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The aging male: Relationship between male age, sperm quality and sperm DNA damage in an unselected population of 3124 men attending the fertility centre for the first time

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Summary *Objective: the aim of our study was to put forward insights to treat any possible correlation among sperm quality, sperm DNA damage and male age as they may have fertility implications for men who choose to delay fatherhood.*

Materials and methods: Our study is a non-interventional retrospective analysis of 3124 semen samples from patients that were investigated for the conventional semen parameters. Tunel test assay was set up for the evaluation of the sperm DNA fragmentation index (DFI). We applied the Kappa index to compare both the 1999 and the 2010 World Health Organization (WHO) reference criteria to evaluate the competence of such semen parameters categorization during the standard routine of our laboratory.

Results: With regards to our findings, it is possible to underline a significant relationship between aging and semen volume ($p = 0.001$), motility ($p = 0.009$), semen viscosity ($p < 0.003$) and sperm DNA damage ($p < 0.009$). We found a trend when focusing on the semen concentration ($p = 0.05$). The analysis of sperm morphology did not show any influence with advancing age ($p = 0.606$). When comparing both the 1999 and the 2010 WHO scales we found no accordance in the appraisal of sperm morphology but a very good one in the evaluation of the other parameters.

Conclusions: Conventional semen analysis represents the opportunity to draw up a proxy insight on the male fertility status even if semen quality can only indirectly assess the probability of pregnancy. Several studies have verified a decay in the male reproductive system, sperm quality and fertility with advancing age although the reported results are not yet conclusive. Our results substantially agree with those findings outlined in the literature. Moreover we find that the discrepancy between the two WHO reference scales would eventually lead to an improper diagnosis of infertility.

KEY WORDS: *Aging; Male; Semen; Analysis; WHO; Infertility.*

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INTRODUCTION

Infertility has a wide impact on public health. In developed countries, where expectancy of a prolonged life is well established, modern trends have enforced the delay of first parenthood (1). It partly reflects the complications of an overwhelming course marked by the achievement of individual goals such as education and economic solidity prior to attempting conception (2). Aging is explained by biological and demographic topics characterized by the impairment of several physiological functions and to what concern human reproduction, age related decrease of couples fertility potential is usually associated to female aging (1, 3). Female germ cells are lessened during life span and do not replace (4). Therefore, as ovary grow older, the total number of oocytes and their quality decrease, lowering female fecundity (5). Female age has been explained as a predictor of poor reproductive success resulting in decreased fertilization and implantation rates as well as in increased abortion rates (5). Efficient reproduction and early embryonic development mostly rely on oocyte quality with those from older women being more prone to nondisjunction caused by meiotic errors and therefore impaired by an increased occurrence of aneuploid abnormalities (4, 5). Male reproductive functions do not suddenly come to an end as spermatogenesis continues into advanced ages. Consequently men can conceive children at later ages (6). Anyway a large number of studies have marked a linkage between advanced male age and the decrease in fertility potential status (1, 3, 7). The effect of advanced paternal age on embryo quality, miscarriage rate or pregnancy rate has been assessed for the general population and for infertile patients outlining an increased time-to-pregnancy disorder (2, 8). Older fathers are reported to imply higher rates of miscarriages and several diseases in the new generations: altered designs of genetic expression in aged male are related to a wide range of genetic disorders through descendants as the rate of denovo inheritable mutations is strictly linked to the male age (8). Substantial interest exists in studying effects of aging on semen quality and sperm DNA damage (1, 3, 7, 9). Conventional semen analysis represents the opportunity to draw up an insight on the male fertility status even if semen quality is an indirect measure of the probability of pregnancy (7). Advanced paternal age is related to a decline in sperm quality and to an increased sperm DNA damage referring to a combination

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of genetic and environmental factors which can lead to a condition of oxidative stress impairing the integrity of sperm DNA (1, 10). Several studies tried to fix an age effect on semen quality indicating a broad trend in age ranges (1) but the effect of advanced paternal age on semen parameters is not yet conclusive and its impact on fertility is still debated (1, 2). Our study is then meant to put forward stronger insights to help clarify any possible correlation among sperm quality, sperm DNA damage and male age as they may have fertility implications for men who choose to delay fatherhood.

