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# Measuring free radicals with relaxometry: Pioneering steps for measurements in human semen

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ABSTRACT

A possible biological mechanism for unexplained male infertility is due to the effect of oxidative stress (OS), defined by the imbalance of reactive oxygen species (ROS) production, and the capacity of the antioxidant defence system to counteract it. In physiological concentrations, ROS and especially free radicals play an essential role in sperm maturation and fertilization, while an overabundance could lead to OS-induced damage to spermatozoa. To date, there are no direct detection techniques available that can measure the total amount of free radicals real time and identify where and when free radicals are generated. This study applies a quantum sensing technique using fluorescent nanodiamonds (FNDs), called $T_1$ relaxometry, which is uniquely sensitive and specific for free radicals allowing measurements of the current radical load for nanoscale detection in living cells and body fluids. This proof-of-principle study investigates if we can use this technique to detect the free radical generation in human whole and separated, using density gradient centrifugation, semen. This method could be potentially used as new diagnostic measure for unexplained infertility or to track the effect of therapeutic interventions such as lifestyle changes. We adapted the existing relaxometry technique to measure free radicals in semen. The measured relaxation time ($T_1$ time) was correlated to sperm concentration and progressive motility. Additionally, we explored the influence of the oxidative trigger hydrogen peroxide and the antioxidant glutathione on the free radical concentration measured. No significant correlations were found, which indicates that measurements in more proximity of the sperm cell are required to use relaxometry as a potential diagnostic tool for unexplained male infertility.

## 1. Introduction

Unexplained male infertility is defined as the inability of a male to conceive with a fertile female partner and no etiological factor can be found (approximately 30–40 % of male infertility cases) [1–3]. Evidence suggests that a major contributor to the aetiology behind 30–80 % of infertile men is oxidative stress (OS)-mediated damage to spermatozoa [4]. OS is defined by the imbalance of reactive oxygen species (ROS) production and the capacity of the antioxidant defence system to counteract it [5]. In physiological concentrations, ROS and especially free radicals (the most reactive ROS) play an essential role for spermatozoa to acquire fertilizing capacity and are involved in the initiation and development of sperm maturation processes, including sperm hyperactivation, capacitation, acrosome reaction (AR), and spermatozoa-oocyte fusion [6]. However, an overabundance of free radicals could lead to decreased motility, decreased capacitation, and impaired membrane fluidity and permeability [7,8]. This is the result of free radical-induced damage to spermatozoa membrane lipids through lipid peroxidation or oxidative DNA damage through sperm DNA fragmentation (SDF) [7,8]. Therefore, measuring free radicals could be an additional alternative to assess sperm quality and function.

Due to the destructive nature of free radicals and their potential to damage biomolecules, free radical measurements could act as a measure for oxidative stress [9]. Because free radicals are short-lived and excessively reactive, it is challenging to detect them with existing methodologies. Current techniques used to measure free radicals in cells can be divided into direct and indirect methods. Direct methods usually rely on nonspecific fluorescent or chemiluminescent compounds to

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produce optically detectable signals in reaction with ROS. However, most of these compounds react with all kinds of reactive species and are not specific for free radicals [10]. In addition, these compounds can only be used in short-term experiments as they suffer from photo-bleaching or are consumed in the reaction with ROS, giving an indication of the accumulated ROS production often over hours rather than the current state [11]. On the other hand, indirect methods evaluate OS by measuring the response of the spermatozoa towards certain ROS. A commonly applied method is to detect the gene expression of enzymes involved in the OS mechanism. However, in this case, one needs to know which enzyme is involved in ROS-induced mechanisms, which is often unknown. Next to this, these techniques represent the history of the sample, rather than the current situation, as it takes time for cells to change their gene expression [9]. OS can also be evaluated using indirect methods quantifying the antioxidant concentration representing the defence capacity of the spermatozoa, or the concentration of certain OS-damage molecules, such as malondialdehyde, which is a product of lipid peroxidation [12,13]. Furthermore, the majority of these methods only measure a specific group of antioxidants or damage products but do not provide an overview of the free radicals involved.

