., 2017). The importance of MI for the development of spermatozoa clearly appears considering that at its concentration peak in the epididymis, this molecule is 28 times than in the rest of body (Vitali, Parente, & Melotti, 1995) and a reduction in this value has been correlated with a decrease in male fertility such as asthenozoospermia, a condition characterised by an increased activity of MI synthesis enzymes as compensatory mechanism. In vitro studies have also reported the capacity of MI to induce a meaningful increase of sperm motility in oligoasthenoteratozoospermic samples after an incubation with this molecule. Regarding the importance of MI for the male fertility, finally, in the context of in vitro fertilisation (IVF), several data demonstrating the improving of in vitro sperm motility and of the fertilisation rate in ICSI after incubation with MI were also collected (Artini et al., 2017; Gulino et al., 2016). MI is involved in several processes inside the male reproductive tract as capacitation, acrosomal reaction, regulation of sperm motility, restoration of mitochondrial crests, increase in mitochondrial membrane potential (MMP) and migration of spermatozoa (Chauvin & Griswold, 2004; Robinson & Fritz, 1979). Considering the MI importance for spermatozoa, the effects of a MI supplementation in patients with idiopathic infertility have been also evaluated. Data collected during the study have demonstrated the capacity of this treatment to improve sperm concentration, sperm total number, acrosomal reactive rate and sperm progressive motility (Condorelli, Vignera, Bari, Unfer, & Calogero, 2011). In view of these data, AT was developed to classify, in a qualitative way, semen samples evaluating the changes of sperm motility after an incubation with MI. Indeed, MI in vitro exposure will induce changes in sperm progressive motility, proportionally to MI levels in seminal fluid before the incubation. For example, samples with a greater deficiency of MI will respond to the incubation with a greater increase in sperm progressive motility corresponding to a worst fertilising quality of spermatozoa. If this test confirms its properties, considering cost and simplicity, it might be a test complementary to the spermiogram for a preliminary qualitative evaluation of semen samples. The aim of this study was to evaluate the predictive power of AT in comparison with methods currently used in the sperm quality analysis and, consequently, its potential use for a preliminary evaluation of semen samples.

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 87 men with an idiopathic infertility, within couples attending the Alma Res Fertility Center, Rome, Italy, between June 2017 and June 2018, were involved in this study (mean age was 42.14 ± 7.61 years). A physician and instrumental evaluation for all patients was performed. Men with systemic and endocrine diseases, reproductive system infections, history of cryptorchidism or varicocele, heavily smokers, usual consumers of alcohol and/or drugs and subjected to a recent hormonal treatment were excluded. The

protocol was approved by the Local Ethics Committee, and informed consent was obtained from all study participants.

2.2 | Samples

Semen samples were collected by masturbation after 3–5 days of sexual abstinence. All samples were allowed to liquefy at 37°C for 60 min and were then assessed according to World Health Organization Laboratory Manual (WHO, 2010a). The parameters evaluated were semen volume, total sperm number, sperm concentration, viscosity, fluidification, density, total motility, progressive motility, morphology and vitality. After liquefaction, simultaneously with spermiogram, two sample aliquots were recovered from all samples for the qualitative evaluations with AT and SCD.

2.3 | Andrositol® test

In the aliquot recovered for AT, MI (Andrositol® DGN, Lo. Li. Pharma) was added at the final dilution of 2 mg/ml following the manufacturer's instruction to perform AT. After the addition of MI, a 30-min incubation at 37°C was performed and it was subsequently evaluated the change of progressive motility in comparison with the same parameter registered during spermiogram. Samples with increases over 60% (considered with a poor quality) will be defined High Responder (HR), those with increases ranging between 30% and 60% will be considered Medium Responder (MR), while samples with increases below 30% (and then with a good quality) will be considered Low Responder (LR).

