Correlation between proportions of sperm with DNA fragmentation assessed by Halosperm test and values of standard quality parameters of semen and possible impact on embryo quality

Korelacija med deleži semenčic s fragmentirano DNK določeno s Halosperm testom in vrednostmi standardnih parametrov kakovosti semena in mogoči vpliv na kakovost zarodkov

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Key words:
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Izvleček

Izhodišče: Namen naše raziskave je bil ugotoviti povezavo med standardnimi laboratorijskimi parametri semenskih izlivov moških (koncentracija, gibljivost, morfologija in vitalnost semenčic), starostjo moških in deležem fragmentirane DNK v semenčicah. Ugotovili smo tudi vpliv poškodbe DNA semenčic na kakovost zarodka po postopkih IVF/ICSI.

Metode: Vzorce 102 semenskih izlivov kot del diagnostične analize semena in plodnosti moških smo ocenili s pomočjo standarne mikroskopske analize izliva. V vsakem vzorcu smo ocenili koncentracijo, gibljivost, morfologijo in vitalnost semenčic. Poškodbe DNK semenčic smo ugotavljali s testom Halosperm, ki ima mejno referenčno vrednost indeksa DNK poškodb (DFI) pri 30 %. Embrionalno kakovost zarodkov smo ocenili pri 32 parih, ki so bili v postopku asistirane reprodukcije (ART) v naši kliniki.

Rezultati: Rezultati kažejo, da je imela skupina moških z ≥ 30 % DFI statistično značilno nižje vrednosti standardnih parametrov za oceno ejakulata v primerjavi s skupino moških z < 30 % DFI (gibljivost semenčic: 20 ± 16 oz. 35 ± 17; p < 0.001, morfologija: 16 ± 19 oz. 45 ± 15; p = 0.006 in vitalnost: 63 ± 16 oz. 72 ± 12; p = 0.002). Ugotovili smo tudi negativno korelacijo med deležem semenčic s poškodbami DNK in gibljivostjo (r = -0.41; p < 0.001), morfologijo (r = -0.32, p = 0.001) in vitalnostjo (r = -0.36, p < 0.001). Rahlo pozitivno korelacijo smo odkrili tudi med fragmentacijo DNK in starostjo moških (r = 0.27; p = 0.007). Prav tako smo ugotovili, da je v skupini bolnikov z indeksom DFI < 30 % bilo bistveno več zarodkov boljše kakovosti.

Zaključki: Pri moških s slabšimi standardnimi ocenami kakovosti semena je pogosto možno pričakovati tudi višji delež semenčic s poškodovano DNK. Večja poškodba DNA semenčic, ugotovljena s testom Halosperm, vpliva tudi na kakovost zarodkov v tej skupini bolnikov.

Abstract

Background: The aim of the present study was to investigate the correlation between standard laboratory parameters of human semen, men's age and sperm DNA fragmentation. We have also examined an impact of sperm DNA damage on embryo quality after IVF/ICSI procedures.

Methods: Semen samples from 102 men have been analysed as a part of diagnostic semen analysis and fertility evaluation by using conventional microscopic semen analyses. In each sample, the concentration, motility, morphology and vitality of spermatozoa were evaluated. Sperm DNA damage was determined by using Halosperm test with threshold value of DNA fragmentation index (DFI) at 30 %. Embryo quality was evaluated in 32 couples undergoing assisted reproduction technique (ART) procedures at our clinic.

Results: Results indicate that the patient group with DFI ≥ 30 % had significantly lower values of standard semen parameters than the group of patients with DFI < 30 % (sperm motility: 20 ± 16 vs. 35 ± 17; p < 0.001, morphology: 16 ± 19 vs. 45 ± 15; p = 0.006 and vitality: 63 ± 16 vs.

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72 ± 12; p = 0.002, respectively). Negative correlations were found between DNA fragmentation and motility (r = -0.41, p < 0.001), morphology (r = -0.32, p = 0.001) and vitality (r = -0.36, p < 0.001). Weak positive correlation was found between DNA fragmentation and male age (r = 0.27, p = 0.007). We also found that in the group of patients with DFI index < 30 % there were significantly more embryos of better quality. Conclusions: In men with poorer semen quality, evaluated by standard semen parameters, a higher proportion of sperm with damaged DNA can also be expected. Higher sperm DNA damage, established by Halosperm test, also had an impact on embryo quality in this group of patients.

