Oxidative stress level in fresh ejaculate is not related to semen parameters or to pregnancy rates in cycles with donor oocytes

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Abstract

Purpose The purpose of the present study is to study the relationship between oxidative stress (OS) in semen, semen characteristics, and reproductive outcomes in oocyte donation intracytoplasmic sperm injection (ICSI) cycles.

Methods OS was measured in 132 semen samples.

Results OS levels were as follows: very high (1.5 %), high (43.2 %), low (30.3 %), and very low (25.0 %). Overall seminal parameters were as follows: volume (ml)=4.2 (SD 2.1), concentration (millions/ml)=61.6 (SD 59.8), motility (a+b%)=47.4 (SD 18.0), and normal spermatozoa (%)=8.2 (SD 5.1). Of the 101 cycles that reached embryo transfer, 55.4 % evolved in biochemical, 46.5 % in clinical, and 43.6 % in ongoing pregnancy. OS level does not relate to seminal parameters, fertilization rate, or pregnancy outcomes. **Conclusions** OS testing by nitro blue tetrazolium (NBT) in fresh ejaculate might not be useful for all patients. Reproductive results with young oocytes and ICSI do not seem to be affected by OS-level semen.

Keywords ICSI · Oocyte donation · Oxidative stress · Pregnancy rates · Sperm

Capsule Semen oxidative stress was measured in 132 patients' ejaculates by nitro blue tetrazolium testing before performing ICSI with donor oocytes. The measured level did not correlate with either seminal parameters or reproductive outcomes.

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Introduction

Reactive oxygen species (ROS) include oxidative species such as hydroxyl radicals ($\cdot\text{OH}$) and non-radical species such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2). ROS are normal by-products of metabolism, and small amounts of them are required for specific cellular processes in spermatozoa [1], and (O_2^-) is the main ROS produced in spermatozoa [2, 3]. O_2^- and H_2O_2 are needed for functions such as sperm capacitation (the complex series of changes allowing spermatozoa to bind to the zona pellucida), acrosome reaction, and the acquisition of the hyperactivated state [4]. A delicate balance between ROS and antioxidants is required for efficient fertilization. H_2O_2 stimulates capacitation while the enzyme catalase prevents it [5]. It has also been shown that while low concentrations of H_2O_2 are required for capacitation, elevated concentrations reduce hyperactivation, zona pellucida binding, and acrosome reaction [6]. ROS also damage the sperm membrane and, consequently, might affect both its motility and ability to bind to the oocyte membrane [7]. Mitochondrial DNA is another target of ROS, limiting ATP production [8].

Oxidative stress (OS) takes place when the concentration of ROS is too high and/or the antioxidant protection ability is exceeded, and it is considered to be among the main causes of DNA damage in spermatozoa [9]. ROS can also damage DNA directly by producing oxidized DNA adducts leading to DNA sites without bases, causing single-strand DNA (ssDNA) breaks [10]. ssDNA across the genome is related to OS in sperm [10]. DNA repair in spermatozoa occurs during spermatogenesis and stops during nuclear condensation in the epididymis [11, 12]. However, spermatozoa can be exposed to oxidative damage in the vas deferens [13]; from this point onward, spermatozoa DNA repair can only occur after entry into the oocyte; ssDNA seems to be more readily repaired than double-strand DNA (dsDNA) by the oocyte, and in the

mouse, the efficiency of DNA repair decreases with increasing maternal age [14, 15]. Post-testicular oxidative damage targets specific chromatin domains of lower compaction associated with histones and attached to the sperm nuclear matrix [16]. These specific domains of the paternal genome are enriched in genes involved in the control of post-fertilization DNA replication events and in the developmental program of the embryo [17], thus potentially affecting the quality and viability of the resulting embryo.