2.4 | Sperm chromatin dispersion

The second aliquot was used to perform SCD using HaloSperm G2® test (HTHSG2; Selinion Medical) a commercial kit, produced by Halotech DNA. This test was performed within 3 hr following the sample collection, as described in the manufacturer's instruction. The sperm DNA, during this analysis, becomes visible at microscopy on bright field after staining. Spermatozoa with not fragmented DNA form loops visible as halo at microscopy. In case of fragmented DNA, instead, there are not loops and for this reason halos are not visible.

2.5 | Statistical analysis

To understand the capability of AT to predict the SCD result, Cohen's kappa coefficient (κ) was performed in terms of normal or abnormal results (assessed according to World Health Organization Laboratory Manual (WHO, 2010a) for the spermiogram and on the basis of manufacturer instructions of SCD and AT). AT allows qualitative classification of sperm samples in three classes (Low, Medium or High Responders) differently from spermiogram and SCD which use only two possible results (normal or abnormal and not fragmented or fragmented respectively). For this reason, to optimise the comparison between SCD, spermiogram and AT, sperm samples belonging to the Low Responder class, considered by the manufacturer good quality samples, here were considered normal while sperm samples belonging to High Responder and Medium Responder classes, considered by the manufacturer MI-deficient samples, here were considered abnormal although with different severity grade. Considering the complete overlapping between results of AT, SCD and spermiogram related to normal samples, agreement and Cohen's kappa coefficient (κ) were evaluated only for abnormal results. In this regard, agreement and Cohen's kappa coefficient (κ), related to the SCD versus spermiogram and its three main parameters (morphology, total motility and number of spermatozoa) in all patients and in AT classes corresponding to abnormal results (Medium Responder and High Responder), were performed. Moreover, this comparison was depth analysing sensibility, specificity, accuracy and positive/negative predictive value of SCD versus spermiogram and its three main parameters (morphology, total motility and number of spermatozoa) in all patients and in AT classes corresponding to abnormal results (Medium Responder and High Responder). Statistical analyses were implemented using Stata™ version 8.2.

3 | RESULTS

A total of 87 semen samples, obtained by an equal number of men, were evaluated by spermiogram, SCD and AT. Baseline characteristics of patients are reported in Table 1. Data obtained were crossed to analyse the samples characteristics and to validate deriving results (Table 2). In first instance, at spermiogram analysis, 40 samples resulted normospermic (45.9% of total population) while remaining 47 resulted

TABLE 1 Baseline characteristics of normospermia and oligoasthenospermia patients

	Normospermia	Oligoasthenospermia	*t* test (*p*)
Age (Years)	43.71 ± 7.47	41.83 ± 8.41	NS
Weight (kg)	77.82 ± 3.12	78.41 ± 2.74	NS
Volume (ml)	3.16 ± 1.54	3.02 ± 1.32	NS
Concentration (CI/ml)	63.53 ± 20.42	28.50 ± 18.37	<.05
Total motility (%)	54.83 ± 9.20	37.02 ± 12.36	<.05
Morphology (%)	93.37 ± 1.29	96.91 ± 1.23	<.05
Number of Spermatozoa ($\times 10^6$)	190.62 ± 98.40	88.76 ± 41.10	<.05

TABLE 2 Percentages of samples distribution and cross of data obtained by Spermiogram, SCD, AT and Morphological

