

Predictive value of seminal oxidation-reduction potential (ORP) analysis for reproductive outcomes of intracytoplasmic sperm injection (ICSI) cycles



Ralf Henkel , Aqeel Morris , Paraskevi Vogiatzi , Ramadan Saleh , Hassan Sallam , Florence Boitrelle , Nicolas Garrido , Mohamed Arafa , Murat Gül , Amarnath Rambhatla , Israel Maldonado Rosas , Ashok Agarwal , Kristian Leisegang , Thomas Ignatius Siebert

PII: S1472-6483(22)00359-5
DOI: <https://doi.org/10.1016/j.rbmo.2022.05.010>
Reference: RBMO 2997

To appear in: *Reproductive BioMedicine Online*

Received date: 9 February 2022
Revised date: 19 April 2022
Accepted date: 17 May 2022

Please cite this article as: Ralf Henkel , Aqeel Morris , Paraskevi Vogiatzi , Ramadan Saleh , Hassan Sallam , Florence Boitrelle , Nicolas Garrido , Mohamed Arafa , Murat Gül , Amarnath Rambhatla , Israel Maldonado Rosas , Ashok Agarwal , Kristian Leisegang , Thomas Ignatius Siebert , Predictive value of seminal oxidation-reduction potential (ORP) analysis for reproductive outcomes of intracytoplasmic sperm injection (ICSI) cycles, *Reproductive BioMedicine Online* (2022), doi: <https://doi.org/10.1016/j.rbmo.2022.05.010>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo editing, typesetting, and review of the resulting proof before it is published in its final form. Please note that during this process changes will be made and errors may be discovered which could affect the content. Correspondence or other submissions concerning this article should await its publication online as a corrected proof or following inclusion in an issue of the journal.

Predictive value of seminal oxidation-reduction potential (ORP) analysis for reproductive outcomes of intracytoplasmic sperm injection (ICSI) cycles

Ralf Henkel^{a,b,c,d}, ORCID: 0000-0003-1128-2982

Aqeel Morris^a, ORCID: 0000-0002-6438-183X

Paraskevi Vogiatzi^e, ORCID: 0000-0001-9971-1619

Ramadan Saleh^f, ORCID: 0000-0003-0503-3533

Hassan Sallam^g, ORCID: 0000-0003-4662-8445

Florence Boitrelle^{h,i}, ORCID: 0000-0002-5322-0141

Nicolas Garrido^j, ORCID: 0000-0001-8271-5218

Mohamed Arafa^{k,l}, ORCID: 0000-0003-0107-8857

Murat Gül^m,

Amarnath Rambhatlaⁿ, ORCID: 0000-0002-1383-6670

Israel Maldonado Rosas^o, ORCID: 0000-0003-2765-6176

Ashok Agarwal^d, ORCID: 0000-0003-0585-1026

Kristian Leisegang^p, ORCID: 0000-0002-3003-8048

Thomas Ignatius Siebert^q

^aDepartment of Medical Bioscience, University of the Western Cape, Bellville, South Africa

^bDepartment of Metabolism, Digestion and Reproduction, Imperial College London, London, UK

^cLogixX Pharma, Theale, Reading, UK

^dAmerican Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH 44195, U.S.A.

^eAndromed Health & Reproduction, Fertility Diagnostics Laboratory, Maroussi, Greece

^fDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine, Sohag University, Sohag, Egypt

^gAlexandria University Faculty of Medicine, Alexandria, Egypt

^hReproductive Biology, Fertility Preservation, Andrology, CECOS, Poissy Hospital, Poissy, France

ⁱParis Saclay University, UVSQ, INRAE, BREED, Jouy-en-Josas, France

^jIVI Foundation, Health Research Institute La Fe, Valencia, Spain

^kHamad Medical Corporation, Doha, Qatar

^lDepartment of Andrology, Sexology & STIs, Faculty of Medicine, Cairo University, Cairo, Egypt

^mDepartment of Urology, Selcuk University School of Medicine, Konya, Turkey

ⁿDepartment of Urology, Vattikuti Urology Institute, Henry Ford Health System, Detroit, MI, USA

^oCitmer Reproductive Medicine, IVF LAB, Mexico City, Mexico

^pSchool of Natural Medicine, University of the Western Cape, Bellville, South Africa

^qDepartment of Obstetrics and Gynecology, Stellenbosch University, Tygerberg, South Africa

Corresponding Author

Prof. Ralf Henkel, PhD

Department of Metabolism, Digestion and Reproduction, Imperial College London, London,
UK

E-mail: r.henkel@imperial.ac.uk; Tel. : +44 7927 550637

Previous address: Department of Medical Bioscience, University of the Western Cape,
Bellville, South Africa

Abstract:

Research question: Has seminal oxidation-reduction potential (ORP) clinical relevance for reproductive outcome.

Design: In this prospective observational study a total of 144 couples who had an ICSI cycle between June 2018 and December 2020 were included. The study included patients undergoing fresh ICSI cycles with autologous gametes. Cycles that had day-3 embryo transfers and cryopreservation cycles were excluded. There was no restriction to patients with severe male infertility; couples with unexplained infertility and couples with unexplained male infertility were included, those with azoospermia were excluded. Semen analysis, seminal ORP, sperm DNA fragmentation and reproductive outcomes (fertilisation, blastocyst development, pregnancy and live birth) were determined.

Results: Seminal ORP correlated significantly negatively with fertilisation rate ($r=-0.267$; $P=0.0012$), blastocyst development rate ($r=-0.389$; $P<0.0001$), implantation ($r=-0.281$; $P=0.0010$), pregnancy ($r=-0.293$; $P=0.0006$) and live birth ($r=-0.359$; $P<0.0001$). ROC curve analysis showed significant predictive power for ORP for fertilisation ($\geq 80\%$; $AUC=0.652$; $P=0.0012$), blastocyst development rate ($\geq 60\%$; $AUC=0.762$; $P<0.0001$), implantation ($AUC=0.658$; $P=0.0015$), pregnancy ($AUC=0.673$; $P=0.0004$) and live birth ($AUC=0.728$; $P<0.0001$). Comparable results were obtained for SDF (fertilisation: $AUC: 0.678$; blastocyst development: $AUC: 0.780$; implantation: $AUC: 0.649$; pregnancy: $AUC: 0.664$; live birth: $AUC: 0.723$). Normal sperm morphology showed for all reproductive outcome parameters the lowest predictive power. With male age as confounding factor, ORP (cut-off value of $0.51 \text{ mV}/10^6 \text{ sperm/mL}$) has significant ($P<0.04667$) effects on odds ratios for all reproductive outcome parameters. Multivariate logistic regression to investigate potential seminal and female confounding factors revealed that seminal ORP significantly ($P<0.0039$; $P<0.0130$) affects reproductive outcome.

Conclusion: Seminal ORP as determined by means of the MiOXSYS system is relevant for good fertilisation, blastocyst development, implantation, clinical pregnancy and live birth.

Keywords

seminal oxidative stress / oxidation-reduction potential / sperm DNA fragmentation / ICSI / predictive value

Key message

Seminal oxidation-reduction potential is predictive of fertilisation, blastocyst development, pregnancy and live birth after ICSI.

Introduction

Globally, nearly 70 million people are affected by infertility. According to the World Health Organization, an estimated 9% of couples worldwide are unable to conceive and up to 50% of the infertility cases are attributable to male factors (*Boivin et al., 2007*). Despite advances in understanding the causes of male infertility, idiopathic infertility still accounts for about 30% to 50% of the male infertility issues (*Gelbaya et al., 2014*). Many causes of male infertility have been identified and can be classified into pre-testicular, testicular or post-testicular with congenital, acquired or idiopathic factors (*Agarwal et al., 2020; 2021a*). In many of the underlying factors such as lifestyle choices, infections, varicocele, radiation, or environmental pollution, oxidative stress (OS) is involved eventually causing sperm DNA fragmentation (SDF) (*Agarwal et al., 2020*).

OS has been reported to play an important role in various aetiologies of male infertility including male genital tract infection/inflammations, varicocele or adverse lifestyle conditions and related diseases such as obesity or diabetes mellitus (*Agarwal et al., 2006; Atiken et al., 2014; Wright et al., 2014; Abbasihormozi et al., 2019*). OS is induced by the imbalance between reactive oxygen species (ROS) and antioxidants. Under normal conditions, ROS and antioxidants are in balance; antioxidants neutralize excessive amounts of these highly

reactive free radicals and maintain homeostasis (Kothari *et al.*, 2010). Under pathological conditions, when there are higher levels of ROS than antioxidants, ROS-mediated OS ensues. Since sperm plasma membranes have an extraordinary high content of polyunsaturated fatty acids (Parks & Lynch, 1992; Henkel, 2011), male germ cells highly susceptible to lipid peroxidation (Gharagozloo & Aitken, 2011). OS is also regarded as a major cause of sperm DNA fragmentation (SDF) (Aitken & Clarkson, 1987; Muratori *et al.*, 2015a). There is a growing body of evidence indicating important roles for seminal OS and SDF for male infertility (Esteves *et al.*, 2021) and the reproductive outcomes of assisted reproduction (Sakkas & Alvarez, 2010; Esteves *et al.*, 2015; Agarwal *et al.*, 2021b). In a systematic review and meta-analysis including 23 studies with a total of 6771 cycles of treatment with assisted reproductive techniques (ART), Deng *et al.* (Deng *et al.*, 2019) reported that high SDF was associated with a significantly increased risk of lower pregnancy outcome. In another systematic review and meta-analysis of 27 studies with 1941 patients with unexplained recurrent spontaneous abortion, Yifu *et al.* reported a significant association between SDF and recurrent pregnancy loss (Yifu *et al.*, 2020).