Although the molecular mechanisms by which OS is detrimental for spermatozoa are known [19] and OS and severe seminal alterations seem to be correlated [7], there are conflicting data regarding the relationship between sperm OS and assisted reproduction technologies (ART). The concentration of ROS in seminal plasma does not seem to affect the fertilization rate in IVF/intracytoplasmic sperm injection (ICSI) cycles [18], especially when spermatozoa are selected by swim up [19]. Nonetheless, a negative effect of ROS levels on ART outcomes has also been reported: after IVF, fertilization and pregnancy rates were negatively associated with ROS levels, and there was a negative association between ROS and embryo development to the blastocyst stage when using ICSI [20]. The aim of the study is to investigate the relationship between OS in freshly collected ejaculate, semen parameters, and pregnancy outcomes in patients undergoing oocyte donation cycles with ICSI.

Materials and methods

Study design and ethical approval

This is a prospective cohort study, carried out in a private fertility center between October 2013 and December 2014. The study was approved by the local IRB. Semen samples were collected from 132 consecutive patients by masturbation after 2–5 days of abstinence. The sample was frozen (CryoProtec II, Nidacon) for future IVF use, and the excess sample was used for the study. All patients underwent IVF with ICSI and donor oocytes; they received an average of 6.4 mature oocytes (SD 1.7). Oocyte donors were between 18 and 35 years old, while male age was between 24 and 63 years old. The day of the embryo transfer was 2.6 (SD 0.6) and the number of embryos transferred 1.9 (SD 0.4). Semen and cycle characteristics are presented in Table 1.

NBT-based test

After liquefaction, semen was analyzed by SCA software (Sperm Class Analyzer; Microptic) according to the World Health Organization guidelines [21]. The OS level of each sample was established using a colorimetric test based on nitro blue tetrazolium (NBT: OxiSperm, Halotech DNA®). NBT, in the form of a reactive gel (RG), measures the level of O_2^- in

the sample, as O_2^- converts the soluble tetrazolium salt to insoluble blue crystals, producing an increasingly intense color in the RG, turning from yellow to purple-blue.

The OS assay was applied within 60 min from collection, following the manufacturer's instructions. Briefly, the tube of RG was liquefied at 80–90 °C for 5 min; RG temperature was then reduced to 40 °C for 5 min, and the RG was mixed with the 20–100 µl of semen sample. The mixture was allowed to gelify at 4 °C and then incubated for 45 min at 37 °C. The resulting color was compared against a visual palette provided by the manufacturer to assign a category to the level of OS: very low, low, high, and very high. The call was agreed upon by two separate people.

Before ICSI, semen samples were thawed and prepared by swim up. Fertilization was assessed 14 to 19 h post-ICSI. Embryos were classified according to modified scale based on the combined embryo score [22] which takes into account number of blastomeres, percentage of embryo fragmentation, and symmetry of the blastomeres with a maximum score of 10.

Statistical analysis

Semen diagnoses, male age, seminal parameters, fertilization rate, and embryo morphology have been described for the sample. A correlation analysis was performed using Spearman's rho coefficient to evaluate the association between the previous parameters and OS level.

Pregnancy outcomes (biochemical, clinical, and ongoing pregnancy) after the first fresh embryo transfer have been described overall and across OS levels; linear trend was evaluated by linear-by-linear test. In addition, the association between the OS levels and each pregnancy outcome was evaluated using a model of logistic regression, adjusted by day of embryo transfer (ET), and number of transferred embryos.

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 22). A *p* value of <0.05 was set as statistically significant.

Results

The OS assay classified the 132 semen samples into four categories: very low OS (*n*=33; 25.0 %), low OS (*n*=40; 30.3 %), high OS (*n*=57; 43.2 %), and very high OS (*n*=2; 1.5 %). Due to the low number of samples classified into “very high OS,” “high OS” and very high OS were joined into one group for statistical analyses.

