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Estimation of Static Oxidation Reduction Potential in Couples Undergoing ICSI Procedure

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Abstract

There are some evidences suggest that an imbalance between levels of reactive oxygen species and antioxidants in semen plasma results in an oxidative state which leads to male infertility. Using this type of spermatozoa for Assisted Reproduction Techniques / Intra-Cytoplasmic Sperm Injection can result in losses before and after implantation, major / minor birth defects, and even childhood cancer. It is essential to develop techniques to detect cases with high free radical levels; a new promising analysis for assessing seminal oxidative stress is the static Oxidation-Reduction Potential estimation. Our study was conducted to identify clinical value of seminal oxidative stress estimation measured by MiOXSYS™ System and sperm DNA fragmentation in ICSI treatment strategy of male infertility. Semen analysis using World Health Organization (WHO) standard guidelines 2010 , Sperm DNA Fragmentation Indexby Sperm Chromatin Dispersion (SCD)test using Halosperm® Kit and static Oxidation-Reduction Potential by MiOXSYS™ System were assessed in potentially fertile (n = 25) and potentially infertile (n = 75) men. Potentially infertile patients had a significantly lower mean Sperm Count (47.06 10^6 sperm/ml vs. 96.88 10^6 sperm/ml), Normal Morphology (7.39% vs. 9.92%), Total Motility (42.17% vs. 53.92%), and A crosomal Index (45.6% vs. 57.6%). Conversely, potentially infertile patients had significantly higher ORP level (4.63 vs. 0.83) and non-significant (p = 0.388) higher Sperm DNA Fragmentation values than potentially fertile controls. Also potentially infertile patients had significantly higher Cumulative Pregnancy Result (62.67% vs. 36%) than potentially fertile controls. Oxidation Reduction Potential of the potentially infertile males showed a statistically significant negative correlation with each of Sperm Concentration ($p<0.001$), Normal Morphology ($p<0.001$), Total Motility ($p=0.045$), A crosomal Index ($p=0.003$) and statistically significant positive correlation with DNA Fragmentation Index ($p=0.004$). Oxidation Reduction Potential of the potentially fertile males showed statistically significant negative correlation with Sperm Count ($p<0.001$) only. Regarding ICSI outcome no correlation exists between seminal Oxidation Reduction Potential and Cumulative Pregnancy Results. The results of the study highlight the central value of oxidative stress estimation in male infertility, as it shows that assessing the semen antioxidant state and DNA integrity can be useful in the men visiting infertility centers for assessment of their fertility.

Keywoeds: Antioxidants; Oxidative Stress; Male Infertility; Sperm DNA Damage.

1. Introduction

Oxidative stress is a state of disturbance of balance between the antioxidants defense capacity and reactive oxygen species (ROS) production and is believed to be of clinical importance in the pathophysiology of male infertility [1]. Twenty five to forty percent of infertile males were reported to have high levels of seminal reactive oxygen species [2].Sperm produce reactive oxygen species at physiological quantities as they have a vital role in physiological functional processes such as capacitation, a crosomal reaction, and spermatozoon–oocyte fusion [3]. But, high levels of reactive oxygen species production and/or impairment of spermatozoa and seminal plasma antioxidant defense systems results in oxidative stress state [4]. Morphologically abnormal spermatozoa and leukocytes are the main sources of ROS in semen [5]. Oxidative stress can lead to sperm dysfunction in

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different ways such as increasing per oxidation of membrane lipids, damaging spermatozoa DNA, and preventing apoptosis. These consequences can compromise the integrity of the sperm structurally and functionally, alter their concentration, morphology, motility and viability [6], of greatest importance is the impact of oxidative stress on the sperm DNA integrity, in which oxidative stress induces breaks in DNA strands, modifications of nucleotide, and cross linking of chromatin leading to sperm DNA fragmentation (SDF) [7]. Although intra-cytoplasmic sperm injection (ICSI) by passes totally the functional processes related to natural fertilization for example sperm capacitation, a crosomal reaction, fusion and penetration of the zonapellucida [8, 7], Post-fertilization fetal maturation can be severely disrupted due to abnormal chromatin packaging and DNA damage leading to lower rates of fetal division and pregnancy[10]. The measuring of redox potential with the MiOXSYS ™ system offers new measurement tool of oxidative stress in semen. It has many preferences than current measures of sperm quality. The system allows for a wider implementation of seminal oxidative stress testing in clinical and scientific trials. The inclusion of the ORP measurement in the settings of male infertility specialists facilitates the infertile couple's management by detecting who could benefit from the oxidative stress treatment [11].