Recently, a quantum sensing technique, called diamond magnetometry, has been utilised to detect the current radical load in living cells. This technique is based on lattice defects in diamonds, called nitrogen-vacancy (NV) centers. These defects change their fluorescent properties based on their magnetic environment [14,15]. This technique has been already successfully used to detect magnetic nanoparticles or nanostructures, spin labels, or proteins with a metallic core [16–20].

For free radical measurements of cells, a specific mode of diamond magnetometry called $T_1$ relaxometry is used due to its relative simplicity and high sensitivity to spin noise. The free electron spin of free radicals is a source of magnetic noise, which can be sensed by the lattice defects within fluorescent nanodiamonds (FNDs) and measured using relaxometry [21]. Because there is no photobleaching of the FNDs, individual cells can be followed for long-term periods, only limited by biological constrains of the cell rather than the particles themselves [22]. Our group has demonstrated that relaxometry can be used to measure free radical generation in yeast cells, human dendritic cells, cancer cells, and most recently boar sperm [9,11,23,24].

The measurement of free radical generation in human ejaculate might reveal a linking factor for idiopathic male infertility. To date, no study has been published on the real-time detection of free radical generation in human semen. The current study aims to test if such measurements are possible by adapting the existing relaxometry techniques to measure free radicals in human whole and separated semen, using a colloidal silica density gradient. This was done to study the difference in free radical concentration and the influence of separation on the T1 relaxometry. On the one hand, whole semen forces contact between spermatozoa and cell debris, defective spermatozoa, and leukocytes, which can induce ROS generation [4]. On the other hand, the separation process involves centrifugation steps that can also increase ROS production [25]. While separated semen provides good-quality spermatozoa mimicking the situation during assisted reproductive technology (ART), whole semen is a better comparison to the real-life situation within the human body. Both methods were tested, to see the difference in free radical production. In addition, CellROX Green assays were performed to compare the new T1 relaxometry with respect to the currently available fluorescent dye-based techniques.

Furthermore, we hypothesize that there is a negative correlation between free radical concentration in whole and separated semen and sperm concentration and progressive motility. Additionally, we hypothesize that an OS state stimulator results in higher concentration of free radicals in both whole and separated semen.

## 2. Experimental method

### 2.1. Study population

Between May 2022 and August 2022, 17 males of couples seeking fertility treatment at the Centre of Reproductive Medicine (CRM) of the University Medical Centre Groningen (UMCG) were included in this study. Rest material after semen analysis (SA) was anonymously collected after informed consent. Eligible participants were males between 18 and 55 years old, with a planned SA in the context of standard care. We excluded males from the study who were azoospermic, had $>2 \times 10^6$/mL round cells, receive(d) chemo- and/or radiotherapy, use(d) testosterone suppletion and/or anabolic steroids, had an abnormal SA due to genetic causes, or were currently using antibiotics.

The Institutional Review Board (IRB) of the UMCG (in Dutch: “Medische Ethische Toetsingscommissie”, METc IRB approval no. 2022/130) approved this Medical Scientific Research without People Act (nWMO) pilot study.

### 2.2. Semen analysis and preparation

Standard SA was performed by the laboratory staff of the CRM of the UMCG, according to the World Health Organization (WHO) guidelines [26]. The sperm concentration and progressive motility data were used for analysis in the current study. After SA, approximately 1–1.5 h after production, the rest material was collected for this study. During the sperm transportation and separation process, the semen was kept at room temperature to minimize sperm capacitation [27].