In order to predict male fertility and sperm fertilising capacity, especially for assisted reproduction procedures, various semen parameters and sperm functions including normal sperm morphology (Kruger *et al.*, 1988; Obara *et al.*, 2001) or SDF (Simon *et al.*, 2017) are used and shown valuable tools in the clinical work-up of male infertility. For seminal OS and ROS assessment in particular, usually luminometric techniques with luminol or lucigenin as chemiluminescent probes are used (Aitken *et al.*, 1992; Zorn *et al.*, 2003). However, despite the simplicity of the luminometric measurement, the direct determination of ROS in semen is technically challenging, and assays are therefore not validated. For the determination of SDF, several methods with different protocols have been described. The most frequently used techniques to determine SDF and thereby predict the probability of a successful outcome of ART are the TUNEL assay (Henkel *et al.*, 2004; Sharma *et al.*, 2010), the Sperm Chromatin Structure Assay (SCSA; Jerre *et al.*, 2019), the COMET assay (Simon *et al.*,

2011) and the sperm chromatin dispersion (SCD) assay (Muriel *et al.*, 2006). On the other hand, the different methods described for SDF actually measure different aspects of sperm DNA damage (Zini & Sigman, 2009; Henkel *et al.*, 2010) resulting in only moderate correlation between the different methods (Rex *et al.*, 2017). In addition, extensive preparations (TUNEL assay), labour-intensive procedure (COMET assay), small number of evaluated cells (SCD, COMET assay) and poor predictive accuracy for IVF and ICSI outcome (SCSA) result in a lack of strong evidence (Cissen *et al.*, 2016; Rex *et al.*, 2017; Simon *et al.*, 2019) and therefore lack of agreement for using these tests.

A newly introduced method of detecting seminal OS is to measure the oxidation-reduction potential (ORP) which provides the overall balance between oxidants and antioxidants (Agarwal *et al.*, 2016). This method has been shown to be a quick, easy, reliable and cost-effective tool to identify infertile men (Agarwal *et al.*, 2018). In addition, Arafa *et al.* (Arafa *et al.*, 2019) and Elbardisi *et al.* (Elbardisi *et al.*, 2020) have shown a mediocre, but highly significant positive association between SDF and seminal ORP. Because of the reported issues including labour-intensive and costly procedures with the different techniques to determine SDF, there is still a need for diagnostic tools to improve the prediction of the male fertility potential. In contrast to methods for SDF determination, measurement of ORP is easy and quick, but has not yet been evaluated to predict male fertility potential in an assisted reproduction set up. Therefore, the aim of this prospective study was to investigate the predictive capabilities of seminal ORP for reproductive outcomes (fertilisation, blastocyst development, pregnancy and live birth) in patients undergoing intracytoplasmic sperm insemination (ICSI) cycles and compare these with the predictive potential of a standard fluorescent TUNEL assay for SDF.

Materials and Methods

Setting and subjects

The current prospective observational study was approved by the Biomedical Ethics Committee (Reference number: BM/16/5/18) of the University of the Western Cape, Bellville, South Africa, and all participating clinics. The study was conducted from June 2018 to December 2020. A total of 144 patients (one cycle each that was included in the study) undergoing ICSI treatments at Drs. Aevitas Fertility Clinic, Vincent Palotti Hospital, Pinelands, South Africa, Cape Fertility Clinic, Claremont, South Africa, and Tygerberg Fertility Clinic, Institute for Reproductive Medicine, Tygerberg, South Africa, were enrolled in this study and gave informed consent. The study included consenting patients undergoing fresh ICSI cycles with autologous gametes. Cycles that had day 3 embryo transfers and cryopreservation cycles were excluded. There was no restriction to patients with severe male factor infertility; couples with unexplained infertility and couples with unexplained male infertility were also included and those with azoospermia excluded. On the female side, patients with primary and secondary infertility, unexplained infertility, polycystic ovaries, endometriosis, tubal factor, myomectomy, as well as ovarian insufficiency and advanced maternal age were included.

Study procedures

The patients were instructed to abstain from sexual intercourse 2 to 3 days prior to providing the semen sample. The ejaculated semen samples were produced by masturbation, collected in a sterile specimen container and kept at 37°C until liquefaction was observed. Andrological diagnostics (i.e. manual semen analysis, testing for sperm DNA fragmentation (SDF) and determination of seminal oxidation-reduction potential (ORP) were conducted in 170 µL (50 µL for standard semen analysis; 30 µL for ORP; 100 µL for SDF) of the semen samples that were provided for the ICSI procedure within 30 minutes of liquefaction. The rest of the sample was either subjected to density gradient centrifugation or swim-up based on total motile count before the ICSI procedure (Figure 1).

ART Procedure

Stimulation protocol

For ovarian stimulation, the flexible antagonist protocol consisting of daily gonadotropins (300 IU FSH in a step down fashion to 150 IU) for 5 days beginning on day 3 of the menstrual cycle was used. Administration of GnRH antagonist (0.25 mg) was started by subcutaneous injection when the leading follicle measured 14 mm or more. Ovulation was induced by subcutaneous or intramuscular administration of 10,000 IU hCG or GnRH agonist 200 µg when the lead follicle was ≥ 18 mm and at least two other follicles were ≥ 16 mm in size. Oocyte retrieval was performed within 34 to 36 hours after hCG administration. Luteal phase support was utilized by vaginal progesterone preparation if hCG was used.

Semen Processing for ICSI

For sperm processing, standardized methods conforming to WHO 2010 criteria (WHO, 2010) were followed across the participating clinics. The preferred method for sperm processing was determined by the initial evaluation of semen parameters and quality of the semen sample produced. Two basic methods, swim-up (SU) and density gradient centrifugation (DGC; SilSelect; FertiPro, Harrilabs, Cape Town, South Africa), were used. For the swim-up, Origio sperm wash medium was used, while for the density gradient, sperm preparation buffer (SAGE, Cooper Surgical, Ferring, Cape Town, South Africa) was used for wash procedures and Sil-Silect gradient preparation (FertiPro; 40%/80%) for the density gradient centrifugation.

Oocyte retrieval procedure

Oocytes were retrieved under conscious sedation (Dormicum® IV, Hoffmann-La Roche Ltd, Basel, Switzerland) using transvaginal ultrasound guidance. Follicular fluid was aspirated utilizing sonar guided ultrasound, and examined for the presence of corona-oocyte-complexes [COC] both macroscopically and microscopically. The oocytes within the COCs were superficially graded to infer maturity (immature, germinal vesicles [GV] or metaphase I [MI] oocytes; mature, metaphase II [MII]). Post grading, the COCs were collected, washed in

Quinn's Advantage™ Medium with HEPES (SAGE™), and transferred to a Fert™ (ORIGIO®) medium.

ICSI and embryo culture

All embryology procedures were performed by a fully trained and experienced embryologist. For this study, all included oocytes were injected by standard ICSI. For all preparatory and culture procedures, Quinn's Advantage™ (Harrilabs) sequential culture medium range was used including HEPES buffered medium, sperm preparation medium, fertilisation/cleavage and blastocyst medium, oil for tissue culture, hyaluronidase, polyvinylpyrrolidone. A standardised, routine method was used for all patients. After incubation for 16-18 hours, oocytes were assessed for fertilisation (visualization of 2 PN). Embryos were individually cultured and evaluated for embryo quality (blastomere morphology, size and percentage of fragmentation) on day-2 and day-3 post-insemination. Culture to the blastocyst stage (day-4 to day-6 post-insemination) was performed in SAGE one-step culture medium (SAGE, Cooper Surgical, Ferring, Cape Town, South Africa). Embryo culture was performed in Planer BT37 incubators (Planer, Sunbury-On-Thames, UK) under 5% O₂ and 7.2% CO₂.

Embryo Transfer and reproductive outcome parameters

Embryo transfers were performed using Wallace® Classic embryo transfer catheters (Cooper Surgical®) under standard ultrasound guidance (*Sallam & Sadek, 2003*) on day 5. According to the SASREG embryo transfer guidelines, patient history and conforming to the National Legislation requirements, no more than three embryos were transferred by a reproductive medicine specialist and clinical embryologist. Embryos were assessed and graded on day 5 (blastocyst stage). Embryo grading was performed under high magnification according to a modified grading system (*Richardson et al., 2015*).

As study outcome parameters, fertilisation (number of oocytes with 2-pronuclei stage/total metaphase II oocytes) and blastocyst formation rates (developed blastocysts/fertilised

oocytes) as well as the clinical parameters implantation (sacs as seen on ultrasound), pregnancy (as determined by foetal heart beat) and live birth rates (birth of a live baby) were recorded. For the classification for good and poor fertilisation and blastocyst development, the benchmark values for the key performance indicators (fertilisation: $\geq 80\%$; blastocyst development: $\geq 60\%$) according to the Vienna Consensus of the ESHRE (ESHRE, 2017) were used. For implantation, pregnancy and live birth, classification for ROC curve analyses was done on a yes/no basis. Pregnancy and live birth were analysed per embryo transfer.

Andrology Laboratory Procedure

Semen Analysis

All semen samples were assessed for sperm concentration and motility according to World Health Organization criteria (WHO, 2010) with standard quality control measures being applied. For the determination of normal sperm morphology, a smear of 10 μL semen was applied on a slide, stained according to the Papanicolaou staining protocol and evaluated according to strict criteria (Menkveld et al., 1990).

Oxidative stress (OS)

Seminal OS was determined by means of measurement of the oxidation-reduction potential (ORP) using the MiOXSYS system (Aytu Bioscience, Englewood, CO). In brief, 30 μL of liquefied semen were placed on the sample port of the ORP sensor. After about 3 to 4 minutes, the result could be read in millivolts (mV) at the display, was then normalized according to the sperm concentration and expressed as $\text{mV}/10^6 \text{ sperm/mL}$ (Agarwal et al., 2019).

Sperm DNA fragmentation

Sperm DNA fragmentation was measured using the fluorometric TUNEL assay (Promega Corporation, Madison, USA) according to the protocol described previously (Henkel et al., 2004). Three hundred spermatozoa were evaluated for TUNEL-positivity under an

epifluorescence microscope (Zeiss, Oberkochen, Germany) at a $\times 1000$ magnification. Then, the percentage of green fluorescing sperm (TUNEL-positive) was calculated and recorded. Positive and negative controls were run in parallel with the samples.