Ethic approval; Approval for this study was granted by Cairo University Faculty of Medicine Ethics Committee for the conduct of humane research in Andrology department. Ethic approval serial number: I- 161014.

2. Materials and methods

2.1. Patients

This study used a prospective investigational design. Data were collected from Adam fertility and sterility hospital since September 2016 to March 2018. In this study, male participants were divided into two groups, potentially infertile and potentially fertile. Potentially infertile group included those attended "Adam international hospital" for infertility treatment by ICSI procedure (azoospermic patients were excluded) in addition the female partners were free of infertility causes, while the potentially fertile included couples with proved fertility having at least two girls whose mean age of youngest 3 years attended "Adam international hospital" for Pre implantation Genetic Diagnosis to select male embryos for transfer ,social sexing for family balancing, by ICSI procedure also.

Informed written consent was taken from all participants included in this study. Ethical approval was permitted by Cairo University Faculty of medicine Ethics Committee for the Conduct of Human Research in Andrology Department. Ethic approval serial number: I- 161014. From calculation of sample size, a minimum sample size of 100 subjects was determined. Potential factors, like smoking, occupation with high scrotal heat-exposure, history of varicocele, malignancy, genitourinary tract infections were excluded.

2.2. Semen analysis

After 2-7 days of abstinence, a complete pure semen sample was collected in a sterile container in a laboratory room by masturbation. Within the initial thirty minutes post sample collection, spermatozoa concentration was obtained by the hemocytometer count chamber techniques, and a wet preparation technique was used to determine sperm motility. Phase contrast optics was used to examine the slides at a magnification of 400x and only morphologically complete sperm were evaluated. Sperm morphology determination was carried through an air-dried, fixed, and Spermic –stained (Fertil Pro, Industrie park, Noord, Beernem, Belgium) preparation using bright-field optics. In this study semen analysis parameters were sperm count, morphology and motility according to the WHO guidelines 2010 [12].

2.3. Measurement of DNA Fragmentation Index

The spermatozoal DFI was determined by using Sperm Chromatin Dispersion test -Halosperm® Kit. Primarily a heated Eppendorf tube with Agarose was put in a bath of water maintained for a 5 minutes at 100ºC then it was transferred to another bath of water maintained also for 5 minutes at 37ºC.Then a 50 µl of semen sample was added and mixed. Then 10 µl of the mixed solution was putted on a slide and covered by a covering slip then put on a cold surface for 7 minutes to solidify the Agarose. The covering slips were separated cautiously then slides were promptly immersed horizontally in a plate filled with freshly prepared acidic denaturing solution for 7 minutes at

22°C. Then denaturation was discontinued and proteins removed by slides transferal to a dish containing Solution 1 for neutralization and lysis kept at 37°C for 10 minutes, followed by incubation in Solution 2 for neutralization and lysis kept at 37° C for 5 minutes. The slides were washed in Tris-borate-EDTA buffer for 2 minutes, dehydrated by successive ethanol baths of 70%, 90%, and 100% (2 minutes for each), then air dried. Slides staining were done by Eosin for 8 minutes and by Azure B for 8 minutes. The slides were examined by the Oil immersion lens under a simple microscope for detection of halos that present around the sperm. Spermatozoa surrounded by halos of large or medium size, typical of dispersed DNA loops were considered having intact DNA. Spermatozoa surrounded by small degraded halos or absent halos were considered having fragmented DNA. Percent of DNA fragmentation was calculated for each semen sample [13].