Density gradient centrifugation (DGC) was performed on 1 mL of the total semen volume to select for the motile spermatozoa [28]. The rest was kept as whole semen. The DGC method was performed using a discontinuous 40 %/80 % PureSperm® gradient (NidaconTM International, Mölndal, Sweden) in duplo [24,28]. Then the sample was centrifugated at 300*g* for 20 min to separate the motile spermatozoa. The supernatant was removed and the pellet was dispersed with a Ringer solution, followed by a second centrifugation step at 500*g* for 10 min. Hereafter, the supernatant was again discarded until 0.5 mL of the sample was left in the Falcon tube, followed by resuspension in the existing liquid to ensure the detachment of the spermatozoa sediment. Relaxometry was performed in either whole or separated semen. CellROX green assays were also performed in either whole or separated semen (See supplementary method).

### 2.3. Study parameters and data collection

The main study parameter is the relaxation time (or $T_1$ time) of human semen samples measured during relaxometry. The relaxation time reflects the current free radical concentration in the surrounding of the FND measured in the presence of either whole semen or separated semen.

### 2.4. Fluorescent nanodiamonds

In this study, oxygen-terminated FNDs with a hydrodynamic diameter of 70 nm (Adámas Nano, Raleigh, NC, USA) were used. The manufacturer created these FNDs by grinding high-pressure high-temperature (HPHT) diamonds followed by particle irradiation with an electron beam at 3 MeV and a fluence of $5 \times 10^{19}$ e/cm$^2$, followed by high-temperature annealing (>600 °C). This resulted in the particles hosting approximately 500 NV centers each [29]. The shape and surface chemistry of the FNDs have already been characterized thoroughly in previous studies [21,30].

### 2.5. FND-coated dishes

For free radical measurements in whole and separated semen, clean

4-chamber glass-bottom Petri dishes were coated with oxygen-terminated FNDs. The FNDs were suspended in distilled water (1 μL/mL) and 200 μL of suspension was added to every chamber of the dish. The dishes were incubated at 37 °C for approximately 2 weeks to let them dry completely before use.

### 2.6. $T_1$ relaxometry measurements

$T_1$ relaxometry enables the visualization of the free radical concentration in the surroundings of the FND measured in whole or separated semen. For these measurements, oxygen-terminated FND-coated dishes were used.

A homemade magnetometry setup, consisting of a confocal microscope equipped with a laser that is pulsed at varying intervals and an avalanche photodiode detector (Excelitas, SPCM-AQRH), was used for $T_1$ relaxometry [9]. A suitable FND (photon count between 1 and 3 million without any bleaching) attached to the coated chamber was selected using confocal scanning and widefield microscopy. The free electron spin of free radicals is a source of magnetic noise, which can be sensed by the NV centers within the FNDs. 5 μs long laser pulses (532 nm) are used to pump NV centers into their ms = 0 state of the ground state, which is bright and polarized. After dark times (τ) ranging between 200 ns to 10 ms, the photoluminescence signals are determined indicating how many NV centers lasted in this polarized state or had returned to the (dark) equilibrium between ms = 0 and ms = +1 or −1. The depolarization of the NV centers occurs faster when spin noise from surrounding free radicals is present [9,22]. Therefore, when more free radicals are produced in the semen surrounding the FND, a larger drop in relaxation time is seen. The pulse sequence was set to be repeated 10,000 times to achieve a sufficient signal-to-noise ratio for each $T_1$ measurement. At the location of sample measurement, the laser power was set to 50 μW (measured in continuous illumination) to prevent affecting the cell viability, but strong enough to polarize the NV centers within the FND.

Firstly, a $T_1$ measurement was performed on the dry diamond particle. Secondly, 200 μL of either whole or separated semen was added and $T_1$ measurements were performed again on the same FND. The difference in $T_1$ time measured before and after adding the sample gives a quantitative indication of the free radical concentration in the semen sample surrounding the FND. One measurement internally contained 12 repetitions, and the measurements were performed in triplicate in the whole and separated semen.

### 2.7. Oxidative trigger and antioxidants

A selection of four semen samples with a sperm concentration >15 × 10 [6]/ml and progressive motility >32 % were treated with hydrogen peroxide ($H_2O_2$) to stimulate maximal OS, followed by treatment with the antioxidant glutathione (GSH). GSH is added to counteract the free radicals stimulated by the $H_2O_2$ to simulate the biological antioxidant defence system against OS [31,32].