Materials and methods

Patients enrolled in this study were recruited from January 2009 to December 2012. Semen samples were obtained from unselected males attending, for the first time in their life, the andrology laboratory both for infertility problems as well as for a spontaneous screening provided for free, during the so-called "*Fertility Days*" arranged at our *Centre*. Each patient produced a semen sample by masturbation into a sterile plastic container. As the number of days of abstinence may have influence on semen parameters, patients included in this study were previously taught to observe 2 up to a maximum of 5 days of abstinence from intercourse before their planned analysis. Semen samples were collected in a room next to the laboratory. All the samples were allowed to liquefy for at least 20 minutes at 37°C, prior to be processed. Analysis of semen samples, liquefaction, viscosity and volume were recorded. A 10 µL drop was examined using phase contrast microscopy to assess sperm concentration, motility and morphology according to WHO guidelines for the examination and processing of human semen (11). We both used the 2010 and the 1999 reference cut-off values to evaluate the competence of such semen categorization during the standard routine of our laboratory (12). A raw portion of the semen sample was then taken out at the time of the semen evaluation. It was immediately processed for the subsequent assessment of the DFI (DNA fragmentation index) setting up the Tunel test assay. Visualization of fragmented sperm nuclear DNA was performed with the use of Cell Death Detection Kit with tetramethylrhodamine-labelled dUTP (*Roche, Monza, Italy*), according to the manufacturer's instructions. Ejaculated sperm samples were washed from seminal plasma by low-speed centrifugation, smeared on microscope slides, air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C for 25 minutes, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Spermatozoa with fragmented DNA were detected in an epifluorescence microscope with a X100 oil immersion objective and the percentage of TUNEL-positive spermatozoa was determined (13).

Statistical analysis

Shapiro-Wilk test was used to test normality assumption. Chi square test (or Fisher exact test when appropriate), was used to compare categorical variable. To set up the evaluations, records were ranked by age brackets to create three consecutive age groups each containing suitable observations to be analysed as described in previous studies. Bonferroni correction was used to adjust multiple comparisons. Non parametric Mann-Withney test was used for continuous variables. To compare classification of same subjects with two different scale, Mc Nemar test was used. Kappa index was then calculated to evaluate accordance between the two scale using the Landis-Koch reference model (14). Such model ranges from 0 to 1 to point out respectively "*no-accordance*" (value = 0) and "*overlapping conformity*" (value = 1) between the used examiners (Table 1). A p *value* < 0.05 was considered statistically significant.

Results

Population characteristics

Our study is a non-interventional retrospective analysis of 3124 semen samples from patients that were investigated for the conventional semen parameters and the DFI. None of the men included in the study revealed having or having suffered cryptorchidism, hypospadias or testicular cancer. All the patients enrolled in the study were investigated for smoking habits and none of them has disclosed a heavy consumption of cigarettes. When considering the whole sample, a share of around 65% of the men investigated were found to be pathological for at least one of the features investigated: concentration, motility or morphology as well. The remaining 35% was normozoospermic. The age of the patients ranged from 21 to 65 with a mean of 40 years (SD 5, 9). The results of our study were allocated to three age groups: younger than 35 years (n = 775, 25%); 36-40 years (n = 1110, 35%); 41 years and older (n = 1239, 40%).

Semen volume

The median semen volume was 3 ml (Table 2). Patients with normal volume values were 87% of the entire sam-

Table 1.
Landis-Koch model.

< 0	Poor
0-20	Slight
0.2-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.00	Almost perfect

Table 2.
Median and interquartile range (IQR, 25th-75th percentile) of semen parameters.

N	Volume (ml)	Sperm concentration 10^6/ml	Sperm motility % (a+b+c)	Sperm morphology %	Sperm DNA damage
3124	3 (2,4)	24 (12, 38)	60 (50, 70)	4 (3, 7)	5 (2, 10)

Table 3.
Median and interquartile range (IQR, 25th-75th percentile) of semen parameters.

N	< 35 years		36-40 years		>= 41 years		
	N	%	N	%	N	%	*p value*
N	775	-	1110	-	1239	-	-
Volume	77	9.9	130	11.7	189	15.3	0.001
Concentration	281	36.3	396	35.7	390	31.5	0.037
Motility	138	17.8	202	18.2	279	22.5	0.009
Morphology	397	51.2	546	49.2	631	50.9	0.606
Viscosity (augmented)	195	25.2	287	25.9	398	32.1	< 0.001

ple. Those with abnormal ones were 13%. When referring to the whole sample we found a weak relationship between the two variables investigated (r = 0,1). On the contrary, when we focused attention only on the subjects who were estimated having abnormal volume values, we found a significant trend toward age groups (p = 0,001) and the number of patients with abnormal semen volume raising up as age increased.

Sperm concentration and total sperm count
The median sperm concentration was $24*10^6$/ml (Table 2). Patients with normal sperm concentration were 66% of the entire sample, those with abnormal ones were 34%. We found a trend when focusing on the whole population (r = 0.04, p = 0.05) and a trend (p = 0.037) across the three groups but no clear post-hoc analysis confirmation emerged. Total sperm count analysis failed to point out any relationship with age (p = 0.769) among groups.