			SG		SCD		AT			MO		AT/MO					
		N	NO	AB	<30%	>30%	LR	MR	HR	NO	AB	LR/NO	MR/NO	HR/NO	LR/AB	MR/AB	HR/AB
		87	**45.9**	**54.1**	**49.4**	**50.6**	**10.3**	**23.0**	**66.7**	51.7	48.3	8.1	11.5	32.1	2.3	11.5	34.5
SG	NO	40	–	–	87.5	**12.5**	17.5	**20.0**	**62.5**	100.0	0.0	17.5	20.0	62.5	0.0	0.0	0.0
	AB	47	–	–	17.1	82.9	4.3	25.5	70.2	10.6	89.4	0.0	4.3	6.4	4.3	21.2	63.8
SCD	<30%	43	81.4	18.6	–	–	20.9	25.6	53.5	88.4	11.6	16.3	20.9	51.2	4.6	4.6	2.4
	>30%	44	11.4	88.6	–	–	**0.0**	**20.5**	**79.5**	15.9	84.1	0.0	2.3	13.6	0.0	18.2	65.9
AT	LR	9	77.8	22.2	**100.0**	**0.0**	–	–	–	77.8	22.2	77.8	0.0	0.0	22.2	0.0	0.0
	MR	20	40.0	60.0	55.0	45.0	–	–	–	50.0	50.0	0.0	50.0	0.0	0.0	50.0	0.0
	HR	58	43.1	56.9	39.7	60.3	–	–	–	48.3	51.7	0.0	0.0	48.3	0.0	0.0	51.7
MO	NO	45	88.9	11.1	**84.4**	15.6	15.6	22.2	62.2	–	–	15.6	22.2	62.2	0.0	0.0	0.0
	AB	42	0.0	100.0	11.9	**88.1**	4.8	23.8	71.4	–	–	0.0	0.0	0.0	4.8	23.8	71.4
AT/MO	LR/NO	7	100.0	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0	–	–	–	–	–	–
	MR/NO	10	80.0	20.0	90.0	10.0	0.0	100.0	0.0	100.0	0.0	–	–	–	–	–	–
	HR/NO	28	89.3	10.7	78.6	21.4	0.0	0.0	100.0	100.0	0.0	–	–	–	–	–	–
	LR/AB	2	0.0	100.0	100.0	0.0	100.0	0.0	0.0	0.0	100.0	–	–	–	–	–	–
	MR/AB	10	0.0	100.0	20.0	**80.0**	0.0	100.0	0.0	0.0	100.0	–	–	–	–	–	–
	HR/AB	30	0.0	100.0	3.3	**96.7**	0.0	0.0	100.0	0.0	100.0	–	–	–	–	–	–

Abbreviations: AB, Abnormal; AT, Andrositol Test; HR, High Responder; LR, Low Responder; MO, Morphology; MR, Medium Responder; *N*, Number of samples; NO, Normal; SCD, sperm chromatin dispersion (Sperm DNA Fragmentation); SG, Spermiogram; TN, Total.
Values indicated in bold are percentages cited.

TABLE 3 Agreement analysis of Sperm DNA fragmentation (SCD) versus spermiogram and its three main parameters (morphology, number of spermatozoa and total motility) in all patients and in Medium and High Responder classes for AT

	Agreement (%)	Expected agreement (%)	κ	*SE*	*p*-Value
All patients					
SCD versus spermiogram	85.06	50.05	.7009	0.107	<.0001
SCD versus morphology	86.21	49.98	.7242	0.1071	<.0001
SCD versus number of spermatozoa	60.92	49.66	.2236	0.0863	.0048
SCD versus total motility	63.22	49.72	.2685	0.0927	.0019
Medium responder class					
SCD versus spermiogram	85.00	49.00	.7059	0.2137	.0005
SCD versus morphology	85.00	50.00	.7000	0.2225	.0008
SCD versus number of spermatozoa	75.00	52.00	.4792	0.2124	.012
SCD versus total motility	55.00	53.00	.0426	0.1893	.4111
High responder class					
SCD versus spermiogram	86.21	51.43	.7160	0.716	<.0001
SCD versus morphology	87.93	50.36	.7569	0.1293	<.0001
SCD versus number of spermatozoa	51.72	43.58	.1444	0.0893	.0529
SCD versus total motility	63.79	45.36	.3373	0.1051	.0007

oligoasthenospermic (54.1% of total population). Subsequently, the same population at SCD analysis, resulted fragmented in 44 cases of 87 (50.6%) and not fragmented in the remaining 43 cases (49.4%). Instead, at AT analysis, samples were reclassified based on the correlation between quality and MI level, highlighting an important shortage of MI in 78 sperm samples of 87 (89.7% of total population), of which 20 were Medium Responder (23.0%) and 58 were High Responder (66.7%), while only in nine cases of 87 (10.3%) sperm samples appeared to have a good quality. Furthermore, combining AT and spermiogram results, it appeared that, of 40 normospermic