Cells were treated with 0.3 % $H_2O_2$ (1 μL $H_2O_2$/100 μL semen) and $T_1$ measurement was performed. Lastly, 50 μL GSH (10 μM) was added and $T_1$ was measured. The same FND was measured for the different timepoints. The treatment of both $H_2O_2$ and GSH was performed in both whole and separated semen. Using this method, the free radical concentration was visualized in the surrounding of the FND following the course of the OS state.

### 2.8. Statistical analysis

The obtained data were analysed using MatLab software version R2018b. Statistical analysis was performed using IBM SPSS Statistics 27.0.1.0 (SPSS Inc., Chicago, IL, United States). To assess normal distribution for all continuous variables, the Shapiro-Wilk test was used.

The change in $T_1$ time was compared to the baseline $T_1$ time, which is the $T_1$ time measured in a dry FND. The baseline of dry FND measurement was set at 100 % and $T_1$ time measured after addition of the semen, $H_2O_2$, and GSH were determined relatively. A paired *t*-test was performed to compare $T_1$ time in whole and separated semen. The $T_1$ time was analysed against sperm concentration and progressive motility. For this, linear regression with a Pearson correlation was used on the normalized $T_1$ value after correction for the baseline $T_1$ value of the dry FND as mentioned above. Analysis was performed with a one-way ANOVA and Tukey's post hoc test. Significance was defined as: ns $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

## 3. Results

### 3.1. Whole vs. separated semen

A boxplot was used to visualize the $T_1$ times of whole and separated semen. No significant difference was found between the $T_1$ time of whole and separated semen ($p = 0.119$) (Figs. 1, S1). Furthermore, both conditions did not significantly differ from the negative control (dry FNDs vs. whole semen, $p = 0.236$, and dry FNDs vs. separated, $p = 0.342$, respectively). For the CellROX Green assay, a significant difference was observed for both whole and separated semen compared to the negative controls (cells without staining (separated $p \leq 0.001$ and whole $p \leq 0.001$)) as well as between whole and separated semen compared to each other ($p \leq 0.001$) (Fig. S2).

### 3.2. T1 vs. sperm concentration and motility

Linear regression lines were plotted of the $T_1$ time of whole and separated semen against sperm concentration and progressive motility (Fig. 2). This was done to check if in this study population, the $T_1$ time was not distinctive for all different concentrations and progressive motility. No significant correlation was found between the $T_1$ time of whole and separated semen and the sperm concentration ($r = 0.156$, $p = 0.594$ and $r = 0.130$, $p = 0.658$, respectively) (Fig. 2A, B). Additionally, no significant correlation was found between the $T_1$ time of whole and separated semen and the sperm progressive motility (r = 0.379, p = 0.182 and r = 0.451, $p = 0.105$, respectively) (Fig. 2C, D).

### 3.3. T1 after oxidative trigger and antioxidant protection

No significant difference was seen in $T_1$ times after the addition of $H_2O_2$ and the GSH in whole semen (Figs. 3, S3) ($p = 0.325$). There is a trend of lower $T_1$ time after the addition of $H_2O_2$ followed by the increase of $T_1$ time after the addition of GSH in separated semen. However, the trends seen were not statistically significant (Figs. 4, S3) ($p = 0.102$).

## 4. Discussion

This study showed pioneering steps of the use of $T_1$ relaxometry measuring free radical generation in whole and separated human semen [24].

We observed no significant drop in $T_1$ time measured in the dry FND after the addition of both whole or separated semen, therefore no significant difference in free radical concentration was measured in these samples. For the CellROX Green assay, we did see a significant difference between whole and separated semen, which could suggest that there is a difference in total ROS production within these two conditions (Fig. S2). Additionally, this study explored whether the sperm cells concentration and progressive motility had an influence on the $T_1$ time measured in whole and separated semen. No significant correlations were seen between either whole or separated semen and sperm cells concentration or motility (Fig. 2).