2.4. Measurement of static oxidation-reduction potential

The oxidation reduction potential value was measured with MiOXSYS. A 30 µl sample was loaded into the MiOXSYS disposable sensor sample port within 1 hour of liquefaction and loaded into the MiOXSYS analyzer. The test began immediately when the reference cell was filled by the sample and the electrochemical circuit was completed. After a short time, the oxidation reduction potential values measured in millivolts were displayed on a screen. The oxidation reduction potential provides a "snapshot" of the current balance in the oxidation reduction potential system. An elevated oxidation reduction potential level indicates a disturbance of balance between the activities of all available oxidants compared to all available antioxidants in ejaculated semen- which is known as oxidative stress. Absolute oxidation reduction potential (mV) and normalized oxidation reduction potential (mV/106 sperm/ml) values were calculated. All determinations, semen analysis, sperm DFI and oxidation reduction potential were carried out by the same expert.

2.5. Semen Sample Processing

Rich semen samples were prepared using gradient centrifugation and poor samples were washed in sperm washing medium only.

2.6. ICSI procedure and embryo scoring:

Briefly, Long or fixed antagonist protocols were used for all patients. Using trans-vaginal needle guided ultrasound; oocytes were brought within 34-36 hours post HCG. After collection in Global total medium with HEPES with HSA (Life Global USA, HGTH-050, 50 ml), the oocytes were treated with Hyaluronidase (Life Global USA, LGHY-010, 10 ml). The oocytes were washed in fresh drops of Global total medium with HEPES with HSA (Life Global USA, HGTH-050, 50ml) and were incubated in culture medium Global total medium with HSA (Life Global USA, HGGT-030, 30 ml) till injection [14].

ICSI was carried out in an injection dish (BD Falcon™ USA1006) that contained small drops of Global total medium with HEPES with HSA (Life Global USA, HGTH-050, 50 ml), and PVP 10% (Sage ART 4005-A) which is a viscous sperm handling solution. Sperm collection and injection was done using an Eppendorf micromanipulator (Germany) mounted on a Nikon TE 2000 inverted microscope (Japan) with the aid of Humagen Micro pipette (Origio MIC 50-30 USA) attached to an Eppendorf Cell Tram syringe. The sperm motility and morphology were the selecting criteria for ICSI. Group embryo culture was done in 30/40 µl drops of Global total medium with HSA (Life Global USA, HGGT-030, 30 ml) covered by Life Guard Oil (Life Global USA, LGUA-500, 500 ml) using 60 mm Tissue culture dish [14].

Fertilization assessment by the pronucli presence was done after about 16 to 18 hours after ICSI. Then the fertilization rate was calculated from the ratio between the fertilized oocytes and the total number of survived injected metaphase II oocytes multiplied by 100. The Cleavage rate was calculated from the ratio between the cleaved embryos and the number of fertilized oocytes. The quality of the embryos was assessed on the second or third day after oocyte retrieval, using a four point score as described by Racowsky *et al* (2010)[15]. Blast cyst grading and embryo transferal was done on the third day or the fifth day after injection depending on the number of fertilized oocytes as when at least 5 fertilized embryos were noticed embryo transfer was scheduled at D5 or else D3 embryo transfer was done. PGD is done on Day 5 for those ICSI cases who request it. Biopsy slides are sent to the Genetic lab and suitable embryos for transfer (male) are reported to the IVF lab.

Embryo transfer was done according to Madani *et al* 2010[16].Embryo / Blast cyst vitrification was done for good and fair embryos using the Irvine vitrification

medium (Irvine Scientific USA 90133-SO) and Rapid I as a vitrification vehicle (Rapid-i™ Kit 14420 Vitrolife, Sweden) while thawing was done using Irvine warming medium (Irvine Scientific USA 90137-SO).

2.7. Clinical follow-up:

After15 days of embryo transfer a pregnancy test was done. And every woman with positive test was given a trans-vaginal ultrasound examination after 3 weeks. A clinical pregnancy was determined when the fetal heartbeat was found out. Implantation rate was defined by the number of embryos with fetal heart beats per number of embryos transferred. Pregnancy rates were calculated per transfer. Abortion was defined as loss of pregnancy before 20 weeks.