Introduction

Infertility presents a growing health and social problem, which affects about 15 % of couples, furthermore, the male factor infertility accounts for 50 % of infertile couples.[1] The causes of male factor infertility are gene mutations, aneuploidy, varicocele, infectious diseases, infection of male sex glands, immune disorders, radiation, chemotherapy, systemic and iatrogenic disorders, lifestyle, etc.[2] Sperm is a highly specialized cell, the fertilization potential of which depends on the functional capability of sperm and integrity of sperm DNA, indispensable for the correct transmission of paternal genetic information.[3-5] Semen quality is frequently used as an indirect measure of male fertility, and the majority of laboratories use the standard semen analysis based on conventional parameters in order to assess male fertility. The analysis includes organoleptic assessment of volume, pH, concentration, motility, morphology and vitality according to the World Health Organization (WHO) manuals, and sometimes additional sperm function tests such as acrosome reaction.[6-8] However, conventional parameters provide only an approximate evaluation of the functional competence of sperm, so men with normal semen parameters may still be infertile.[9,10] One of the reasons of unexplained infertility may be DNA fragmentation.[11,12] Therefore, many studies have pointed to the need for the introduction of sperm DNA fragmentation tests in addition to the standard semen testing to obtain more reliable information on the reproductive potential of man.[13] DNA fragmentation includes single and double strand breaks and is frequent in subfertile men.[14] Sperm DNA damage has a major impact on fertility, pregnancy loss and malformations of the offspring.[15] Studies have shown a negative correlation between DNA fragmentation and fertility in natural and medically assisted cycles.[16,17] Fertilization achieved by a sperm with fragmented DNA after IVF/ICSI procedures negatively impacts on the embryo development and quality, implantation and pregnancy rates and positively on the spontaneous miscarriage rates and pregnancy losses after IVF/ICSI procedures.[18-20] Some other authors have found no correlation between sperm DNA fragmentation and embryo quality or pregnancy outcome.[21,22] Today, there are a number of tests used for the evaluation of sperm cromatine structure. Some of the most common tests in laboratory practice are: TUNEL (Terminal dUTP Nick-End Labeling) test, Comet test (Single Cell Gel Electrophoresis), AO (Acridine Orange) test, CMA3 (Chromomycin A3) test, SCSA (Sperm Chromatin Structure Assay) test and SCD (Sperm Chromatin Dispersion) test.[22-28]

Halosperm is an improved, economical and simple SCD test that measure susceptibility of sperm DNA to acid denaturation. Classification of spermatozoa is based on the principle that sperm with fragmented DNA fails to produce halos of dispersed DNA, which are characteristic for sperm with intact DNA.[29]

In this study, we wanted to examine the correlation of semen parameters (concentration, motility, morphology and vitality) and DNA fragmentation, as well as the possible impact of male aging on DNA integrity, using the Halosperm test. We have also examined the relationship between DNA fragmentation and embryo quality after IVF/ICSI procedures.

Materials and methods

Population

The research included 102 men with both normal and abnormal semen parameters as a part of diagnostic semen analysis and fertility evaluation during the period from February to July of 2011. Embryo quality was assessed in 32 couples undergoing ART procedures at our clinic. All participants in the study signed informed consent. Study was approved by the etical committee of the University Hospital Center Split.

Assessment of semen quality

The standard parameters of semen analysis

Samples were collected by masturbation after 2–4 days of sexual abstinence and examined with light microscope at 200X magnification in a Makler chamber. All samples were allowed to liquefy (30 min, at 37 °C). Volume, motility, concentration, morphology and vitality were estimated according to the manual of the World Health Organization.[1] Smears were made for morphological analysis of sperm, dried at room temperature and treated with May-Grünwald and Giemsa dye (Polysciences Europe GmbH, Eppelheim, Germany) with previous dehydration in 96 % ethanol. Each slide was examined with the light microscope for morphology determination. Hypoosmotic test (HOS)–was used for sperm viability estimation after setting the standard semen parameters. Semen (100 µl) was added to 1 ml of thawed hypoosmotic solution, mixed and incubated for 5 min at 37 °C. After incubation for 30 min at 37 °C slides were made and examined with a phase-contrast microscope at 400 x magnification, and 200 sperm were counted. Vital sperm with intact membranes create different flagellar shapes in hypoosmotic medium, while nonvital sperm have damaged membrane and tails that do not change shape.[30]