Furthermore, the measurements of the oxidative trigger hydrogen peroxide ($H_2O_2$) and the antioxidant glutathione (GSH) treatments on the free radical concentration showed a trend to lower $T_1$ time after the

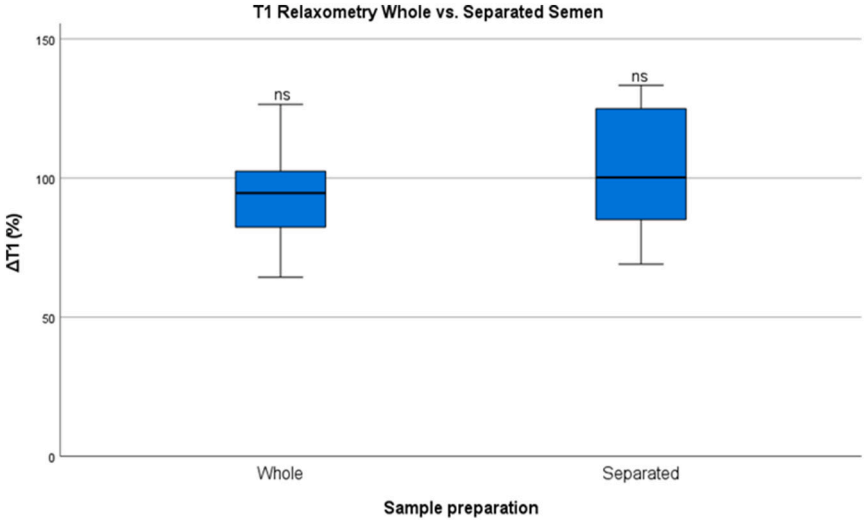


**Fig. 1.** $T_1$ time of whole and separated semen after normalization for the baseline $T_1$ time of the dry FND. Bars depict the spread. No significant correlation was found between the $T_1$ time measured in dry FND and $T_1$ time after the addition of whole or separated semen ($p = 0{,}236$ and $p = 0{,}342$ respectively). Association of $T_1$ time between whole and separated semen show no significant correlation ($p = 0{,}119$).