TABLE 4 Sensitivity, specificity, accuracy, positive and negative predictive values of SCD versus spermiogram and its three main parameters (morphology, number of spermatozoa and total motility) in all patients and in Medium and High Responder classes for AT

	Sensitivity	Specificity	Accuracy	Positive predictive values	Negative predictive value
All patients					
SCD versus spermiogram	82.98	87.50	0.85	81.40	88.64
SCD versus morphology	88.10	84.44	0.86	88.37	84.09
SCD versus number of spermatozoa	77.78	56.52	0.61	90.70	31.82
SCD versus total motility	77.27	55.46	0.63	88.37	38.64
Medium responder class					
SCD versus spermiogram	75.00	100.00	0.85	100.00	72.73
SCD versus morphology	80.00	90.00	0.85	88.89	81.82
SCD versus number of spermatozoa	83.33	71.43	0.75	55.56	90.91
SCD versus total motility	50.00	56.25	0.55	22.22	81.82
High responder class					
SCD versus spermiogram	90.91	80.00	0.86	85.71	86.96
SCD versus morphology	96.67	78.57	0.88	82.86	95.65
SCD versus number of spermatozoa	81.82	44.68	0.52	25.71	91.30
SCD versus total motility	93.75	52.38	0.64	42.86	95.65

patients at spermiogram analysis, 82.5% were Medium Responder (20.0%) or High Responder (62.5%). Moreover, crossing AT and SCD data, it was possible to highlight that in all good quality samples for AT analysis, the DNA fragmentation was <30% (100% of samples). On the contrary, fragmented samples were presented only in the Medium Responder group (20.5%) and in the High Responder group (79.5%) confirming a worse sperm quality. Finally, combining AT data with one spermiogram parameter, the spermatozoa morphological evaluation, it was possible to predict accurately sperm DNA fragmentation. Based on data collected in this work, samples morphologically normal were not fragmented in 38 of 45 cases (84.4%) while samples morphologically abnormal were fragmented in 37 cases of 42 (88.1%). These data were strengthened also in the subpopulations deriving by crossing of AT and morphological evaluations results. In 30 High Responder samples, morphologically abnormal, 29 cases were also fragmented, previewing SCD results with a sensitivity of 96.7%. These data confirm the usefulness of AT to classify sperm quality thanks to its high sensibility. This last consideration is supported by statistical analysis of data collected: the reliability of this test was calculated considering agreement and Cohen's kappa coefficient (κ) (Table 3), and calculating accuracy, sensibility, specificity and predictive power (Table 4) of SCD versus spermiogram and its three main parameters (morphology, total motility and number of spermatozoa) in all patients and in AT classes corresponding to abnormal results (Medium Responder and High Responder).