Motility
The median motility was 60% (Table 2). Patients with normal motility values were 80% of the entire sample, those with abnormal ones were 20%. No clear trend was revealed for the whole sample analysis (r = -0.01). Quite the opposite emerged when we analyzed only the subjects who were estimated having abnormal values. We found a significant trend toward age groups (p = 0,009) and the number of subjects with abnormal assessments raised up as age increased.

Morphology
The analysis of sperm morphology, with the sample equally divided in normal and abnormal estimations, (median value = 4, r = 0.01) did not show any influence by advancing age (p = 0.606).

Semen viscosity
Twenty-eight percent of our sample reported an increased semen viscosity estimation. It was possible to point out a clear deviation within age groups (p < 0.001) toward the older one (p < 0.003).

Sperm DNA damage
The analysis for the three age groups point out a clear trend toward older subjects (p < 0.009). When we have analysed the relationship between sperm morphology linked to sperm DNA fragmentation, we found a negative correlation between the variables investigated both for the whole sample (r = -0.16, p < 0.001) and for all the three age groups respectively: r = -0.17; r = -0.16; r = -0.18 (p < 0.001).

Comparison between WHO 2010 and WHO 1999
Once the primary data analysis was concluded (Table 3), we have reloaded the data of the whole population. To this, we have applied the older WHO reference values scale (12) for semen analysis (Table 4). In this subsequent investigation we found again that the third group, that including men at older ages, still issued a significant difference regarding semen values if referred to the other two groups and mainly, when evaluating concentration and motility, toward the first group of younger subjects. Considering the amount of difference between the scale currently in use and the 1999 replaced one, we have decided to match them up, so to evaluate any possible divergence which could reveal a clinical weight in the IVF treatment of couples. To this purpose, McNemar test

Table 4.
Number of subjects with abnormal values (WHO, 1999).

N	< 35 years		36-40 years		>= 41 years		
	N	%	N	%	N	%	*p value*
N	775	-	1110	-	1239	-	-
Volume	170	21.9	260	23.4	359	29.0	0.001
Concentration	376	48.5	524	47.2	531	42.9	0.023
Motility	216	27.9	332	29.9	416	33.6	0.018
Morphology	758	97.8	1072	96.6	1203	97.1	0.295
Viscosity (Augmented)	195	25.2	287	25.9	398	32.1	< 0.001

Table 5.
Two scale comparison (WHO, 1999 vs. WHO, 2010).

			WHO (1999)		WHO (2010)			
		Total	**n**	**%**	**n**	**%**	***p value****	**Kappa**
Whole sample								
	Volume	3124	789	25.3	396	12.7	< 0.001	0.601
	Concentration	3124	1431	45.8	1067	34.2	< 0.001	0.761
	Motility	3124	964	30.9	619	19.8	< 0.001	0.713
	Morphology	3124	3033	97.1	1574	50.4	< 0.001	0.059
< 35 years								
	Volume	775	170	21.9	77	9.9	< 0.001	0.564
	Concentration	775	376	48.5	281	36.3	< 0.001	0.753
	Motility	775	216	27.9	138	17.8	< 0.001	0.719
	Morphology	775	758	97.8	397	51.2	< 0.001	0.046
36-40 years								
	Volume	1110	260	23.4	130	11.7	< 0.001	0.605
	Concentration	1110	524	47.2	396	35.7	< 0.001	0.766
	Motility	1110	332	29.9	202	18.2	< 0.001	0.685
	Morphology	1110	1072	96.6	546	49.2	< 0.001	0.066
> =41 years								
	Volume	1239	359	29.0	189	15.3	< 0.001	0.612
	Concentration	1239	531	42.9	390	31.5	< 0.001	0.760
	Motility	1239	416	33.6	279	22.5	< 0.001	0.730
	Morphology	1239	1203	97.1	631	50.9	< 0.001	0.060

**Mc Nemar test.*

was applied to the entire sample and then to the three different groups (Table 5). Kappa index was then used to evaluate the accordance between the two different WHO reference values scales (Table 5). It is possible to highlight greater percentages of subjects with pathological semen evaluations when the 1999 WHO scale is applied due to its stringent criteria of estimation. Moreover, Kappa index exhibits a valuable accordance between the two guidelines if referred to the estimation of volume, concentration and motility. On the contrary, such correspondence failed when we analyzed the morphology parameters determining the misidentification of a remarkable infertility male factor (Table 5).