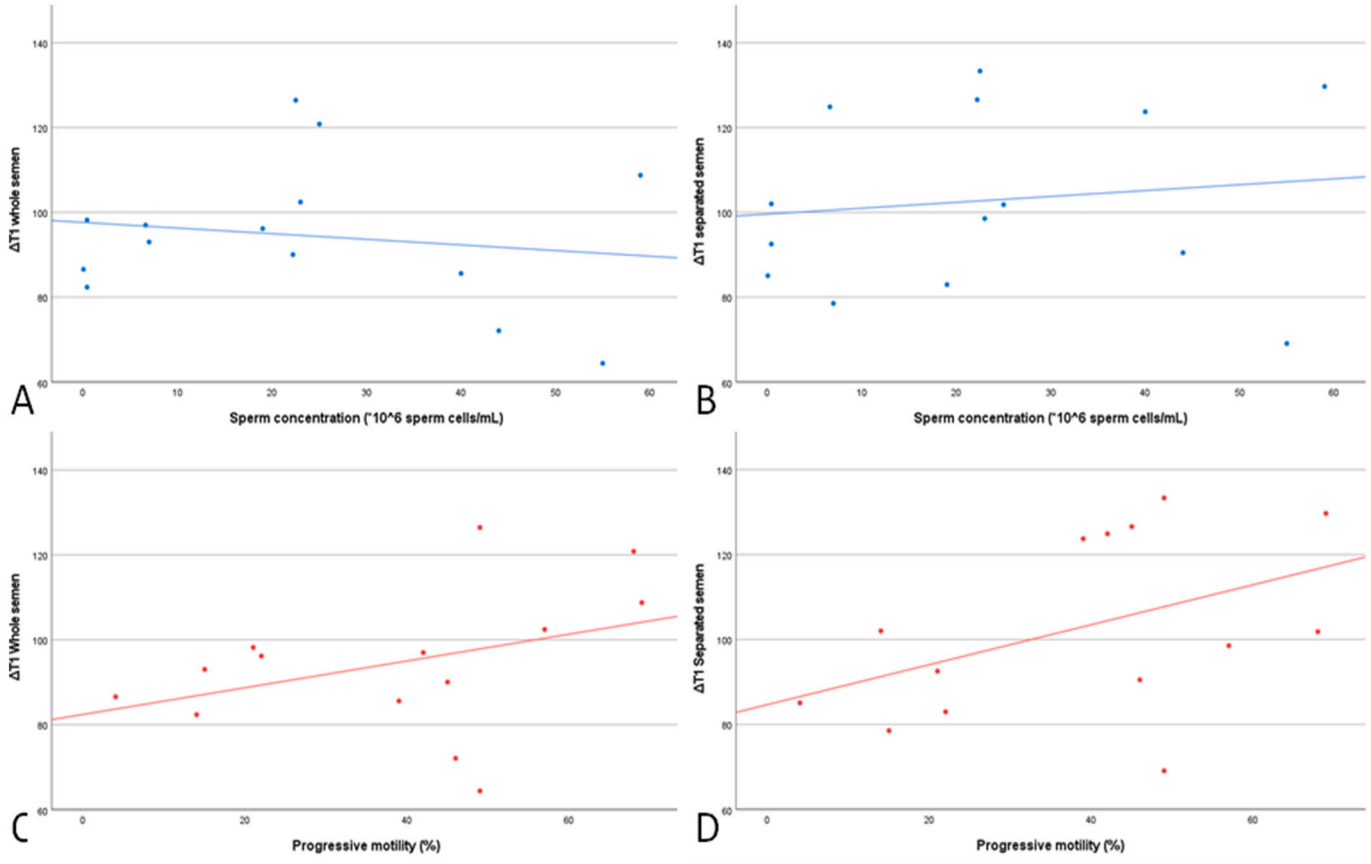


**Fig. 2.** Association between normalized $T_1$ time in whole and separated semen and sperm parameters: (A) No significant correlation between $T_1$ time of whole semen and sperm concentration ($r = 0.156$, $p = 0.594$); (B) no significant correlation between $T_1$ time of separated semen and sperm concentration ($r = 0.130$, $p = 0.658$); (C) no significant correlation between $T_1$ time of whole semen and sperm progressive motility ($r = 0.379$, $p = 0.182$); (D) no significant correlation between $T_1$ time of separated semen and sperm progressive motility ($r = 0.451$, $p = 0{,}105$). Lines represent the best fitting lines from linear regression.

addition of $H_2O_2$, in separated semen (Fig. 4). However, this was not seen in whole semen (Fig. 3).

In general, lower $T_1$ values correspond to higher concentrations of free radicals measured and reversed [22–24]. If there is a change in the radical concentration, we would expect that the $T_1$ values measured in the dry FNDs state were higher than the $T_1$ time measured when adding the biological sample containing free radicals [9]. However, our results suggest that there was no change in free radical concentration before and after adding the semen sample in both cases of the whole and separated semen. This could be explained by the lack of free radical-