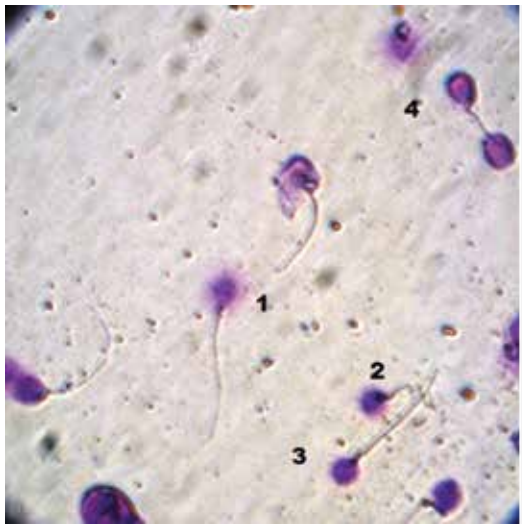
Sperm DNA Fragmentation

Halosperm test was used to assess the level of DNA fragmentation (Halotech Madrid, Spain).[29] Semen samples of each patient were diluted with Quinn´s Sperm Washing Medium (SAGE In Vitro Fertilization, Inc.,Trumbull, USA) to a concentration of 5–10 x 10^6/ml. Agarose gel from the kit was incubated for 5 min at 90–100 °C to fuse agarose and then 5 min at 37 °C in controlled water bath after which 25 µl of semen sample, together with the gel, was added into the Eppendorf tube and mixed carefully. Twenty micro liters of the mixture was placed onto a supercoated slide from the kit, placed on a cold surface, and covered with a 22 x 22 mm coverslip. Slides were kept for 5 min at 4 °C in the refrigerator in order to create a microgel with an implanted sperm.

Coverslips were carefully removed, and slides were then immersed into the previously prepared acid solution (80 µl HCl in 10 ml of distilled water) for 7 min. After that, slides were transferred to the tray with a lysing solution from the kit and incubated for 25 min. Rinsing with distilled water was followed by dehydration for 2 min in increasing concentrations of ethanol (70 %, 90 % and 100 %). After drying, slides were stained with Giemsa dye (Polysciences Europe GmbH, Eppelheim, Germany), rinsed under tap water and allowed to dry at room temperature. Each slide was examined under the light microscope at x 100 magnification and 200 sperm were scored.

Sperm classification

Sperm without fragmented DNA, immersed into agarose matrix and exposed to lysing solutions to deproteinize nuclei, create the halos of dispersed DNA. The halos correspond to relaxed DNA loops attached to the residual nuclear structure (core). The sperm nuclei with fragmented DNA produce small or no halos of dispersed DNA; in contrast, nuclei without DNA fragmentation release DNA creating big halos. Five SCD patterns are possible: (i) sperm cells with large halos (thickness equal to or greater than the length of the minor diameter of the core), (ii) sperm cells with medium halos (thickness smaller than the length of the minor diameter of the core and greater than 1/3 of the minor diameter of the core), (iii) sperm cells with small halos (thickness equal to or



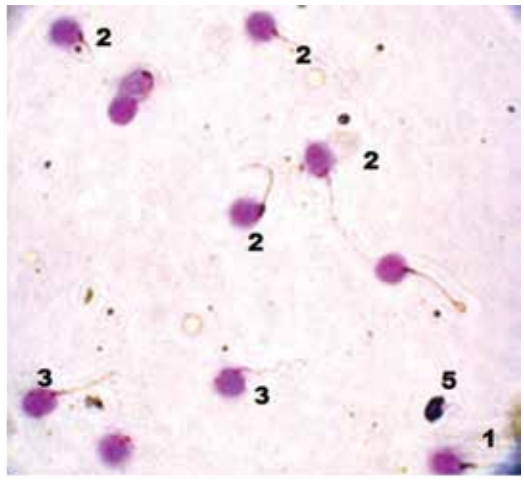


Figure 1 and 2: Sperm assessed by Halosperm test (image taken from the microscope). *Sperm without DNA fragmentation: sperm with big halo (1) and sperm with medium halo (2). Sperm with DNA fragmentation: sperm with small halo (3) and sperm without halo (4), and degraded sperm (5).*

less than 1/3 diameter of the minor diameter of the core), (iv) sperm without halos, and (v) sperm cells with degraded halos. SCD patterns (i) and (ii) are defined as 'sperm without DNA fragmentation and patterns (iii), (iv) and (v) as sperm with DNA fragmentation (Figure 1).