4 | DISCUSSION

The increasing reduction in male fertility is a problem that increasingly worries the scientific community and the lack of analytical tools able to give patients a sure diagnosis is exacerbating this issue. The impossibility to achieve pregnancy within 12 months of unprotected sexual intercourses represents a condition that involves about 100 million of persons in the world (Agarwal, Mulgund, Hamada, & Chyatte, 2015; Hamada, Esteves, Nizza, & Agarwal, 2012) but actually a real and complete estimate of infertility burden is not possible because a unique distinction between normal and abnormal cases is not always possible (Duca, Calogero, Cannarella, Condorelli, & Vignera, 2019). In fact, there is a rate of men, characterised by normal spermiogram parameters, according to WHO, unable to achieve pregnancy. One limit of the standard evaluation methods is the application of threshold values not completely functional to identify the real fertilising capability of patients because based on the statistical distribution of males and not on objective characteristics related to sperm quality (Agarwal et al., 2019). To improve this anomaly, several tests for qualitative evaluations were developed and one of these was reported in the WHO guidelines (WHO, 2010a), the sperm DNA fragmentation test called also SCD. This test allows to highlight breaks and lesions in the sperm DNA. Indeed, in literature, it is known that sperm samples, with an excessive number of breaks, hardly can induce a pregnancy. The fragmentation threshold value (>30%) represents in a reliable way the tolerance limit of breaks inside of sperm DNA. This evaluation allows to understand the functionality of a sample and not only to know the quantitative sample parameters. Despite this new qualitative method, till today, the identification of sub-fertile male patients (i.e., patients with idiopathic infertility defined as the impossibility to start a pregnancy within 12 months of unprotected sexual intercourses with a normal partner despite normal spermiogram parameters), following WHO guidelines, is very difficult (Agarwal et al., 2019; Duca et al., 2019). In the last years, several functional tests were developed, like SCD analysis or the evaluation of Mitochondrial Membrane Potential, complex methods determining unavoidably an increase in processing costs. In this context, AT is proposed as a linking bridge between different analytics dimensions, being a functional test, easy to use, with a weak economic burden, that allows a qualitative evaluation of sperm samples analysing myo-inositol level of spermatic fluids, a fundamental molecule for spermatozoa motility (Artini et al., 2017; Calogero, Gullo, Vignera, Condorelli, & Vaiarelli, 2015; Colone et al., 2010; Condorelli, Vignera, Bellanca, Vicari, & Calogero, 2012; Condorelli et al., 2011, 2017). Thanks to AT, it is possible to distinguish three spermatic classes evaluating the modifications of linear progressive motility, a useful parameter to understand the real fertilising capacity of spermatozoa (Scarselli et al., 2016). Aim of the present study was to evaluate AT potentiality to highlight sperm samples with a good quality, taking as gold standard the analytics capacities of spermiogram and SCD. The previously reported spermiogram limits, in identifying sperm samples with a good quality, emerged clearly considering a partial overlapping with SCD data. Indeed, this last method has identified an important rate of fragmented samples (12.5%) inside of the group of normospermic samples for spermiogram. Moreover, thanks to SCD, eight cases of 47 abnormal at spermiogram were found to have a good quality. Passing then to AT analysis, a better stratification is possible, with a reclassification of samples that highlights an important shortage of MI in 78 cases of 87 (89.7%), of which 20 were Medium Responder (23.0%) and 58 High Responder (66.7%) while nine cases of 87 (10.3%) were Low Responder samples accordingly with a good quality. In this regard, crossing data obtained from SCD and AT, the capability of the latter test to identify samples with a good quality clearly appeared. Indeed, all Low Responder samples were not fragmented (100%) while fragmented samples were reported in the Medium Responder group (20.5%) and in the High Responder group (79.5%). The predictive power of AT resulted even stronger crossing AT data with the morphological evaluation of samples. From data collected in this study, the samples that were normal at morphological evaluation, resulted not fragmented in 38 cases out of 45 (84.4%); furthermore, the samples that were abnormal at the morphological evaluation resulted fragmented in 37 cases out of 42 (88.1%). As anticipated, these data were even more evident crossing AT and morphological evaluation. Indeed, it emerged that between the morphologically abnormal samples, in 29 cases of 30 (96.7%) of High Responder samples and in eight cases of 10 (80%) of Medium Responder samples also a DNA fragmentation >30% was found, while in the Low Responder group no cases of DNA fragmented were observed. For this reason, the application of AT can be very useful for a preliminary sample evaluation, predicting an absence of DNA fragmentation in case of Low Responder

samples, and a presence of DNA fragmentation in case of Medium or High Responder samples with abnormal morphology, predicting SCD results with a probability of 80% for Medium Responder sample and of 96.7% for High Responder sample. These data show the advantages deriving from AT application as preliminary analysis, a method useful to integrate the spermiogram and to anticipate SCD results. Indeed, thanks to its high sensibility, AT can complete the spermiogram evaluation with a qualitative data quickly, without weigh down the laboratory practice but rather simplifying it, giving a reliable forecast of SCD results.

In conclusion, then, thanks to AT, it was possible to analyse, in a reliable way, the sperm samples, allowing a qualitative analysis already in the preliminary evaluations and, for this reason, its application right from the start should be promoted. Obviously, our observations will be confirmed in further studies.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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