2.8. Statistical analysis

Statistical Package for the Social Sciences software (SPSS_ version 25; SPSS Inc., IBM Corp., Armonk, NY, USA) were used for Statistical analysis, with significance set at $P < 0.05$. Summaries of quantitative variables are reported as mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data for the infertile patients (ICSI group) and potentially fertile controls (Social Sex Selection group). The data were not normally distributed as shown by descriptive analysis; so the Mann–Whitney test was used to compare means of sperm count, total and progressive motility, normal morphology, acrosomal indices, SDF, and oxidation reduction potential between infertile patients and fertile controls [17]. Spearman rank correlation examined the correlation (s) between oxidation reduction potential, SDF, Cumulative Pregnancy Result and different indices for both groups [18]. Chi square (x2) test was performed for comparing categorical data. When the expected frequency was less than 5-fisher exact test was used instead [19].

3. Results

3.1. Sample Characteristics (Table 1).

Conventional semen analysis and advanced sperm function tests in potentially infertile group A (n = 75) and potentially fertile controls in group B (n = 25). Potentially infertile patients had significantly lower mean values for conventional sperm parameters (lower sperm count, lower total sperm motility, and lower sperm with normal morphology). Conversely, a crosomal index and oxidation reduction potential values were significantly higher in potentially infertile patients than potentiallyfertile controls ($P < 0.001$ for all). DNA fragmentation index values in group A patients were higher than those in group B, but not statistically significant ($P = 0.388$).

3.2. Correlation between seminal ORP and other sperm parameters in group A (Table 2).

A statistically significant negative correlation between oxidation reduction potential and each of Sperm Count ($p < 0.001$), Sperm Total Motility ($p = 0.045$), Sperm with Normal Morphology ($p < 0.001$), A crosomal Index ($p = 0.003$) and statistically significant positive correlation with Sperm DNA Fragmentation index ($p = 0.004$).

3.3. Correlation between seminal oxidation reduction potential and other sperm parameters in group B (Table 3).

No statistically significant correlation was founded between oxidation reduction potential and sperm parameters except Sperm Count ($p < 0.001$).

Table 1; Sample Characteristics

	Group A (potentially infertile patients)					Group B (potentially fertile controls)					P value
	Mean	SD	Median	Mini.	Maxi.	Mean	SD	Median	Mini.	Maxi.	
Sperm Count (Million)	47.06	40.99	35	0.8	164	96.88	75.2	77	10	265	**0.001**
Total Motility (%)	42.17	19.99	45	1	75	53.92	19.09	60	10	90	**0.015**
Progressive Motility (%)	5.51	6.52	4	0	30	11.12	11.90	10	1	60	**0.001**
Normal Morphology (%)	7.39	5.69	6	0	32	9.92	5.21	10	2	22	**0.015**
Acrosomal Index (%)	45.6	15.8	44	2	84	57.6	16.02	58	20	84	**0.002**
Normalized ORP	4.63	9.31	1.3	-0.16	50.9	0.83	0.87	0.45	0.05	3.13	**0.001**
DFI	14.55	11.17	12.3	1.7	59	11.59	5.38	10.9	5	29	**0.388**

Table 2; Correlation between seminal ORP and other sperm parameters in group A

Group A		Normalized ORP
Sperm Count (Million)	Correlation Coefficient	**-0.861**
	P value	**< 0.001**
Total Motility (%)	Correlation Coefficient	**-0.232**
	P value	**0.045**
Progressive Motility (%)	Correlation Coefficient	**-0.148**
	P value	**0.206**
Normal Morphology (%)	Correlation Coefficient	**-0.566**
	P value	**< 0.001**
Acrosomal Index (%)	Correlation Coefficient	**-0.339**
	P value	**0.003**
DNA Fragmentation Index	Correlation Coefficient	**0.328**
	P value	**0.004**

Table 3; Correlation between seminal oxidation reduction potential and other sperm parameters in group B