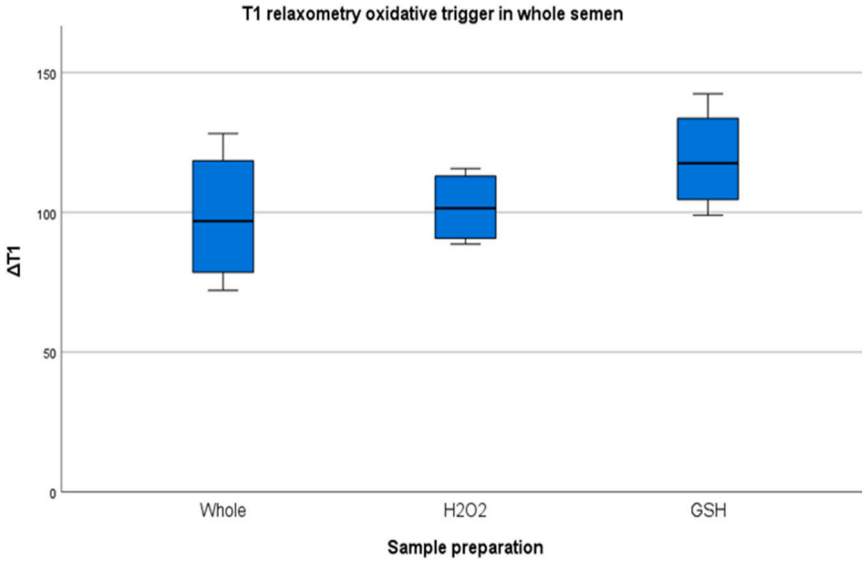


**Fig. 3.** Course of $T_1$ times after normalization for the baseline $T_1$ time of the dry FND. Bars depict the spread. No significant difference was found in the course of the addition of different compounds with whole semen ($p = 0.325$). $H_2O_2$ = hydrogen peroxide, GSH = glutathione.

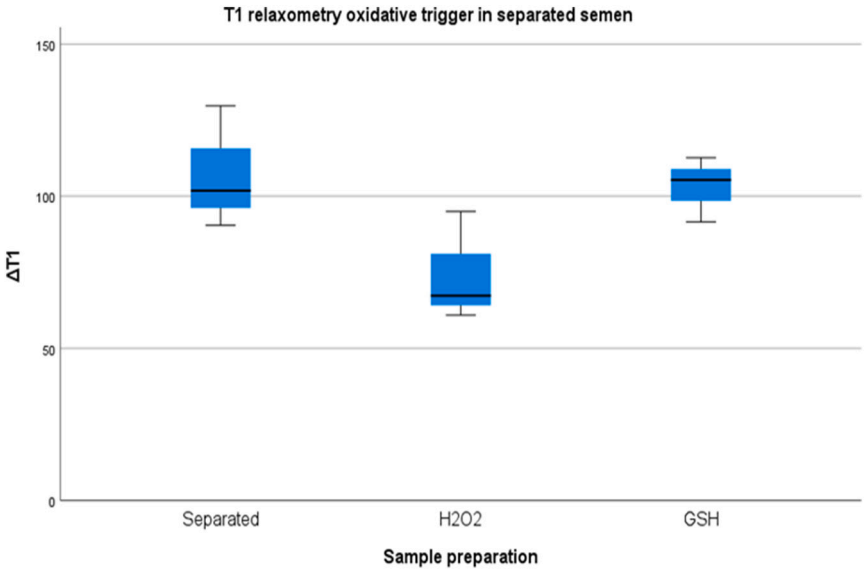


**Fig. 4.** Course of $T_1$ times after normalization for the baseline $T_1$ time of the dry FND. Bars depict the spread. No significant difference was found in the course of the addition of different compounds with separated semen ($p = 0.102$). $H_2O_2$ = hydrogen peroxide, GSH = glutathione.

producing sperm cells in the proximity of the FND probe. As an FND can only sense magnetic noise from radicals that are within a couple of nanometres, the free radicals could have already reacted with other molecules before reaching the proximity of the FND to sense it [23].

Using the CellROX Green fluorescence assay a significant difference between whole and separated semen was observed in contrary to the T1 relaxometry. Therefore, this suggests that the total ROS production in whole semen is significantly higher than the total ROS production in separated semen. This could be explained by the fact that whole semen forces contact between spermatozoa and cell debris, defective spermatozoa, and leukocytes, which induces more ROS generation than the centrifugation steps involved in the separation process. That this was not seen in the T1 relaxometry could be due to the fact that this fluorescence assay measures the total ROS production, while T1 relaxometry focuses on sensing specifically the free radical load. Next to this, the T1 relaxometry measures the free radical load in proximity of the FND probe, while the fluorescence intensity measured in the CellROX Green assay is based on a whole batch of cells producing ROS.