We found no correlation between OS in the ejaculate and male age, seminal parameters (volume, concentration, motility, and normal spermatozoa), fertilization rate, or embryo morphology (Table 2). Mean semen volume was 4.2 ml across the OS groups, and a+b motile spermatozoa were on average 47–

Table 1 Semen diagnoses overall and by OS levels

	Overall <i>n</i> = 132	Very low OS <i>n</i> = 33	Low OS <i>n</i> = 40	High and very high OS <i>n</i> = 59
Normozoospermia; <i>n</i> (%)	84 (63.6)	23 (69.7)	26 (65.0)	35 (59.3)
Oligozoospermia; <i>n</i> (%)	9 (6.8)	1 (3.0)	6 (15.0)	2 (3.4)
Asthenozoospermia; <i>n</i> (%)	14 (10.6)	2 (6.1)	4 (10.0)	8 (13.5)
Teratozoospermia; <i>n</i> (%)	9 (6.8)	2 (6.1)	2 (5.0)	5 (8.5)
Oligoasthenozoospermia; <i>n</i> (%)	8 (6.1)	2 (6.1)	2 (5.0)	4 (6.8)
Oligoteratozoospermia; <i>n</i> (%)	5 (3.8)	1 (3.0)	0	4 (6.8)
Asthenoteratozoospermia; <i>n</i> (%)	1 (0.8)	1 (3.0)	0	0
Oligoasthenoteratozoospermia; <i>n</i> (%)	2 (1.5)	1 (3.0)	0	1 (1.7)

48 % in all categories. The fertilization rate was similar in all groups (69–75 %).

From the 132 patients whose samples were analyzed, 30 (22.3 %) did not start their IVF cycle at the time of analysis, and there was 1 (0.8 %) fertilization failure (level of OS: high). Of the 101 cycles that reached ET, 56 (55.4 %) resulted in biochemical, 47 (46.5 %) in clinical, 44 (43.6 %) in ongoing pregnancy, and 37 (37.4 %) in live birth (Table 3), without significant differences across OS levels. Overall miscarriage rate was 27.5 %.

Regression-adjusted analyses showed no effect of OS level on any of the pregnancy outcomes (Table 4).

Discussion

Oxidative stress (OS) is produced by an imbalance between the production of ROS and the antioxidant capacity of the biological system. OS can harm fertilization both by affecting directly the sperm's membrane and by causing DNA damage.

The OS in the ejaculate can damage the spermatozoa motility first and its DNA integrity later.

There are different methods to identify OS in semen: direct methods measure damage created by excess free radicals against the sperm lipid membrane or DNA, while indirect ones detect ROS production. Chemoluminescence and light microscopy quantification of nitroblue tetrazolium (NBT) activity are two indirect methods, the latter being simple and inexpensive [7]. The NBT assay has already been applied to measure ROS in semen samples, where the presence of ROS was positively correlated with sperm DNA fragmentation and negatively correlated with motility [23]. The NBT assay that we used, Oxysperm, resulted in a colorimetric read which was assessed by the naked eye. Although the results were read by two people for each sample analyzed, the subjective call diminishes the reliability of the results. In order to minimize subjectivity when assigning a sample to one of two adjacent categories, an automated reading machine would improve the application of the test.

Table 2 Seminal parameters, fertilization rate, and embryo morphology, overall and by OS level, with Spearman's rho correlation coefficient

	Overall <i>n</i> = 132	Very low OS <i>n</i> = 33	Low OS <i>n</i> = 40	High and very high OS <i>n</i> = 59	Spearman's rho
Male age (years); mean (SD)	41.7 (7.8)	43.1 (8.8)	42.7 (7.6)	40.2 (6.9)	0.13
Volume (ml); mean (SD)	4.2 (2.1)	4.2 (1.8)	4.2 (1.8)	4.2 (2.4)	0.07
Concentration (million/ml); mean (SD)	61.6 (59.8)	46.3 (37.6)	63.0 (66.8)	69.4 (64.2)	−0.15
Motility a+b (%); mean (SD)	47.4 (18.0)	46.6 (17.4)	47.8 (17.9)	47.5 (18.7)	−0.01
Normal spermatozoa (%); mean (SD)	8.2 (5.1)	7.1 (4.2)	7.5 (3.4)	9.2 (6.2)	−0.12
Fertilization (%); mean (SD)	71.2 (22.6)	74.9 (20.8)	71.1 (25.3)	69.0 (22.0)	0.10
Embryo morphology; mean (SD)	7.9 (1.2)	7.9 (1.2)	8.0 (1.3)	7.8 (1.2)	0.04
MII attributed to each recipient; mean (SD)	6.4 (1.7)	6.8 (1.6)	6.5 (2.3)	6.1 (0.9)	0.16 ^a
Number of embryos transferred to each recipient; mean (SD)	1.9 (0.4)	1.9 (0.3)	1.9 (0.4)	1.8 (0.5)	0.69 ^a
Quality of transferred embryos (1 to 10 scale); mean (SD)	7.9 (1.2)	7.9 (1.2)	8.0 (1.3)	7.8 (1.2)	0.04