Statistical Analysis

For the statistical analysis, MedCalc[®] Statistical Software version 20.009 (MedCalc Software Ltd, Ostend, Belgium) was used. After testing for normal distribution using the Chi-squared test, non-parametric tests were employed for the statistical evaluation. Spearman's rank correlation coefficient was used to determine the numerical correlations for analysed parameters. To determine the predictive capabilities of ORP, SDF and normal sperm morphology for fertilisation, blastocyst development rates, pregnancy and live birth, receiver operating characteristics (ROC) curve analyses were generated according to the DeLong method (DeLong *et al.*, 1988).

In order to investigate whether seminal ORP is independently influencing the reproductive outcome, the female variables (age, primary and secondary infertility, unexplained infertility, polycystic ovaries, endometriosis, tubal factor myomectomy, ovarian insufficiency and advanced maternal age) were included in a stepwise logistic regression model. In addition, a stepwise logistic regression model was also used to determine the influence of the semen factors (sperm concentration, motility, normal sperm morphology and seminal ORP) on fertilisation, blastocyst development, implantation, pregnancy and live birth.

As a sub-analysis, men were categorized according to their age as <37 years or ≥ 37 years by using the median of the male age. The Cochran-Mantel-Haenszel test was used to calculate odds ratios for male age. A P-value of <0.05 was considered significant.

Results

A total of 144 patients were recruited for the study, out of whom only 143 underwent ICSI treatment and were included in the analysis; in one case no oocytes were obtained. In addition, cryopreservation cycles and day 3 embryo transfers were excluded. The patient

cohort comprised of 20 couples with primary infertility, 12 with secondary infertility, 45 with unexplained infertility, 43 women with polycystic ovary syndrome, 31 with endometriosis, 25 with tubal factor, 3 after myomectomy, 14 with ovarian insufficiency, 21 poor responders and 73 women at advanced maternal age (>35 years). Ninety three patients presented with multiple of these conditions. The summary statistics of patient age, semen parameters (sperm concentration, total motility, and normal sperm morphology), SDF and ORP along with fertilisation and blastocyst development rates are presented in Table 1. Overall, in 134 women out of 143 (93.7%) implantation was observed, 53 (39.6%) became pregnant and 39 (29.1%) delivered a live baby, while 13 (9.1%) miscarriages occurred. In 5 (3.5%) cases, no fertilisation occurred and in 9 (6.3%) cases no blastocysts were obtained.

Table 2A compares the mean values of seminal ORP and SDF in the cases where no fertilisation, blastocyst formation, implantation, pregnancy and live birth, respectively, occurred with those from patients with fertilisation, blastocyst formation, implantation, pregnancy and live birth. All comparisons except for ORP (fertilisation and blastocyst development) and SDF (fertilisation), showed significant differences. In Table 2B, the mean values of seminal ORP and SDF of cases with no fertilisation and blastocyst formation, respectively, compared with those cases where the fertilisation and blastocyst formation rates were $\geq 80\%$ and $\geq 60\%$, respectively. While no difference between the groups was found for ORP, the differences for SDF were significant.

Sperm preparation of the 144 semen samples was done in 43 (29.9%) samples with standard swim-up and in 101 (70.1%) with density gradient centrifugation. No difference in fertilisation (SU: 70.9% vs. DGC: 74.6%; $P=0.3113$), blastocyst development (SU: 68.6% vs. DGC: 64.2%; $P=0.3819$), and implantation rates (SU: 35.4% vs. DGC: 29.0%; $P=0.2681$) as well as for pregnancy (SU: 0.5 vs. DGC: 0.3; $P=0.1484$) and live birth (SU: 0.4 vs. DGC: 0.2; $P=0.0945$) was recorded between the two sperm separation techniques.

The correlations between the male parameters analysed in this study are shown in Table 3. As expected, significant positive correlations were found between sperm concentration, motility and normal morphology, as well as between normal morphology and motility. There were also highly significant positive correlations between the percentage of TUNEL-positive sperm and seminal ORP ($r=0.665$; $P<0.0001$) as well as male age ($r=0.328$; $P=0.0001$). Furthermore, seminal ORP showed a weak, but significant ($r=0.268$; $P=0.0012$) positive association with male age. On the other hand, significant negative correlations were found between male age and sperm concentration, normal morphology and motility, between sperm concentration and ORP and the percentage of TUNEL-positive sperm, between ORP and motility, and normal morphology, as well as between the percentage of TUNEL-positive sperm and normal morphology and motility (Table 3).

Correlations between male age, standard semen parameters, advanced sperm function test (ORP and SDF) and reproductive outcomes (fertilisation rate, blastocyst development rate, pregnancy and live birth) in patients undergoing ICSI treatment are reported in Table 4. In the current study, all assisted reproduction parameters (fertilisation, blastocyst development, implantation, pregnancy and live birth) were significantly negatively correlated with ORP ($r=-0.267$; $P=0.0012$, $r=-0.389$; $P<0.0001$, $r=-0.281$; $P=0.0010$, $r=-0.293$; $P=0.0006$ and $r=-0.359$; $P<0.0001$, respectively). Similarly, the percentage of TUNEL-positive sperm was significantly negatively associated with these parameters (fertilisation: $r=-0.341$; $P<0.0001$, blastocyst development: $r=-0.465$; $P<0.0001$, implantation: $r=-0.313$; $P=0.0002$, pregnancy: $r=-0.278$; $P=0.0011$ and live birth: $r=-0.351$; $P<0.0001$, respectively). While male age was not correlated with the fertilisation rate, weak but significant negative associations were observed for the blastocyst development rate ($r=-0.272$; $P=0.001$), implantation ($r=-0.297$; $P=0.0005$), pregnancy ($r=-0.250$; $P=0.0035$) and live birth ($r=-0.188$; $P=0.0295$).

Receiver operating characteristic (ROC) curve analyses for seminal ORP, the percentage of TUNEL-positive sperm and normal sperm morphology were carried out to evaluate the

predictive power of seminal ORP, SDF and normal morphology with regard to the benchmark cut-off values for fertilisation ($\geq 80\%$) (Figure 2A) and blastocyst development rate ($\geq 60\%$), (Figure 2B), implantation (Figure 2C) as well as pregnancy (Figure 2D) and live birth (Figure 2E) after ICSI. The calculations were significant for all parameters (ORP, percentage of TUNEL-positive sperm and normal morphology) with respect to all endpoints (fertilisation and blastocyst development rates, implantation, pregnancy and live birth) except for the prediction of fertilisation with normal sperm morphology (AUC: 0.572; $P=0.1320$) (Tables 5A-E). When comparing the predictive power of the tests by evaluating the areas under the curve (AUC), it appeared that the AUC for normal morphology was the lowest ranging between 0.572 and 0.704, whereas the AUC for ORP was ranging from 0.652 to 0.762 and that for the percentage of TUNEL-positive sperm from 0.649 to 0.780. When the AUCs for these different parameters were directly statistically compared, no differences were found for all endpoints (fertilisation, blastocyst development, implantation, pregnancy and live birth) except for the comparison TUNEL-positive sperm vs. normal sperm morphology for fertilisation ($P=0.0400$; Table 5A). Furthermore, while the predictive power for the three parameters testes were well below 70% for fertilisation, implantation and pregnancy, it was well above 70% for ORP and the percentage of TUNEL-positive sperm for blastocyst development and live birth. For normal morphology, the predictive power was highest with about 70% for blastocyst development. The relevant calculated cut-off values for seminal ORP predicting good fertilisation ($>80\%$), blastocyst formation ($>60\%$), implantation, pregnancy and live birth were ≤ 0.709 , ≤ 0.53 , ≤ 0.465 , ≤ 0.465 and ≤ 0.393 mV/ 10^6 sperm/mL, respectively, with an average value of 0.51 mV/ 10^6 sperm/mL (Table 5).

Logistic regression with fertilisation, blastocyst development, implantation, pregnancy and live birth as dependent parameters and high/low seminal ORP (≤ 0.51 mV/ 10^6 sperm/mL), and primary and secondary infertility, unexplained infertility, polycystic ovaries, endometriosis, tubal factor, myomectomy, ovarian insufficiency and advanced maternal age as independent variables showed overall model fits ($P<0.0017$) with a significant ($P<0.0130$)

influence of ORP on all reproductive outcome parameters. Together with seminal ORP, female age was only included in the stepwise logistic regression model for blastocyst development, implantation and pregnancy ($P < 0.0120$). Among the semen factors, high ORP values proved significant ($P < 0.0039$) in the overall model fit for fertilisation, implantation, pregnancy and live birth.

Calculation of odds ratios using the cut-off point for a normal ORP of ≤ 0.51 mV/ 10^6 sperm/mL obtained in this study and the various reproductive endpoints (fertilisation, blastocyst development, implantation, pregnancy and live birth) resulted in significantly higher odds for patients achieving $\geq 80\%$ fertilisation, ≥ 60 blastocyst development, implantation, pregnancy and live birth, if the ORP was low, i.e. ≤ 0.51 mV/ 10^6 sperm/mL (Tables 6). If the previously published cut-off value of 1.34 mV/ 10^6 sperm/mL (Agarwal *et al.*, 2019) was used, the results were only significant for fertilisation and blastocyst development, but not for pregnancy and live birth (not shown).

A similar trend was observed in the odds ratios when the male age was taken into consideration as confounding factor (Table 7). It was also obvious that older men (≥ 37 years) with high ORP (> 0.51 mV/ 10^6 sperm/mL) not only have a lower chance of fertilising oocytes, but also a lower chance of good ($\geq 60\%$) blastocyst development. Consequently, this will result in a lower chance of implantation, pregnancy and live birth than in men with low ORP or young men. Overall, younger men have higher chances of a successful reproductive outcome.