Discussion

Several studies have demonstrated a decline in the male reproductive system, sperm quality and fertility with advancing age but many of the biological mechanisms of such processes are poorly understood and the reported results are not conclusive (2). In the present analysis, methods, personnel, and instrumentation were consistent throughout the study period because study settings represent a topic of wide importance: earlier attempts to draw conclusions from pooled data from different publications or from the same investigators at different locations have been troubled by differences in methods, personnel, and instrumentation. The lack of standardization in analytic techniques weakened statistical analysis of pooled data by compounding the existent inherent variability in measured semen characteristics. Moreover, in our study we enrolled subjects at older ages preventing the loss of statistical power as occurred in some of the past clinical studies, where older patients (e.g. > 50 years) were under-represented (3, 15). In this way a limited age range may have improved the possibility to ignore the threshold age at which the biological consequences of aging are disclosed. With regards to our current findings, it is possible to underline a significant relationship between advancing age and semen volume, viscosity, motility and sperm DNA damage and a trend for sperm concentration. Despite other previous findings we could underline no significant relationship emerging when age and sperm morphology were linked (3). Quite a lot of mechanisms have been suggested to explain how advancing age may influence semen quality (3). For instance abnormal semen volume alterations could be caused by seminal vesicle deficiency as vesicle fluid itself largely provides most of the ejaculate volume (16). Variations in the prostate arise with aging and the related decay in protein and water contents can clarify the negative impact on semen volume, motility and viscosity (16). Once more, the epididyms have a valuable importance: considering sperm acquiring its straight motility only during the transition through the epididymal tract, any alteration may negatively restrict this parameter (3). Aging may regulate tubular lumen suffering a condition of sclerosis determining a decline in the spermatogenic activity, loss of germ cells proliferation and a deficiency of Leydig cells functions (7). Testes compromised responsiveness to the endocrinological stimuli can possibly explain the alterations of sperm concentration and the increase in sperm count with age may be also explained by urogenital infections (9). Spermatozoa are constantly produced by the testes as germ cells and go through enduring replication, meiosis and spermiogenesis. Cell selection is a major process during early years. It has been speculated that when DNA damage is induced in cells of young men equally levels of apoptosis also increase to eliminate cells with

irreversible DNA damage (17). On the contrary, when DNA damage is induced in cells from older men, apoptosis do not increase, implying that the ability to remove damaged cells decrease (17). In such a way it can be assumed the existence of age-induced anomalies in the apoptotic pathway leading to an age-linked increase in sperm cells with higher DNA damage (17). Confirming the contradiction of the literature published results and in spite of what other authors reported (2, 3, 16, 17), we found no decline in sperm concentration levels with advancing age. Sperm DNA damage has been associated to both male aging and infertility and it has been linked to several factors such as oxidative stress, abortive apoptosis and deviations in the process of recombination and protamine replacement during spermatogenesis (8). Oxidative stress occurs when the generation of free radicals and the scavenging ability of antioxidants become unbalanced. The performance of antioxidant enzymes from older men may be reduced if compared to those of younger ones leading spermatozoa to be more suitable for mutational changes due to the endogenous activity of ROS (8). High DNA fragmentation is linked to diminished sperm concentration, motility and morphology. Decreased fertilization rates and implantation rates are associated to DNA fragmentation (8). Higher levels of sperm DNA damage are associated to decreased embryo quality and pregnancy rates (10). Several studies dealt with the relationship between sperm damaged DNA and the impairment of embryonic developmental stages and it has been marked a strong evidence of lowered quality in embryo development at later stages (4). As a final point, it has been long recognized that the female aging increases the odds for meiotic errors in oogenesis resulting in offspring with chromosome affected by aneuploid abnormalities (4, 5, 18). Therefore, there seems to be a lesser impact for paternal than maternal age on the couple fertility potential (4, 18). Nevertheless, various authors have pointed out that the oocytes are able to mend up DNA damages of paternal origin thus saving the aging spermatozoa (19). Consequently, repairing mechanisms of oocytes from older women may be less competent and may give rise to a condition of infertility or to a prolonged time to pregnancy (19). In conclusion there are lots of difficulties in using semen parameters to screen and clear male health condition when considering the wide impact of the physiological variations of spermatogenesis combined with the distinct lifestyle habits and the growing effects of a lifelong exposure to those factors usually considered as exogenous pollutants (20).

Conclusions

The semen analysis stands for one of the first essential steps in the assessment of the male fertility condition (3). Starting from 2010 renewed semen standard thresholds has been defined for the WHO reference manual (11). This resulted in the statement of lower cutoffs than the previously practiced to handle semen analysis. Comparing both the 1999 and the 2010 reference values in the evaluation of broad semen parameters, we found no accordance in the appraisal of sperm morphology but a very good one in the evaluation of the other parameters. We can therefore speculate that, based on such innovation, a large number of couples previously detected with male factor infertility would now be ranked as having normozoospermia. In such a way, we find that the discrepancy between the two WHO reference scales would eventually lead to an improper diagnosis of infertility. As the new reference values are almost similar to the previous ones, it has become quite difficult to clearly set apart fertile from infertile male patients and it therefore seems of pressing utility to manage a more comprehensive clinical approach when couples with problems of infertility come up to a fertility center.

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