^a One-way ANOVA

Table 3 Pregnancy rates (biochemical, clinical, and ongoing), live birth, and miscarriage rates overall and by OS level

	Overall (<i>n</i> = 101)	Very low OS (<i>n</i> = 27)	Low OS (<i>n</i> = 31)	High and very high OS (<i>n</i> = 43)	<i>p</i> value ^a
Biochemical pregnancy; <i>n</i> (%)	56 (55.4)	16 (59.3)	15 (48.4)	25 (58.1)	0.97
Clinical pregnancy; <i>n</i> (%)	47 (46.5)	14 (51.9)	13 (41.9)	20 (46.5)	0.73
Ongoing pregnancy; <i>n</i> (%)	44 (43.6)	14 (51.9)	13 (41.9)	17 (39.5)	0.33
Live birth; <i>n</i> (%)	37 (36.6)	11 (40.7)	9 (29.0)	17 (39.5)	0.71
Miscarriage; <i>n</i> (%)	14 (13.9)	2 (7.4)	4 (12.9)	8 (18.6)	0.31

A *p* value <0.05 is considered statistically significant

^a Linear-by-linear test

Relationship between ROS and seminal parameters

As expected, we found that the percentage of patients with high and very high OS in the ejaculate was not significantly different in the teratozoospermic group compared to the normozoospermic (58.8 vs 41.7 %; Table 1). This is most likely due to the fact that our approach detects the OS present in the seminal fluid, which is unrelated to testicular and epididymal OS damage.

OS has been found to promote a dose-dependent increase of tyrosine nitration and S-glutathionylation in the

sperm, and this change relates to an alteration in motility and the ability of spermatozoa to undergo capacitation [24]. Lipid peroxidation (an indicator of oxidative status) and decreased antioxidant capacity in the ejaculate lead to low motility in asthenoteratozoospermic and oligoasthenoteratozoospermic men; morphology and sperm count were also lower [25]. We found no significant differences in sperm motility between asthenozoospermic and normozoospermic patients among the cases with high and very high OS (52 vs 41.7 %; Table 1), in agreement with a recent study, where no significant

Table 4 Multivariate analysis for association of OS levels to pregnancy outcomes

		95 % CI			
		OR	Lower	Upper	<i>p</i> value ^a
Biochemical pregnancy	Low vs. very low OS	0.82	0.28	2.40	0.72
	High and very high vs. very low OS	1.39	0.53	3.66	0.51
	1 embryo vs. 2	1.87	0.58	6.03	0.30
	3 embryos vs. 2	0.65	0.05	9.12	0.75
	Day of ET (3 vs. 2)	1.02	0.44	2.32	0.97
Clinical pregnancy	Low vs. very low OS	0.86	0.30	2.53	0.79
	High and very high vs. very low OS	1.15	0.43	3.04	0.79
	1 embryo vs. 2	2.40	0.68	8.47	0.17
	3 embryos vs. 2	1.22	0.08	17.75	0.88
	Day of ET (3 vs. 2)	1.13	0.50	2.58	0.78
Ongoing pregnancy	Low vs. very low OS	0.83	0.28	2.44	0.74
	High and very high vs. very low OS	0.88	0.33	2.36	0.80
	1 embryo vs. 2	1.95	0.55	6.91	0.30
	3 embryos vs. 2	1.22	0.08	17.71	0.89
	Day of ET (3 vs. 2)	1.41	0.61	3.25	0.42
Live birth	Low vs. very low OS	0.67	0.21	2.16	0.50
	High and very high vs. very low OS	1.49	0.52	4.27	0.46
	1 embryo vs. 2	2.47	0.62	9.86	0.20
	3 embryos vs. 2	1.68	0.11	26.71	0.71
	Day of ET (3 vs. 2)	1.60	0.65	3.85	0.31

A *p* value <0.05 is considered statistically significant

^a Logistic regression

correlation was reported between ROS production and sperm motility [26].