Discussion

Although the WHO has standardised the procedures for conventional standard semen analysis (WHO, 2010; WHO, 2021), it cannot predict male fertility and fertilising potential of spermatozoa (Nagler, 2011; Wang & Swerdloff, 2014). Since the fertilisation process is a multifactorial process (Henkel *et al.*, 2005), any given test can only provide a limited probability for successful fertilisation and even less forecast a positive reproductive outcome. Consequently, conventional semen parameters provide only a limited prognosis for adequate sperm function and do not identify the cause of the infertility in about 30% to 50% of the cases (Jungwirth *et al.*, 2015; Chehab *et al.*, 2015). Hence, these cases remain idiopathic or unexplained and frequently do not receive the necessary treatment. Due to the problems of standard semen analysis in predicting male fertilising potential, additional sperm function tests including tests for sperm DNA damage and OS, i.e. tests for sperm DNA fragmentation (SDF) and reactive oxygen species (ROS), were developed.

While the clinical value of SDF testing is gradually being acknowledged and recommended (Tharakan *et al.*, 2021; WHO, 2021), tests for OS are still regarded as research tests. Traditionally, seminal OS is measured either by direct determination of the ROS using luminescent probes such as luminol or lucigenin (Aitken *et al.*, 1992). Alternatively, the determination of the total antioxidant capacity (TAC; Sharma *et al.*, 1999) has been suggested as an indirect measure for OS. Yet, these techniques suffer from high variation of results and are not generally accepted for routine use. A novel galvanostatic technique measures the balance between oxidation and reduction, the ORP. The technique was shown to distinguish between sperm donors and infertile patients (Agarwal *et al.*, 2016). However, it is still considered experimental, as there is insufficient evidence to correlate seminal ORP with reproductive outcomes. To the best of our knowledge, the current study is the first presenting evidence for the predictive value of seminal ORP measurement with regard to reproductive endpoints, namely fertilisation, blastocyst development, implantation, pregnancy and live birth after ICSI.

As expected, seminal ORP was significantly negatively correlated with sperm concentration, motility and normal morphology (Agarwal *et al.*, 2018; Cicek *et al.*, 2021) and significantly positively with male age (Nago *et al.*, 2021) and sperm DNA fragmentation (Arafa *et al.*, 2019). While the former associations indicate the negative impact of seminal OS on sperm functions, the latter associations, though only weak, are in line with the age-related increase of seminal ROS levels (Cocuzza *et al.*, 2008). Yet, logistic regression revealed that ORP is independent from female and other semen parameters. This not only explains the significant associations between male age and the reproductive outcomes, but also our observation that older men (≥ 37 yrs.) with high seminal OS (> 0.51 mV/ 10^6 sperm/mL) have lower chances for their sperm resulting in successful reproductive outcome than men of the same age group with low seminal ORP values (≤ 0.51 mV/ 10^6 sperm/mL). This further implies that an antioxidant treatment or lifestyle changes leading to a decrease in OS may result in increased fertilisation and improved blastocyst development rates.

ROC curve analyses for normal sperm morphology, SDF and seminal ORP were performed in this study for fertilisation, blastocyst development, implantation, pregnancy and live birth. All tested sperm/semen parameters significantly predicted the reproductive outcome parameters included in this study with predictive powers between 61.8% (normal morphology for implantation) and 78.0% (SDF for good blastocyst development). Notably, normal sperm morphology showed the worst predictive power for all classification variables. This might be because in this study all patients were treated with ICSI where a selection of the best morphologically appearing vital sperm for injection is performed. When the areas under the curve (AUC) were statistically compared, no differences between the AUCs of the sperm/semen parameters, except between SDF and morphology for fertilisation, were observed. In fact, the AUCs of SDF and ORP were comparable with those reported for SDF in previous studies (Muratori *et al.*, 2015b; Cissen *et al.*, 2016; Wiweko & Utami *et al.*, 2017).

Interestingly, while cut-off values for SDF and normal sperm morphology were calculated for all reproductive outcome points in the range of what was previously published, between 19% and 36.5% for SDF and 4% for normal morphology, respectively, the cut-off point for ORP with an average of $0.51 \text{ mV}/10^6 \text{ sperm/mL}$ was markedly lower than those ($1.34 \text{ mV}/10^6 \text{ sperm/mL}$, $1.36 \text{ mV}/10^6 \text{ sperm/mL}$ and $1.38 \text{ mV}/10^6 \text{ sperm/mL}$) published previously (Agarwal *et al.*, 2017; Arafa *et al.*, 2018; Agarwal *et al.*, 2019). A possible explanation for this discrepancy could be that these values were not calculated by using reproductive outcomes in an assisted reproduction program, but by distinguishing between men with normal/abnormal semen parameters and donors/patients, respectively. However, when considering the clinical value of a specific test in andrological diagnostics, reproductive outcome parameters should be used. Therefore, a lower cut-off value for seminal ORP than the previously published ones seems reasonable and we propose a cut-off of $\leq 0.51 \text{ mV}/10^6 \text{ sperm/mL}$. However, at this stage, it must also clearly be said that this value still needs to be validated in larger, more controlled studies. This value might also differ for IVF or for intrauterine insemination (IUI) because for IVF and IUI all sperm functions have to be in the optimum range to achieve a pregnancy and live birth.

In many studies, trying to evaluate sperm functional tests, fertilisation is used as the primary reproductive endpoint without further exploring possible adverse effects in early embryo development, implantation, pregnancy and live birth. However, considering that sperm with DNA damage can still fertilise oocytes (Henkel *et al.*, 2004), this might not be the best endpoint to assess sperm functional capability. In addition, one has also to consider that besides the fertilising capacity of sperm, blastulation depends on the quality of the oocyte as well as the culture conditions. Moreover, the occurrence of pregnancy and live birth depend on a good embryo transfer, endometrial receptivity as well as on female variables such as preeclampsia, systemic diseases or preterm labour. Based on this, it is obvious that the AUC and the odds ratios diminished steadily from fertilisation to live birth. This can clearly be seen in our results. Therefore, later reproductive endpoints were used in this study.

Since no difference between the AUCs for ORP and SDF was observed, the question arises which assay might be clinically most suited. In this study, the association between SDF and seminal OS measured as ORP was confirmed (*Homa et al., 2019*), but one should not forget that DNA damage can also occur due to defective or failing DNA repair mechanisms (*Puzuka et al., 2021*). Hence, both tests complement each other. However, when considering time, labour and cost, these factors seem to be in favour of the determination of ORP. As reported previously (*Rex et al., 2017*), the TUNEL assay is labour-intensive. In contrast, the analysis of seminal ORP requires only about 4 minutes following liquefaction. Hence, the result can be discussed with the patient on the same day alongside possible treatment options.

Although this study clearly shows the impact of seminal OS on reproductive outcome in a wider range of male patients, the study has limitations. First, the use of ICSI might have contributed to the fact that the calculated cut-off value of ORP was lower than the previously published values because sperm functions such as motility or acrosome reaction did not play a role in the fertilisation process. In contrast, for IUI and IVF, these sperm functions play an essential role as capacitation has to be triggered by a small amount of ROS. Therefore the cut-off value of ORP may be different for IUI or IVF. Second, the use of a fluorescence microscopic TUNEL assay, which limited the number of sperm that were evaluated. Third, this study was conducted using a non-select cohort of patients in whom seminal ORP was determined and related to reproductive outcomes. Further prospective studies need to confirm the current findings to see if the management of patients on the basis of the ORP results can improve clinical outcomes on infertile patients.

Conclusions

In conclusion, this is the first study showing the impact of seminal ORP on fertilisation, blastocyst development, implantation, pregnancy and live birth as reproductive outcome

parameters with an average cut-off value of 0.51 mV/10⁶ sperm/mL in an ICSI program. However, further evaluation not only by carefully establishing a physiological range of ORP for normal sperm functions, but also by conducting larger studies including IVF and IUI, are necessary to clearly establish the predictive capabilities of ORP in andrological diagnostics for assisted reproduction. An early and reliable detection of seminal OS can help to further personalize ART treatment in male infertility patients and guide clinicians in counselling patients. Such an approach could then lead to an increase in successful fertility treatments or the utilization of less invasive approaches, thereby leading to better cost-effectiveness in healthcare systems. However, at this stage, it must also be pointed out that the measurement of seminal ORP cannot be offered to patients with severe oligozoospermia or azoospermia. Based on these findings, it can be concluded that ORP is a useful parameter to be incorporated in the evaluation of male infertility. In order to uncover the impact of seminal ORP on other sperm functional parameters, which play an essential role in natural conception as well as for IVF or IUI, relevant studies are underway.

Availability of data

The data underlying this article will be shared on reasonable request to the corresponding author.

Funding

The authors are grateful to Aytu Bioscience, Englewood, CO, for providing the MiOXSYS system and the relevant sensors.

AM was financially supported by a DAAD-NRF Joint in-Country Scholarship Program (grant number: 118279).

Conflict of interest

RH is employed by LogixX Pharma Ltd.

Journal Pre-proof

Author's roles

Conceptualization: RH, TIS. Data curation: RH, AM. Formal analysis: AM, RH. Investigation: AM. Methodology: RH, TIS. Project administration: RH. Supervision: RH, TIS. Validation: RH, AM. Visualization: RH. Writing—original draft: RH. Review of manuscript: AM, RH, TIS, PV, RS, HS, FB, NGP, MA, MG, AR, IMR, RSS, AA, KL. Final editing: RH

Acknowledgements

The authors wish to thank Dr. Rupin Shah, Division of Andrology, Department of Urology, Lilavati Hospital and Research Centre, Mumbai, India, for reviewing the manuscript.