Embryo development and quality

Sydney Fertilization and Cleavage medium (Cook Medical Inc., Bloomington, USA) was used for the gamete, zygote and embryo culture. To estimate the development and quality of embryos, we used the 3rd day embryo scoring system.[31,32] Embryos were observed 68 ± 1 hour after insemination/microinjection. Given the number, blastomeric symmetry and cytoplasm fragmentation, the development and quality of embryos was evaluated and described as embryo score (ES). The assessment of embryos used in this study is based on the grading system proposed by Veeck with the number of blastomeres, as an additional parameter to the evaluation of embryos. Embryos are given grades from 1–5: (i) grade 5 - embryos with 8 equal blastomeres without cytoplasm fragmentation, (ii) grade 4 -embryos with equal 7–8 blastomeres and with small cytoplasm fragmentation, (iii) grade 3 -embryos with 6–7 unequal blastomeres, small or medium fragmentation, (iv) grade 2 -significantly fragmented 4–6 cell embryos of equal or unequal blastomeres, (v) grade 1 -embryos with a few blastomeres (< 4) of any size with a strong or complete cytoplasm fragmentation.[33]

Statistical analysis

The studied population of men (n = 102) was divided in two groups regarding the DNA fragmentation index (DFI) value. As threshold value of DFI we used 30 % as this value was set in some other studies using SCD and SCSA fragmentation tests.[10,34] According to this value, we split the patients into two study groups: Group 1 with DFI < 30 % and Group 2 with DFI ≥ 30 %.

All data sets were tested for normality by D'Agostino-Pearson test. Values for each parameter are presented as a mean ± standard deviation or median and intrequartile range, as appropriate. Independent-samples t--test or Mann-Whitney U-test were used for each variable separately in order to examine whether there was a significant difference between the values of standard semen parameters in two groups. The association of the standard parameters and the DFI was measured by Spermans's correlation coefficient (r). The results were obtained with 95 % certainty, and statistical significance was shown as $p < 0.05$. The development and quality of embryos is assessed on the 3rd day and shown in ES values. According to ES, two groups were determined: group 1 included embryos of good and medium quality (ES 1–3), and group 2 low quality embryos (ES 4 and 5). We used chi-square test to determine whether there is a difference in the distribution of embryos regarding quality, expressed

as embryo score, between the two groups of patients with different value of DFI. Statistical analysis was performed by MedCalc® statistical software (MedCalc 12.2.1).

Results

Table 1 shows mean values ± standard deviations or median and intrequartile range for two DFI groups: group 1 with DFI < 30 % and group 2 with DFI ≥ 30 %. For each semen parameter we tested whether there was a significant mean difference between the obtained values for two groups. the obtained p values were statistically significant for motility ($p < 0.001$), morphology ($p = 0.006$), vitality ($p = 0.002$) and age ($p = 0.008$) between the two groups, while there was no statistically significant difference ($p = 0.374$) for concentration.

The dependence of parameter variables (concentration, motility, morphology, vitality, and age) on the DFI variable is shown in Table 2. DFI correlated negatively with motility ($r = -0.41$, $p < 0.001$). There was a weak negative correlation between DFI and vitality ($r = -0.36$, $p < 0.001$) and DFI and morphology ($r = -0.32$, $p = 0.001$). A very weak positive correlation ($r = 0.27$, $p = 0.007$) was found between variables DFI and age. Correlation was not statistically significant ($p = 0.069$) for DFI and concentration.

A significant difference was found between the two groups in terms of number of embryos of good/medium quality and low quality embryos. We found that in the group with DF index ≤ 30 there were significantly more embryos of better quality (Table 3).