Relationship between ROS and reproductive outcomes

A possible confounding variable when comparing different studies is semen handling and preparation: oxidative DNA damage is induced in spermatozoa prepared on discontinuous colloidal silicon gradients, because the medium employed contains metals promoting free radical generation [27]. The semen samples in our study were prepared using a centrifugation of 10 min at 1200 rpm followed by swim up; therefore, the possible negative effect of gradients did not intervene in our reproductive outcomes.

Although OS is an important source of sperm DNA fragmentation [9], especially ssDNA [13, 28], OS and DNA damage are not necessarily related, as sperm DNA can be damaged by non-oxidative mechanisms like aberrant apoptosis and incomplete sperm protamination [29]; as mentioned, OS also affects the sperm's membrane. Moreover, spermatozoa with various degrees of DNA damage can fertilize [30, 31], and if the spermatozoa carry limited DNA damage, the oocyte could repair the DNA with little consequence for embryo development [32]. The use of ICSI in the current study might have mitigated the effect of OS on fertilization rate bypassing the effect of OS on motility, in accordance with a report where no significant effects of elevated ROS levels on fertilization rates were found when using ICSI [18].

We did not find a significant relationship between embryo quality and OS levels in the ejaculate; there is very scarce literature comparing directly OS in semen and reproductive outcomes; however, in order to put our results in perspective, we analyzed reports assessing both DNA fragmentation and DNA oxidations, as admittedly imperfect proxies for OS evaluation. In the light of the mitigating effect of the oocyte on ssDNA damage in the sperm, the level of OS should become especially relevant to fertility with increased oocyte age; for example, damaged paternal DNA has been related to poor blastocyst formation in a study where the woman age was ≈ 35 [33]. Using a patient's own oocytes, for every 10 % increase in sperm DNA fragmentation, the probability of not becoming pregnant increased by 1.31. On the other hand, using donor oocytes (18–35 years old), no significant differences between DNA fragmentation and reproductive outcomes were found [34]. In contrast, an inverse relationship between sperm DNA oxidation (levels of 8-oxoDG) and monthly fecundity rate has been found in a naturally conceiving population where the average woman age was 25 [35], similarly to a donor conception cycle, while Esbert and colleagues [36] found that DNA damage in sperm was unrelated to fertilization, embryo morphology, and pregnancy rates in IVF or ICSI with own or donor oocytes.

We acknowledge a few shortcomings in our study: first of all, Oxysperm has not been clinically validated against other tests of OS in semen; therefore, our reported results should not be considered representative of other tests nor interpreted to be the gold standard for OS measurement. Nonetheless, Oxysperm is a simple test of easy implementation in any IVF laboratory, characteristics that make it more amenable to clinical workflow than other assays. Secondly, we measured OS in the ejaculate, thus mostly assessing the balance of ROS and antioxidants in the seminal plasma. Although our approach provides less exact assessment of the OS damage during spermatogenesis and maturation, the level of OS in the ejaculate of a patient does give useful clinical information: a high OS level could indicate the presence of an infection or of high amounts of superoxide anions which are probably going to affect spermatozoa's motility first and its DNA integrity later. In this last case, the longer the time between sample collection and preparation, the worst the quality of the sample available for ICSI.

In conclusion, the relationship between sperm OS and reproductive outcomes is likely the result of several factors, and based on our results measuring OS by Oxysperm and in agreement with Ko et al. [37], we propose that routine OS measurement in fresh ejaculate should not be recommended for all patients indiscriminately, especially when the oocytes for the ART cycle come from women younger than 35 years old.

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