References

1. Abbasihormozi, S., Babapour, V., Kouhkan, A., Naslji, A.N., Afraz, K., Zolfaghary, Z., Shahverdi, A., 2019. **Stress hormone and oxidative stress biomarkers link obesity and diabetes with reduced fertility potential**. Cell J. 21, 307-313. DOI: 10.22074/cellj.2019.6339
2. Agarwal, A., Baskaran, S., Parekh, N., Cho, C.L., Henkel, R., Vij, S., Arafa, M., Panner Selvam, M.K., Shah, R., 2021a. **Male infertility**. Lancet 397(10271): 319-333. DOI: 10.1016/S0140-6736(20)32667-2
3. Agarwal, A., Henkel, R., Sharma, R., Tadros, N.N., Sabanegh, E., 2018. **Determination of seminal oxidation-reduction potential (ORP) as an easy and cost-effective clinical marker of male infertility**. Andrologia 50. Epub 2017 Oct 23. DOI: 10.1111/and.12914
4. Agarwal, A., Leisegang, K., Majzoub, A., Henkel, R., Finelli, R., Panner Selvam, M.K., Tadros, N., Parekh, N., Ko, E.Y., Cho, C.L., Arafa, M., Alves, M.G., Oliveira, P.F., Alvarez, J.G., Shah, R., 2021b. **Utility of antioxidants in the treatment of**

- male infertility: Clinical guidelines based on a systematic review and analysis of evidence.** World J. Mens Health 39, 233-290. DOI: 10.5534/wjmh.200196
5. Agarwal, A., Majzoub, A., Parekh, N., Henkel, R., 2020. **A schematic overview of the current status of male infertility practice.** World J. Mens Health 38, 308-322. DOI: 10.5534/wjmh.190068
 6. Agarwal, A., Panner Selvam, M.K., Arafa, M., Okada, H., Homa, S., Killeen, A., Balaban, B., Saleh, R., Armagan, A., Roychoudhury, S., Sikka, S., 2019. **Multi-center evaluation of oxidation-reduction potential by the MiOXSYS in males with abnormal semen.** Asian J. Androl. 21, 1-5. DOI: 10.4103/aja.aja_5_19
 7. Agarwal, A., Prabakaran, S., Allamaneni, S.S.R., 2006. **Relationship between oxidative stress, varicocele and infertility: a meta-analysis.** Reprod. Biomed. Online 12, 630-633. DOI: 10.1016/s1472-6483(10)61190-x
 8. Agarwal, A., Roychoudhury, S., Bjugstad, K.B., Cho, C.L., 2016. **Oxidation-reduction potential of semen: what is its role in the treatment of male infertility?** Ther. Adv. Urol. 8, 302-318. DOI: 10.1177/1756287216652779
 9. Agarwal, A., Roychoudhury, S., Sharma, R., Gupta, S., Majzoub, A., Sabanegh, E., 2017. **Diagnostic application of oxidation-reduction potential assay for measurement of oxidative stress: clinical utility in male factor infertility.** Reprod. Biomed. Online 34, 48-57. DOI: 10.1016/j.rbmo.2016.10.008
 10. Aitken, R.J., Buckingham, D.W., West, K.M., 1992. **Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence.** J. Cell Physiol. 151, 466-477. DOI: 10.1002/jcp.1041510305
 11. Aitken, R.J., Clarkson, J.S., 1987. **Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa.** J. Reprod. Fert. 81, 459-469. DOI: 10.1530/jrf.0.0810459

12. Aitken, R.J., Smith, T.B., Jobling, M.S., Baker, M.A., De Iuliis, G.N., 2014. **Oxidative stress and male reproductive health**. *Asian J, Androl.* 16, 31-38.
DOI: 10.4103/1008-682X.122203
13. Arafa, M., Agarwal, A., Al Said, S., Majzoub, A., Sharma, R., Bjugstad, K.B., AlRumaihi, K., Elbardisi, H., 2018. **Semen quality and infertility status can be identified through measures of oxidation–reduction potential**. *Andrologia* 50, e12881; Aug 3. DOI: 10.1111/and.12881.
14. Arafa, M., Henkel, R., Agarwal, A., Majzoub, A., Elbardisi, H., 2019. **Correlation of oxidation-reduction potential with hormones, semen parameters and testicular volume**. *Andrologia* 51, e13258. DOI: 10.1111/and.13258
15. Boivin, J., Bunting, L., Collins, J.A., Nygren, K.G., 2007. **International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care**. *Hum. Reprod.* 22, 1506-1512.
DOI: 10.1093/humrep/dem046
16. Chehab, M., Madala, A., Trussell, J.C., 2015. **On-label and off-label drugs used in the treatment of male infertility**. *Fertil. Steril.* 103, 595-604.
DOI: 10.1016/j.fertnstert.2014.12.122
17. Cicek, O.S.Y., Kaya, G., Alyuruk, B., Doger, E., Girisen, T., Filiz, S., 2021. **The association of seminal oxidation reduction potential with sperm parameters in patients with unexplained and male factor infertility**. *Int. Braz. J. Urol.* 47, 112-119. DOI: 10.1590/S1677-5538.IBJU.2019.0751
18. Cissen, M., Wely, M.V., Scholten, I., Mansell, S., Bruin, J.P., Mol, B.W., Braat, D., Repping, S., Hamer, G., 2016. **Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: A systematic review and meta-analysis**. *PLoS One* 11, e0165125. DOI: 10.1371/journal.pone.0165125
19. Cocuzza, M., Athayde, K.S., Agarwal, A., Sharma, R., Pagani, R., Lucon, A.M., Srougi, M., Hallak, J., 2008. **Age-related increase of reactive oxygen species in**

- neat semen in healthy fertile men. *Urology* 71, 490-494.
DOI: 10.1016/j.urology.2007.11.041
20. DeLong, E.R., DeLong, D.M., Clarke-Pearson, D.L., 1988. **Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach.** *Biometrics* 44, 837-845. PMID: 3203132
21. Deng, C., Li, T., Xie, Y., Guo, Y., Yang, Q.Y., Liang, X., Deng, C.H., Liu, G.H., 2019. **Sperm DNA fragmentation index influences assisted reproductive technology outcome: A systematic review and meta-analysis combined with a retrospective cohort study.** *Andrologia* 51, e13263. DOI: 10.1111/and.13263
22. Elbardisi, H., Finelli, R., Agarwal, A., Majzoub, A., Henkel, R., Arafa, M., 2020. **Predictive value of oxidative stress testing in semen for sperm DNA fragmentation assessed by sperm chromatin dispersion test.** *Andrology* 8, 610-617. DOI: 10.1111/andr.12743
23. ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017. **The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators.** *Reprod. Biomed. Online* 35, 494-510. DOI: 10.1016/j.rbmo.2017.06.015
24. Esteves, S.C., Sanchez-Martin, F., Sanchez-Martin, P., Schneider, D.T., Gosálvez, J., 2015. **Comparison of reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm.** *Fertil. Steril.* 104, 1398-1405. DOI: 10.1016/j.fertnstert.2015.08.028
25. Esteves, S.C., Zini, A., Coward, R.M., Evenson, D.P., Gosálvez, J., Lewis, S.E.M., Sharma, R., Humaidan, P., 2021. **Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations.** *Andrologia* 53, e13874. DOI: 10.1111/and.13874

26. Gelbaya, T.A., Potdar, N., Jeve, Y.B., Nardo, L.G., 2014. **Definition and epidemiology of unexplained infertility**. *Obstet. Gynecol. Surv.* 69, 109-115. DOI: 10.1097/OGX.0000000000000043
27. Gharagozloo, P., Aitken, R.J., 2011. **The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy**. *Hum. Reprod.* 26, 1628-1640. DOI: 10.1093/humrep/der132
28. Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W.-B., Kruger, T.F., 2004. **Influence of deoxyribonucleic acid damage on fertilization and pregnancy**. *Fertil. Steril.* 81, 965-972. DOI: 10.1016/j.fertnstert.2003.09.044
29. Henkel, R., Hoogendijk, C.F., Bouic, P.J., Kruger, T.F., 2010. **TUNEL assay and SCSA determine different aspects of sperm DNA damage**. *Andrologia* 42, 305-313. DOI: 10.1111/j.1439-0272.2009.01002.x
30. Henkel, R., Maaß, G., Bödeker, R.-H., Scheibelhut, C., Stalf, T., Mehnert, C., Schuppe, H.C., Jung, A., Schill, W.-B., 2005. **Sperm function and assisted reproduction technology**. *Reprod. Med. Biol.* 4, 7-30. DOI: 10.1111/j.1447-0578.2005.00087.x
31. Henkel, R. 2011. **Leukocytes and oxidative stress: dilemma for sperm function and male fertility**. *Asian J. Androl.* 13, 43-52. DOI: 10.1038/aja.2010.76
32. Homa, S.T., Vassiliou, A.M., Stone, J., Killeen, A.P., Dawkins, A., Xie, J., Gould, F., Ramsay, J.W.A., 2019. **A comparison between two assays for measuring seminal oxidative stress and their relationship with sperm DNA fragmentation and semen parameters**. *Genes (Basel)* 10, 236. DOI: 10.3390/genes10030236.
33. Jerre, E., Bungum, M., Evenson, D., Giwercman, A., 2019. **Sperm chromatin structure assay high DNA stainability sperm as a marker of early miscarriage after intracytoplasmic sperm injection**. *Fertil. Steril.* 112, 46-53. DOI: 10.1016/j.fertnstert.2019.03.013