Discussion

Semen analysis by testing the conventional parameters is the primary method for assessing men fertility. It is clear that routine semen analysis can provide a limited prediction of man fertility potential and is not always able to explain the cause of male infertility. An increasing amount of data demonstrate an association between sperm DNA damage and fertility.[7,35,38] Some authors have recommended introduction of DNA fragmentation tests as a complementary test in male infertility work-up arguing that it can add to the information obtained by routine semen analysis. It is still unclear whether sperm DNA damage assessment should be introduced as a routine test in male infertility or only applied in selected cases.[35]

While healthy fertile men with normal conventional semen parameters (concentration, motility and morphology) typically have low rates of DNA damage, infertile man are reported to have a higher rates of sperm with DNA fragmentation than fertile controls.[36] Different tests for measuring DNA fragmentation are used in laboratories today. SCSA is well standardized, flow cytometric test that measure susceptibility

Table 1: Age and semen characteristics of male patients with DNA fertility index (DFI) < 30 % (Group 1) and patients with DFI ≥ 30 % (Group 2).

Variables	Whole studygroup (n = 102)	Group 1 (n = 58)	Group 2 (n = 44)	
DFI (%)	28 (24–32)	25 (22–28)	34 (30–44)	
Age	36 (19–54)	34 (19–49)	38 (24–54)	p = 0.008
Sperm concentration (× 106/ml)	27.1 ± 14.3	28.2 ± 12.3	25.7 ± 16.6	p = 0.374
Normal forms (%)	41 ± 17	45 ± 15	36 ± 18	p = 0.006
Motility (%)	29 ± 18	35 ± 17	20 ± 16	p < 0.001
HOS (%)	69 ± 15	72 ± 12	63 ± 16	p = 0.002

Note: Values are mean ± standard deviation for parametars with normal distribution and median (25th and 75th percentile) for parametars that were not distributed normaly.
DFI (DNA fragmentation index)

of sperm DNA to acid-induced DNA denaturation in situ, followed by staining with fluorescent dye. Chohan et al. published a research paper in which results obtained by TUNEL, SCD and AO tests were compared with SCSA results and a good correlation was found between the results.[37] Studies have demonstrated that sperm integrity tests generally correlate moderately with each other (coefficient of correlation 0.4–0.7), which indicates that the tests are probably expressing different aspects of DNA damage.[38]

We measured the DNA fragmentation with Halosperm test, which is an improved version of the SCD test.[29] We used 30 % as threshold value of DFI , as this value was set in some other studies using SCD and SCSA fragmentation tests.[10,34] With respect to the DFI value, we divided subjects into two groups. Statistically significant difference of mean values between the two groups was obtained for motility, morphology, vitality and age (Table 1). Results indicate that the group with DFI ≥ 30 % has poorer results of standard semen parameters; motility, morphology and vitality of sperm which correspond to the results of some studies showing that men with asthenozoospermia have significantly more sperm with fragmented DNA, and those with teratozoospermia have a higher degree of DNA damage than men with normal morphology.[39-41] Some studies using AO test found the sperm DNA damage only with severe cases of oligoasthenozoospermia and teratozoospermia.[42]

Negative correlation between the DNA fragmentation and standard parameters has been found by a number of authors who applied different tests.[11,12,43,44] Our finding is consistent with the results of these authors. In this study, we demonstrated the existence of negative correlations between DFI and motility, morphology, and vitality. Correlation between DFI and concentration was not statistically significant. Velez de la Calle et al. found a significant negative correlation between DNA fragmentation and conventional parameters.[45] Yilmaz et al. also used Halosperm test to compare parameters in fresh semen and found a statistically significant difference in concentration between low fragmentation group (DFI ≤ 30 % ± SD) and high fragmentation group (DFI > 30 % ± SD).[22] This is in contrast to our results according to which there was no statistically significant difference in concentration between the two groups (Table 1). The vitality of sperm expressed as a percentage of cells with intact or functional plasma membrane measured by hypoosmotic test is the semen parameter particularly important in cases with less than 40 % progressively motile sperm.[6] Our results have shown a statistically significant negative correlation between the percentage of vital sperm and DNA fragmentation. Association between these two parameters was also found in the studies of other authors (,46). Abnormal sperm chromatin condensation, sperm apoptosis, oxidative stress and reduced antioxidant protection are currently considered as causes of DNA strand breaks.[2,47-49] Studies suggest that negative correlation of DNA damage and motility, morphology and vitality as standard semen parameters could be explained as the result of elevated production of ROS by immotile, morphologically or functionally abnormal spermatozoa. Increased ROS level in seminal plasma could damage sperm DNA.[39,44]

Table 2: Correlation between the values of standard semen parameters (concentration, morphology, motility, vitality), men`s age and sperm DNA fragmentation indexes (DFI) in 102 examined semen samples.