Group B		Normalized ORP
Sperm Count (Million)	Correlation Coefficient	**-0.875-**
	P value	**< 0.001**
Total Motility (%)	Correlation Coefficient	**-0.280-**
	P value	**0.175**
Progressive Motility (%)	Correlation Coefficient	**-0.204-**
	P value	**0.328**
Normal Morphology (%)	Correlation Coefficient	**-0.160-**
	P value	**0.443**
Acrosomal Index (%)	Correlation Coefficient	**0.154**
	P value	**0.464**
DNA Fragmentation Index	Correlation Coefficient	**-0.107-**
	P value	**0.609**

3.4. Comparison between both groups' Cumulative Pregnancy Result (Table 4).

Although ROS has statistically significant correlations with almost all tested parameters in group A patients, it has statistically significant correlations with only one parameter of group B patients. Group A had significantly higher Cumulative Pregnancy Result (P= 0.02) than Group B. It is worth to be mentioned that group B were subjected to additional Pre implantation Genetic Diagnosis to select male embryos which means more manipulations.

Table 4; Comparison between both groups' Cumulative Pregnancy Result

		Group B		Group A		P value
		Count	%	Count	%	
Cumulative Pregnancy Result (Fresh&1st thawing result)	Positive	9	36%	47	62.67%	**0.02**

4. Discussion

In this prospective study oxidation-reduction potential levels correlates adversely with semen parameters and directly with sperm DNA fragmentation. Like our data other scientists showed elevated levels of ORP in potentially infertile patients which were significantly higher than levels in potentially fertile control group [20-25]. Several

studies also showed that elevated levels of oxidation reduction potential were correlated with poor quality of spermatozoa and oxidation reduction potential correlated negatively with sperm concentration, motility and normal morphology [20, 23-27]. Our findings also indicated that oxidation reduction potential was significantly negatively correlated with sperm parameters. Al Said *et al*. (2017) sought to correlate between the seminal oxidation reduction potential with the total number of motile spermatozoa, in the hope that these two parameters could offer more information in male infertility assessment and they found a significant negative association [28].Also Toor *et al*. (2016) noted that oxidation reduction potential was increased in semen with low sperm concentration, low total sperm count, and low sperm motility [29].

Regarding sperm DNA fragmentation, a significant positive correlation between DNA fragmentation and oxidation reduction potential in potentially infertile patients was showed. In a prospective study conducted by Arafa et al., (2017) including 312 patients, DNA fragmentation was shown to significantly correlate negatively with total / progressive sperm motility and correlate positively with abnormal sperm morphology, oxidation reduction potential, and paternal age. High levels of oxidation reduction potential were observed in the semen of the elevated SDF group compared to that of the normal SDF group [27]. Majzoub *et al*. (2017) conducted a cross-sectional study of 1,162 patients and reported that there was a significant positive correlation between the percentage of abnormal sperm heads and oxidation reduction potential and SDF levels [30]. Regarding reproductive outcomes, unlike in our study, Ayaz *et al*., (2017) pointed out that the clinical pregnancy rate was higher in patients with a low oxidation reduction potential than in those with a high oxidation reduction potential [31]. So, monitoring oxidation reduction potential for ART purposes allows for better selection and preparation of sperm20. Many studies demonstrate that sperm quality has a direct effect on embryo development; therefore sperm selection is very important. The most common techniques used to select sperm are:

4.1. IMSI – Intra-cytoplasmic morphologically selected sperm injection

Studies have shown that sperm classified as morphologically "normal" at conventional optical resolution and magnification (x200-x400) may carry ultra-structural defects. Many of these abnormalities have been associated with hidden chromosomal defects, high levels of DNA fragmentation, abnormal centriolar function, etc. These aberrations may impede fertilization, lead to post-fertilization arrest or even disrupt embryo development. In order to overcome the above-mentioned defects, observations can be performed using an inverted light microscope with high-power optics intended, at x6000-x6600 magnification, which allows the embryologist to assess sperm morphology in real time [32].