34. Jungwirth, A., Diemer, T., Dohle, G.R., Giwercman, A., Kopa, Z., Krausz, C., Minhas, S., Tournaye, H., 2015. **EAU Guidelines on male infertility**. Eur. Urol. <https://uroweb.org/wp-content/uploads/EAU-Guidelines-Male-Infertility-20151.pdf>
35. Kothari, S., Thompson, A., Agarwal, A., du Plessis, S., 2010. **Free radicals: Their beneficial and detrimental effects on sperm function**. Indian J. Exp. Biol. 48, 425-435. PMID: 20795359
36. Kruger, T.F., Acosta, A.A., Simmons, K.F., Swanson, R.J., Matta, J.F., Oehninger, S., 1988. **Predictive value of abnormal sperm morphology in in vitro fertilization**. Fertil. Steril. 49, 112-117. DOI: 10.1016/s0015-0282(16)59660-5
37. Menkveld, R., Stander, F.S.H., Kotze, T.J.W., Kruger, T.F., van Zyl, J.A., 1990. **The evaluation of morphological characteristics of human spermatozoa according to stricter criteria**. Hum. Reprod. 5, 586-592. DOI: 10.1093/oxfordjournals.humrep.a137150
38. Muratori, M., Marchiani, S., Tamburrino, L., Cambi, M., Lotti, F., Natali, I., Filimberti, E., Noci, I., Forti, G., Maggi, M., Baldi, E., 2015b. **DNA fragmentation in brighter sperm predicts male fertility independently from age and semen parameters**. Fertil. Steril. 104, 582-590. DOI: 10.1016/j.fertnstert.2015.06.005
39. Muratori, M., Tamburrino, L., Marchiani, S., Cambi, M., Olivito, B., Azzari, C., Forti, G., Baldi, E., 2015a. **Investigation on the origin of sperm DNA fragmentation: Role of apoptosis, immaturity and oxidative stress**. Mol. Med. 21, 109-122. DOI: 10.2119/molmed.2014.00158
40. Muriel, L., Garrido, N., Fernandez, J.L., Remohi, J., Pellicer, A., de los Santos, M.J., Meseguer M., 2006. **Value of the sperm deoxyribonucleic acid fragmentation level, as measured by the sperm chromatin dispersion test, in the outcome of in vitro fertilization and intracytoplasmic sperm injection**. Fertil. Steril. 85, 371-383. DOI: 10.1016/j.fertnstert.2005.07.1327
41. Nagler, H.M., 2011. **Male factor infertility: A solitary semen analysis can never predict normal fertility**. Nat. Rev. Urol. 8, 16-17. DOI: 10.1038/nrurol.2010.226

42. Nago, M., Arichi, A., Omura, N., Iwashita, Y., Kawamura, T., Yumura, Y., 2021. **Aging increases oxidative stress in semen.** *Investig. Clin. Urol.* 62, 233-238. DOI: 10.4111/icu.20200066
43. Obara, H., Shibahara, H., Tsunoda, H., Taneichi, A., Fujiwara, H., Takamizawa, S., Idei, S., Sato, I., 2001 **Prediction of unexpectedly poor fertilization and pregnancy outcome using the strict criteria for sperm morphology before and after sperm separation in IVF-ET.** *Int. J. Androl.* 24, 102-108. DOI: 10.1046/j.1365-2605.2001.00275.x
44. Parks, J.E., Lynch, D.V., 1992. **Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes.** *Cryobiology* 29, 255-266. DOI: 10.1016/0011-2240(92)90024-v
45. Puzuka, A., Alksere, B., Gailite, L., Erenpreiss, J., 2021. **Idiopathic infertility as a feature of genome instability.** *Life (Basel)* 11, 628. DOI: 10.3390/life11070628
46. Rex, A.S., Aagaard, J., Fedder, J., 2017. **DNA fragmentation in spermatozoa: a historical review.** *Andrology* 5, 622-630. DOI: 10.1111/andr.12381
47. Richardson, A., Brearley, S., Ahitan, S., Chamberlain, S., Davey, T., Zujovic, L., Hopkisson, J., Campbell, B., Raine-Fenning, N., 2015 **A clinically useful simplified blastocyst grading system.** *Reprod. Biomed. Online* 31, 523-530. DOI: 10.1016/j.rbmo.2015.06.017
48. Sakkas, D., Alvarez, J.G., 2010. **Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis.** *Fertil. Steril.* 93, 1027-1036. DOI: 10.1016/j.fertnstert.2009.10.046
49. Sallam, H.N., Sadek, S.S., 2003. **Ultrasound-guided embryo transfer: a meta-analysis of randomized controlled trials.** *Fertil. Steril.* 80, 1042-1046. DOI: 10.1016/s0015-0282(03)01009-4
50. Sharma, R.K., Pasqualotto, F.F., Nelson, D.R., Thomas, A.J. Jr, Agarwal, A., 1999. **The reactive oxygen species—total antioxidant capacity score is a new**

- measure of oxidative stress to predict male infertility.** Hum. Reprod. 14, 2801-2807. DOI: 10.1093/humrep/14.11.2801
51. Sharma, R.K., Sabanegh, E., Mahfouz, R., Gupta, S., Thiyagarajan, A., Agarwal, A., 2010. **TUNEL as a test for sperm DNA damage in the evaluation of male infertility.** Urology 76, 1380-1386.
52. Simon, L., Emery, B., Carrell, D.T., 2019. **Sperm DNA fragmentation: consequences for reproduction.** Adv. Exp. Med. Biol. 1166, 87-105. DOI: 10.1007/978-3-030-21664-1_6
53. Simon, L., Lutton, D., McManus, J., Lewis, S.E., 2011. **Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success.** Fertil. Steril. 95, 652-657. DOI: 10.1016/j.fertnstert.2010.08.019
54. Simon, L., Zini, A., Dyachenko, A., Ciampi, A., Carrell, D.T., 2017. **A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome.** Asian J. Androl. 19, 80-90. DOI: 10.4103/1008-682X.182822
55. Tharakan, T., Bettocchi, C., Carvalho, J., Corona, G., Jones, T.H., Kadioglu, A., Martínez Salamanca, J.I., Serefoglu, E.C., Verze, P., Salonia, A., Minhas, S., EAU Working Panel on Male Sexual Reproductive Health., 2021. **A clinical consultation guide on the indications for performing sperm DNA fragmentation testing in men with infertility and testicular sperm extraction in nonazoospermic men.** Eur. Urol. Focus Jan 6; S2405-4569(20)30319-9. Online ahead of print. DOI: 10.1016/j.euf.2020.12.017.
56. Wang, C., Swerdloff, R.S., 2014. **Limitations of semen analysis as a test of male fertility and anticipated needs from newer tests.** Fertil. Steril. 102, 1502-1507. DOI: 10.1016/j.fertnstert.2014.10.021

57. Wiweko, B., Utami, P., 2017. **Predictive value of sperm deoxyribonucleic acid (DNA) fragmentation index in male infertility.** Basic Clin. Androl. 27, 1. DOI: 10.1186/s12610-016-0046-3
58. World Health Organization., 2010. **WHO Laboratory Manual for the Examination and Processing of Human Semen.** 5th Edition. World Health Organization, WHO Press, Geneva, Switzerland
59. World Health Organization., 2021. **WHO Laboratory Manual for the Examination and Processing of Human Semen.** 6th Edition; Geneva, Switzerland: World Health Organization; Licence: CC BY-NC-SA 3.0 IGO
60. Wright, C., Milne, S., Leeson, H., 2014. **Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility.** Reprod. Biomed. Online 28, 684-703. DOI: 10.1016/j.rbmo.2014.02.004
61. Yifu, P., Lei, Y., Shaoming, L., Yujin, G., Xingwang, Z., 2020. **Sperm DNA fragmentation index with unexplained recurrent spontaneous abortion: A systematic review and meta-analysis.** J. Gynecol. Obstet. Hum. Reprod. 101740. DOI: 10.1016/j.jogoh.2020.101740
62. Zini, A., Sigman, M., 2009. **Are tests of sperm DNA damage clinically useful? Pros and cons.** J. Androl. 30, 219-229. DOI: 10.2164/jandrol.108.006908
63. Zorn, B., Vidmar, G., Meden-Vrtovec, H., 2003. **Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection.** Int. J. Androl. 26, 279-285. DOI: 10.1046/j.1365-2605.2003.00424.x

Figures:

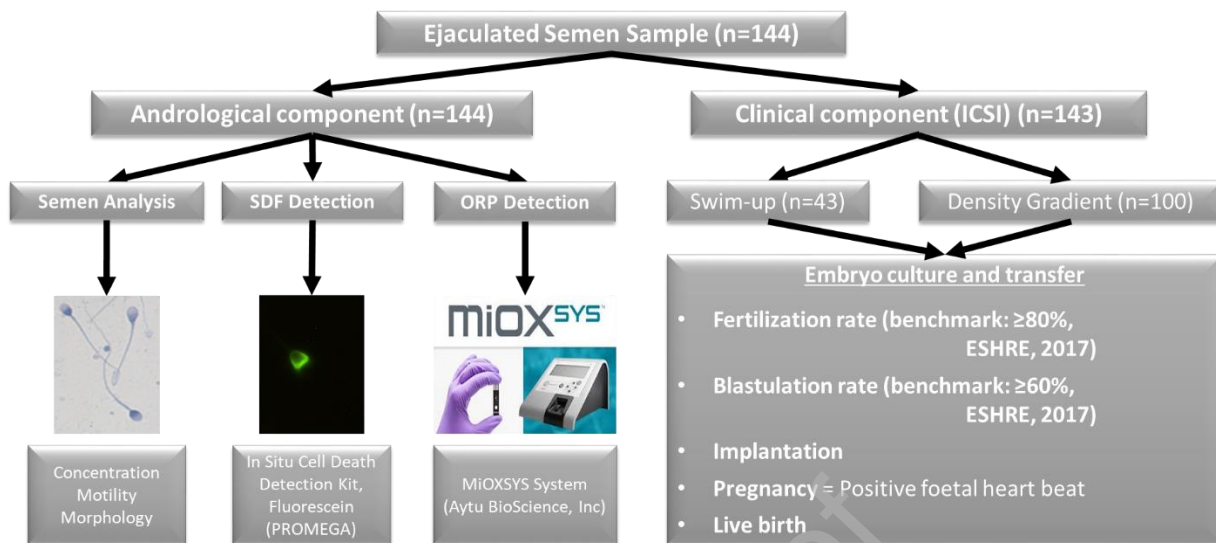
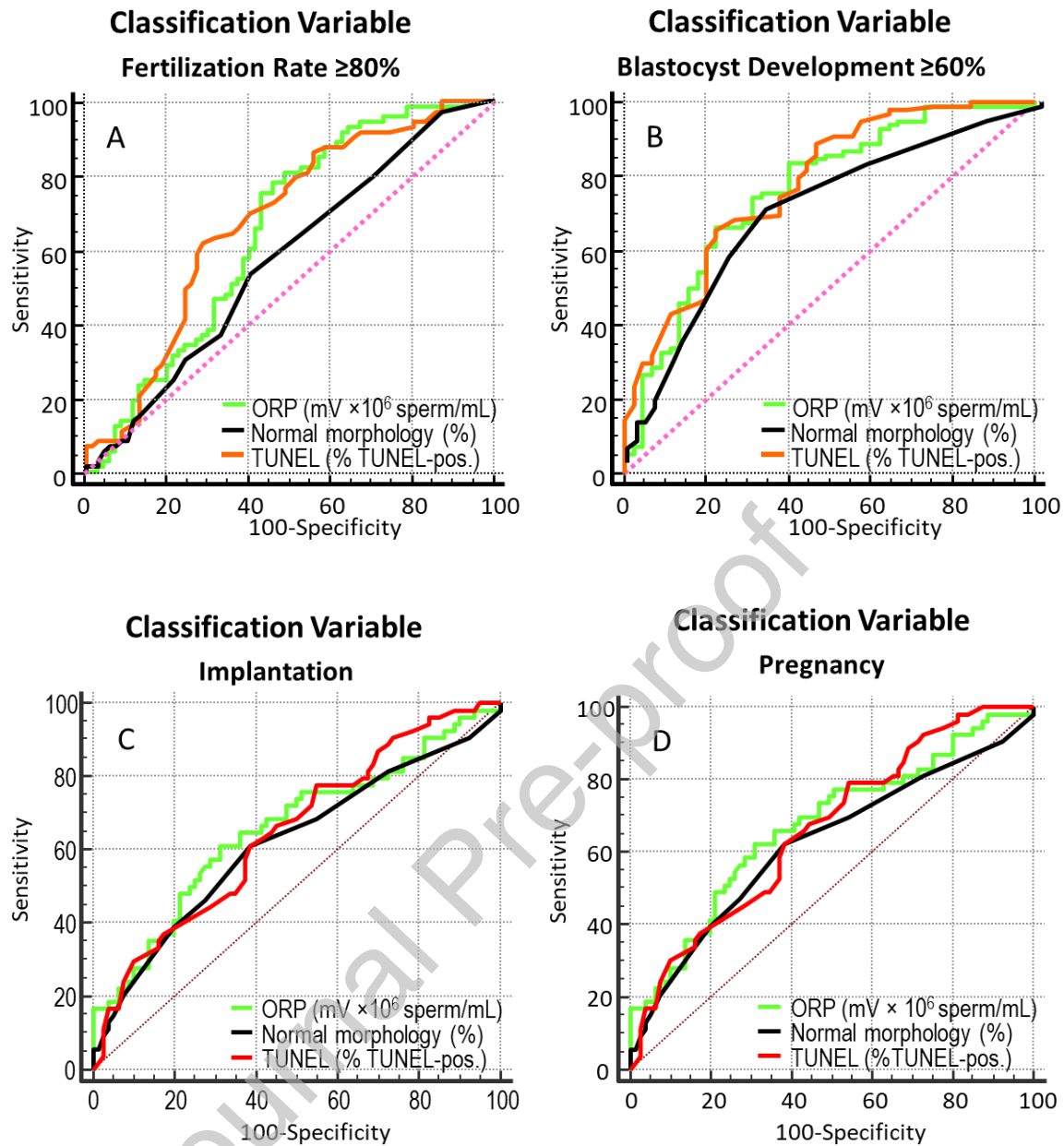


Figure 1: Study design and methodology of the project.



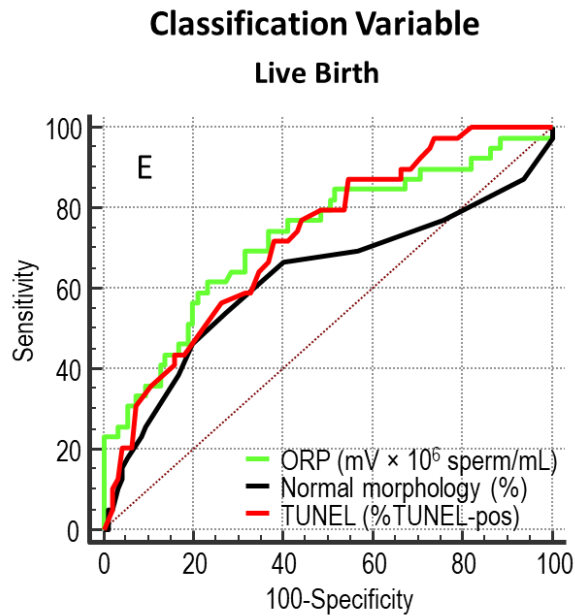


Figure 2: Comparison of ROC curves of seminal ORP and the percentages of TUNEL-positive sperm and sperm normal morphology for fertilization rate ($\geq 80\%$; A), blastocyst development ($\geq 60\%$; B), implantation (C), pregnancy (D) and live birth (E). The data for the fertilization and blastocyst development rates were categorized according to benchmark classification for ICSI (ESHRE Special Interest Group of Embryology; Alpha Scientists in Reproductive Medicine, 2017).

Table 1: Summary statistics of the main parameters analysed in this study.

	N	Rate (%)	Minimum	Maximum	Median	Mean±SD
Female age (yrs.)	144		27.0	46.0	35.0	34.8±3.9
Male age (yrs.)	144		28.0	51.0	37.0	36.5±4.3
Sperm concentration (10 ⁶ /mL)	144		2.0	198.0	50.0	53.5±35.9
Motility (%)	144		5.0	88.0	53.0	51.9±19.0
Normal sperm morphology (%)	144		0.0	23.0	4.0	5.3±4.1
ORP (mV/10 ⁶ sperm/mL)	144		0.02	25.3	0.5	0.9±2.2
TUNEL-pos. sperm (%)	144		4.0	62.0	22.5	24.3±13.4
Fertilisation rate (%; per oocyte injected)	143	96.5	0.0	100.0	80.0	73.5±22.2
Blastocyst development rate (%; per oocyte injected)	143	68.5	0.0	100.0	70.0	65.5±26.8
Implantation (rate per transfer)	134	40.3	0.0	100.0	0.0	30.2±40.2
Pregnancy (rate per transfer)	53	39.6	0.0	1.0	0.0	0.4±0.5
Live birth (rate per transfer)	39	29.1	0.0	1.0	0.0	0.3±0.4

Table 2:

A: Comparison of mean ORP and SDF values in cases with no fertilisation, blastocyst formation, implantation, pregnancy and no live birth, respectively, with cases with fertilisation, blastocyst formation, implantation, pregnancy and live birth. The significance was calculated using the Mann-Whitney test.

	ORP (mV/10 ⁶ sperm/mL)	P-value	SDF (%)	P-value
Fert. rate (0%) (n=5)	0.83±0.82	0.6924	34.20±14.38	0.0914
Fert. rate (>0%) (n=138)	0.98±2.22		23.91±13.34	
Blast. rate (0%) (n=9)	1.66±1.75	0.2689	36.22±12.29	0.0068
Blast. rate (>0%) (n=134)	0.93±2.21		23.47±13.19	
Implantation (no) (n=80)	0.86±0.66	0.0020	26.40±13.86	0.0035
Implantation (yes) (n=54)	1.03±3.40		19.56±11.45	
Pregnant (no) (n=81)	0.87±0.67	0.0007	26.70±14.04	0.0013
Pregnant (yes) (n=53)	1.01±3.43		18.97±10.69	
Live birth (no) (n=95)	0.86±0.65	<0.0001	26.53±13.63	0.0001
Live birth (yes) (39)	1.09±4.00		16.63±9.53	

B: Comparison of mean ORP and SDF values in cases with no fertilisation, blastocyst formation, implantation, pregnancy and no live birth, respectively, with cases where the fertilisation and blastocyst formation rates were higher or equal to the benchmark rates according to the Vienna Consensus of the ESHRE (ESHRE, 2017) (fertilisation ≥80%, blastocyst formation ≥60%).

The significance was calculated using the Mann-Whitney test.

	ORP (mV/10 ⁶ sperm/mL)	P-value	SDF (%)	P-value
Fert. rate (0%) (n=5)	0.83±0.82	0.9593	34.20±14.38	0.0188
Fert. rate (≥80%) (n=73)	0.59±0.43		19.81±10.80	
Blast. rate (0%) (n=9)	1.66±1.75	0.1385	36.22±12.29	0.0007
Blast. rate (≥60%) (n=98)	0.83±2.54		19.87±10.57	

Table 3: Correlation between basic semen parameters (sperm concentration, motility and normal morphology) with the percentage of TUNEL-positive sperm and seminal ORP.

Spearman rank correlation coefficient: r; P-value: P; sample size: n

		Concentration (10 ⁶ /mL)	Normal morphology (%)	Motility (%)	ORP (mV/10 ⁶ /sperm/mL)	TUNEL- pos. (%)
Normal morphology (%)	r	0.466				
	P	<0.0001				
	n	144				
Motility (%)	r	0.522	0.496			
	P	<0.0001	<0.0001			
	n	144	144			
ORP (mV/10 ⁶ sperm/mL)	r	-0.678	-0.472	-0.424		
	P	<0.0001	<0.0001	<0.0001		
	n	144	144	144		
TUNEL-pos. (%)	r	-0.467	-0.415	-0.428	0.665	
	P	<0.0001	<0.0001	<0.0001	<0.0001	
	n	144	144	144	144	
Male age (yrs.)	r	-0.215	-0.252	-0.271	0.268	0.328
	P	0.0097	0.0023	0.0010	0.0012	0.0001
	n	144	144	144	144	144

Spearman rank correlation coefficient

Table 4: Correlation between advanced sperm function test (ORP and SDF) and reproductive outcomes (Fertilisation and blastocyst development rates, implantation, pregnancy and live birth) in patients undergoing ICSI treatment.
Spearman rank correlation coefficient: r; P-value: P; sample size: n