Sperm concentration (×106/ml)	Morphology (%)	Motility (%)	Vitality (%)	Age
r = -0.18	r = -0.32	r = -0.41	r = -0.36	r = 0.27
p = 0.069	p = 0.001	p < 0.001	p < 0.001	p = 0.007

** Spearman´s correlation coefficient*

With age, changes occur in the male reproductive system, i.e. in the function of the testicles, auxiliary glands and the production of reproductive hormones, semen production, sexual activity, fertility, DNA damage, etc.[50] Numerous studies have been carried out recently, testing the potential impact of aging on male fertility, most of them indicating fertility reduction beginning from the late thirties.[51] Sperm DNA has a higher degree of fragmentation in older than younger men. Using TUNEL and CMA3 tests, researchers demonstrated the impact of aging on sperm chromatin integrity and an increase of DNA damage with agein the population of oligoastenozoospermic patients aged 35 and more.[52] Correlation of DFI and age was also found by Singh et al. using Comet test, as well as Wyrobek et al. using SCSA test.[53,54] We found a very weak positive correlation between DFI and age, and our results match those of Vagnini et al., who used TUNEL test, as well those of Moskovtsev et al. who used flow cytometry method.[55,44] Weak correlation could be explained by lower age range of males included in the sample. Possibly, the higher the age range, mainly including much older individuals, the better the chance to obtain a stronger correlation.

Associations between sperm DNA fragmentation and abstinence, varicocele, and sexually transmitted disease assessed by sperm chromatin dispersion test were found in studies of several authors. Gosálvez et al. evaluated duration of sexual abstinence and sperm DNA fragmentation and found lower baseline levels of SDF after shorter periods of abstinence between ejaculations.[56] Also, by using Halosperm test Gallegos at al. found that patients with genitourinary infection with Chlamydia trachomatis and Mycoplasma had increased sperm DNA fragmentation in comparison with fertile controls.[57] With respect to varicicele, Enciso et al., found high relative proportion of sperm with DNA fragmentation in infertile men with varicocele assessed by Halosperm test.[58]

Variations in the association between standard parameters and DNA fragmentation obtained in different studies may be explained by the lack of uniform criteria for selection of patients in those studies as well as using diverse tests for assessing various aspect of DNA damage.[12]

Third-day embryo scoring system has been used for embryo development and quality evaluation, described as embryo score, ES. According to our results there is statistically significant difference between the two DFI groups in terms of number of embryos of good / medium quality and low quality embryos. Muriel et al. have shown that degree of DNA fragmentation, established using the same test, appears to be related to the ability of sperm to fertilize as well as the ability of the embryo to achieve the blastocyst stage until day 6.[59]

Our results indicate that men with level of DNA damage have results of standard semen analysis. There is also a negative association of DNA damage and motility, morphology and vitality of human sperm. According to some authors, level of DNA fragmentation has an impact not only on male fertilization ability but also on embryo development.[17] In our study, higher sperm damage also had an impact on embryo quality, so determination of sperm DNA fragmentation could be of interest in patients with low embryonic quality because DNA damage could be a possible causal factor. Therefore, DNA damage in sperm has important significance in studing male infertility and may be considered as an additional assay in a selected group of

Table 3: Embryo quality distribution in two groups of patients regarding to sperm DNA fragmentation.

DFI index	Embryo quality		Statistics
	good to average (score 1, 2 i 3) (N = 28)	low (score 4 i 5) (N = 45)	
≥ 30 (N = 30)	2	28	$c^2 = 18,67$ df = 1 P < 0.001
< 30 (N = 43)	26	17	

patients.[2] Halosperm test is simple and fast DNA fragmentation assay, which might be applied as a routine sperm function test in an andrology lab.

Abbreviation list

- AO test – Acridine Orange test
- ART–Assisted reproduction techniques
- CMA3 test–Chromomycin A3 test
- Comet test – Single Cell Gel Electrophoresis
- DFI–DNA fragmentation index
- DNA – Deoxyribonucleic acid
- ES – Embryo score
- HOS test – Hypo-Osmotic Swelling test
- SCD test–Sperm Chromatin Dispersion test
- SCSA–Sperm Chromatin Structure Assay
- TUNEL test–Terminal dUTP Nick-End Labeling test
- WHO – World Health Organization

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