4.2. PICSI – Physiological Intra-cytoplasmic sperm injection

This technique is based on the fact that hyaluronic acid (HA) plays an important role in selecting functionally competent sperm during in vivo fertilization. Not only HA is the main component of the cumulus matrix that surrounds the human oocyte, but also a natural selector of developmentally mature sperm [33].

4.3. MACS – Magnetic activated cell sorting

Sperm DNA damage may cause male infertility and lead to sperm cells' apoptosis. Sperm apoptosis is manifested by exposure to phosphatidy-lserineal lowing apoptotic sperm to be detected and separated. This sperm cannot be distinguished from the normal sperm with the naked eye. In order to avoid damaged sperm, we use MACS ART Annexin V system. Before separation, the damaged apoptotic sperm is labeled with magnetic nanoparticles and then passed through a column where the apoptotic sperm is caught. Live intact sperm pass through the column and are collected for later use [34].

As many authors, our findings showed higher levels of DNA- fragmented sperm in potentially infertile men compared to potentially fertile controls but not statistically significant [35-37]. Hosen *et al*. (2015) observed that infertile males had significantly higher levels of 8-hydroxy-2′-deoxyguanosine in seminal plasma compared to fertile controls [38]. Men with oligospermia, as the nozoo spermia and teratozoo spermia showed a significant increase in fragmented DNA sperms compared to controls [39, 40]. Furthermore, significantly higher levels of DNA fragmentation were found in semen from as the nozoospermic patients than in men with normal sperm

motility [41, 42]. Men with normo-zoo spermia showed lower levels of DNA fragmentation than their non-normozoo spermic counterparts.

DNA damage was inversely correlated to total and progressive sperm motility, normal morphology and macrodome index in potentially fertile and potentially infertile patients, although these correlations were statistically significant in infertile group only. Numerous studies have reported a negative correlation between DNA damage and sperm parameters, including sperm count [43-45], motility [36, 45, 46] and normal morphology [45-48]. However, Evgeni *et al*. (2015) showed that DNA damage in non-normozoospermic men did not significantly correlate with sperm parameters other than progressive motility [49]. In addition, no significant correlations were found between DNA fragmentation and sperm concentration [36] and morphology [50].

In this study negative correlations were found between DNA fragmentation and fertilization rate, good embryos, blastocyte rate, implantation rate in both groups but lack statistical significance. Similar studies showed that decrease in fertilization rates were not significantly correlated to elevated levels of sperm DNA fragmentation [51-60].But with embryonic genome activation, the harmful effects of fragmented paternal DNA became obvious. Notably, sperm DNA fragmentation in high levels (30% DFI) manifested as a significant decrease in blast cysts and ongoing pregnancy rates with a tendency towards a lower rate of chemical pregnancies and a higher rate of spontaneous miscarriage [61]. Sperm DNA fragmentation as an indicator of sperm quality has been associated with impaired sperm function and sub fertility [62] as well as lower rates of normal pregnancy [63] and higher rates of pregnancy loss afterIVF and IVF-ICSI treatments [64, 65] and children morbidity [66]. Many studies have been directed to evaluate probable relations between sperm DNA fragmentation and clinical outcomes in ICSI cycles. Some results concluded that DNA fragmentation was not correlated with ICSI outcomes, including fertilization rate [67-70], embryo quality and development [67, 70], the pregnancy rate [67, 69], the live birth rate [71], and pregnancy loss [67]. Conversely, other results have concluded that high sperm DNA fragmentation significantly correlated with decrease of fertilization rate[60, 72], embryo development and quality[41, 60, 61, 68, 73, 74], the pregnancy rate [41, 68, 74], ongoing pregnancy [75-77], as well as increasing pregnancy loss rate [68, 72, 78-81].

5. Conclusion

The present study concluded that high seminal ROS levels are predictive of poor semen quality and DNA fragmentation. Infertile patients had significantly lower semen analysis parameters and significantly higher ORP levels compared to fertile controls. Without a doubt, ICSI bypasses the physiological mechanisms that negatively affected by high seminal ROS. The hope for the future is to be able to sort out the men with sperm oxidative stress caused infertility who potentially would benefit from antioxidant strategies.

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