		ORP (mV/10 ⁶ sperm/mL)	TUNEL- pos. (%)	Normal morphology (%)	Fertilisation rate (%)	Blastocyst development rate (%)	Implantation rate (%)	Pregnancy	Live birth
TUNEL-pos. (%)	r P n	0.665 <0.0001 144							
Normal morphology (%)	r P n	-0.472 <0.0001 144	-0.415 <0.0001 144						
Fertilisation rate (%)	r P n	-0.267 0.0012 143	-0.341 <0.0001 143	0.156 0.0630 143					
Blastocyst development rate (%)	r P n	-0.389 <0.0001 143	-0.465 <0.0001 143	0.273 0.0010 143	0.134 0.1097 143				
Implantation rate (%)	r P n	-0.281 0.0010 134	-0.313 0.0002 134	0.218 0.0115 134	0.091 0.2934 134	0.237 0.0058 134			
Pregnancy	r P n	-0.293 0.0006 134	-0.278 0.0011 134	0.212 0.0138 134	0.104 0.2315 134	0.198 0.0219 134	0.958 <0.0001 134		
Live birth	r P n	-0.359 <0.0001 134	-0.351 <0.0001 134	0.192 0.0261 134	0.104 0.2312 134	0.230 0.0074 134	0.749 <0.0001 134	0.758 <0.0001 134	
Male age (yrs.)	r P n	0.268 0.0012 144	0.328 0.0001 144	-0.252 0.0023 144	-0.086 0.3060 143	-0.272 0.0010 143	-0.297 0.0005 134	-0.250 0.0035 134	-0.16 0.02 134

Spearman rank correlation coefficient

Table 5:

A: ROC curve analysis results for ORP, normal sperm morphology and SDF using the benchmark fertilization rate ($\geq 80\%$) as variable for categorical classification. Direct comparison of the areas under the curve (AUC) show no difference between the AUCs for ORP and TUNEL, and ORP and Morphology. There is only a difference between the AUCs for TUNEL and morphology.

Variable	AUC	Cut-off	Sens. (%)	Spec. (%)	+PV (%)	-PV (%)	P-value
ORP	0.652	≤ 0.709	75.3	57.1	64.7	69.0	0.0012
Morphology	0.572	> 4	53.4	60.0	58.2	55.3	0.1320
TUNEL	0.678	≤ 19	61.6	71.4	69.2	64.1	0.0001
Comp. of AUC	ORP – TUNEL: ORP – Morphology: TUNEL – Morphology:						0.5151 0.0950 0.0400

B: ROC curve analysis results for ORP, normal sperm morphology and SDF using the benchmark blastocyst development rate ($\geq 60\%$) as variable for categorical classification. Direct comparison of the areas under the curve (AUC) show no difference between the AUCs for all parameters tested.

Variable	AUC	Cut-off	Sens. (%)	Spec. (%)	+PV (%)	-PV (%)	P-value
ORP	0.762	≤ 0.53	66.3	77.8	86.7	51.5	<0.0001
Morphology	0.704	> 3	77.6	66.7	83.5	57.7	<0.0001
TUNEL	0.780	≤ 23	65.1	77.8	86.5	50.7	<0.0001
Comp. of AUC	ORP – TUNEL: ORP – Morphology: TUNEL – Morphology:						0.6583 0.2111 0.1365

C: ROC curve analysis results for ORP, normal sperm morphology and SDF using implantation as variable for categorical classification. Direct comparison of the areas under the curve (AUC) show no difference between the AUCs for all parameters tested.

Variable	AUC	Cut-off	Sens. (%)	Spec. (%)	+PV (%)	-PV (%)	P-value
ORP	0.658	≤ 0.465	61.1	68.7	56.9	72.4	0.0015
Morphology	0.618	> 4	61.1	61.2	51.6	70.0	0.0202
TUNEL	0.649	≤ 26	77.8	45.0	48.8	75.0	0.0020
Comp. of AUC	ORP – TUNEL: ORP – Morphology: TUNEL – Morphology:						0.8379 0.4751 0.6001

D: ROC curve analysis results for ORP, normal sperm morphology and SDF using pregnancy as variable for categorical classification. Direct comparison of the areas under the curve (AUC) show no difference between the AUCs for all parameters tested.

Variable	AUC	Cut-off	Sens. (%)	Spec. (%)	+PV (%)	-PV (%)	P-value
ORP	0.673	≤ 0.465	62.3	69.1	56.9	73.7	0.0004
Morphology	0.625	> 4	62.3	61.7	51.6	71.4	0.0148
TUNEL	0.664	≤ 26	79.3	47.7	48.8	77.1	0.0005
Comp. of AUC	ORP – TUNEL: ORP – Morphology: TUNEL – Morphology:						0.8444 0.3861 0.5009

E: ROC curve analysis results for ORP, normal sperm morphology and SDF using live birth as variable for categorical classification. Direct comparison of the areas under the curve (AUC) show no difference between the AUCs for all parameters tested.

Variable	AUC	Cut-off	Sens. (%)	Spec. (%)	+PV (%)	-PV (%)	P-value
ORP	0.728	≤ 0.393	61.5	76.8	52.2	83.00	<0.0001
Morphology	0.621	> 4	66.7	60.0	40.6	81.4	0.0420
TUNEL	0.723	≤ 19	71.8	62.1	43.8	84.3	<0.0001
Comp. of AUC	ORP – TUNEL: ORP – Morphology: TUNEL – Morphology:						0.9100 0.1183 0.1504

Table 6: Odds ratios for various reproductive outcome points using a cut-off value for ORP of 0.51 mV/10⁶ sperm/mL.A: Fertilisation rate ($\geq 80\%$)

	ORP		
	≤ 0.51 mV/10 ⁶ /mL	> 0.51 mV/10 ⁶ /mL	
Fert. rate			
$< 80\%$	28	42	70 (49.0%)
$\geq 80\%$	43	30	73 (51.0%)
	71	72	143
Odds ratio	0.4651		
95% CI	0.2385 to 0.9070		
P-value	0.0247		

B: Blastocyst development rate ($\geq 60\%$)

	ORP		
	≤ 0.51 mV/10 ⁶ /mL	> 0.51 mV/10 ⁶ /mL	
Blast. development			
$< 60\%$	10	35	45 (31.5%)
$\geq 60\%$	61	37	98 (68.5%)
	71	72	143
Odds ratio	0.1733		
95% CI	0.0769 to 0.3907		
P-value	< 0.0001		

C: Implantation

	ORP		
	≤ 0.51 mV/10 ⁶ /mL	> 0.51 mV/10 ⁶ /mL	
Implantation			
No	33	47	80 (59.7%)
Yes	35	19	54 (40.3%)
	68	66	134
Odds ratio	0.3812		
95% CI	0.1866 to 0.7785		
P-value	0.0081		

D: Pregnancy

	ORP		
	≤ 0.51 mV/ 10^6 /mL	> 0.51 mV/ 10^6 /mL	
Pregnancy			
No	33	48	81 (60.4%)
Yes	35	18	53 (39.6%)
	68	66	134
Odds ratio	0.3536		
95% CI	0.1720 to 0.7270		
P-value	0.0047		

D: Live birth

	ORP		
	≤ 0.51 mV/ 10^6 /mL	> 0.51 mV/ 10^6 /mL	
Live birth			
No	39	56	95 (70.9%)
Yes	29	10	39 (29.1%)
	68	66	134
Odds ratio	0.2401		
95% CI	0.1050 to 0.5490		
P-value	0.0007		

Table 7: Odds ratios for various embryological endpoints using the published ORP cut-off value of 0.51 mV/ 10^6 sperm/mL and male age as confounding factor with men <37 years regarded as young and men ≥ 37 years regarded as old.

A: Fertilisation rate ($\geq 80\%$)

Male age	ORP	≤ 0.51 mV/ 10^6 /mL		> 0.51 mV/ 10^6 /mL		Odds Ratio
	Fertilisation $\geq 80\%$	No	Yes	No	Yes	
<37 yrs.		14	26	14	14	0.5385
≥ 37 yrs.		14	17	28	16	0.4706

Total # cases		28	43	42	30	143
Odds ratio	0.5016					
95% CI	0.2545 to 0.9889					
P-value	0.04667					

B: Blastocyst development rate ($\geq 60\%$)

Male age	ORP	≤ 0.51 mV/ 10^6 /mL		> 0.51 mV/ 10^6 /mL		Odds Ratio
	Blast. $\geq 60\%$ Dev.	No	Yes	No	Yes	
<37 yrs.		3	37	7	21	0.2432
≥ 37 yrs.		7	24	28	16	0.1667
Total # cases		10	61	35	37	143
Odds ratio	0.1895					
95% CI	0.0813 to 0.4416					
P-value	0.00006					

C: Implantation

Male age	ORP	≤ 0.51 mV/ 10^6 /mL		> 0.51 mV/ 10^6 /mL		Odds Ratio
	Implantation	No	Yes	No	Yes	
<37 yrs.		19	21	19	9	0.4286
≥ 37 yrs.		14	14	28	10	0.3571
Total # cases		33	35	47	19	134
Odds ratio	0.3926					
95% CI	0.1908 to 0.8081					
P-value	0.0108					

D: Pregnancy

Male age	ORP	≤ 0.51 mV/ 10^6 /mL		> 0.51 mV/ 10^6 /mL		Odds Ratio
	Pregnancy	No	Yes	No	Yes	
<37 yrs.		19	21	19	9	0.4286
≥ 37 yrs.		14	14	29	9	0.3103
Total # cases		33	35	48	18	134
Odds ratio	0.3681					
95% CI	0.1778 to 0.7618					
P-value	0.0067					

E: Live birth

Male age	ORP	≤ 0.51 mV/ 10^6 /mL		> 0.51 mV/ 10^6 /mL		Odds Ratio
	Live Birth	No	Yes	No	Yes	
<37 yrs.		23	17	23	5	0.2941
≥ 37 yrs.		16	12	33	5	0.2020
Total # cases		39	29	56	10	134
Odds ratio	0.2471					
95% CI	0.1074 to 0.5684					
P-value	0.0007					

Vitae

Ralf Henkel studied Biology/Chemistry in Marburg, Germany, and obtained his PhD in 1990. In 2004, he became Professor in the Department of Urology at the University of Jena, and at UWC, Bellville, South Africa, from 2005 until 2020. Since 2020, he is at LogixX Pharma and Imperial College London, UK.