Previously performed research by our group on relaxometry on boar sperm cells successfully measured free radical generation while the FND was attached to the acrosome of the sperm cell, being in utmost proximity of the free radical producing sperm cell [24]. A significant difference in free radical load was found during the capacitation process of the boar sperm cells, which suggests that the relaxometry technique works superior when attaching the FND to the sperm cells, instead of measuring it in the seminal plasma. Due to the heterogeneity of surface characteristics between boar and human sperm cells, it is technically challenging to attach the FNDs to the acrosome of human sperm cells. Therefore, this study aimed to measure free radicals using relaxometry in whole and separated semen, to reduce processing steps to minimize possible OS triggering of sperm cells and reduce complexity for future clinical applications. Furthermore, free radical measurements in whole and separated semen assess the free radical production of a population of sperm cells within the surrounding of the FND, giving a better

understanding of the OS state of the semen, instead on focussing on a single sperm cell.

Another explanation could be that the intrinsic antioxidant defence capacity within the ejaculate could still counteract all the potentially produced free radicals. However, this would suggest that in separated semen, filtering all the debris, leukocytes, and seminal plasma, the antioxidant capacity is certainly deprived. Nevertheless, we did not find a significant difference in $T_1$ time in separated semen.

Moreover, we showed that in our population, the free radical load was not distinctive in either whole or separated semen in relation to sperm concentration and progressive motility. This suggests that even with higher chance of free radicals being in proximity of the FND, due to higher concentration and/or motility of the sperm cells, no signal was achieved. This could be explained by the same arguments concerning either the lack of free radicals producing sperm cells in proximity of the measured FND or that the free radicals could have already reacted with other molecules, for example the intrinsic antioxidants, before the FND is able to sense it.

Regarding literature, poor semen quality (defined as a sperm concentration $<15 \times 10^6$ sperm cells/mL and progressive motility $<32$ %) is suggested to be partly caused by OS-induced damage, where free radicals play an important role, and therefore more free radicals and greater $T_1$ drops are expected in the group with poor semen quality compared to the patients with a normal range of sperm concentration and progressive motility [6,7]. There might be a difference in OS but in the form of molecules that are not paramagnetic and therefore are not detected using relaxometry. Even though no correlations were observed in the small sample size of this study, it does not mean that this is the case in a larger patient population. Literature describes that the main sources of free radicals in seminal plasma are the aerobic metabolism of spermatozoa, activated leukocytes, and immature/functionally abnormal spermatozoa [4]. The free radical load produced by the immature/functionally abnormal spermatozoa, which are expected to be in abundance in semen samples with poor quality, could be atoned for by the free radical load produced by the high concentration of aerobic metabolism of normal spermatozoa in semen samples with good quality. This could potentially explain why there is no correlation in $T_1$ time of whole and separated semen with neither sperm concentration nor progressive motility.

As a positive control we used a state of high OS with the addition of OS-inducing $H_2O_2$ to explore the decrease in $T_1$ time and the antioxidant GSH to counteract the produced free radicals to simulate the defence system and detect an increase in $T_1$ time. There seems to be a trend of a decrease in $T_1$ time after the addition of $H_2O_2$ followed by an increase in $T_1$ time after the addition of GSH in separated semen, but not in whole semen. As the antioxidants are all filtered out in the separated semen using DGC, the effect of the oxidative trigger in stimulating a drop in $T_1$ time and an increase in $T_1$ time by the antioxidant, is seen more clearly. Even though this trend is not significant, it suggests that if the free radicals produced in semen are highly stimulated by an oxidative trigger, it could be potentially measured. In whole semen, the seminal plasma surrounding the sperm cells still has an antioxidant defence capacity, which might explain why no trend in $T_1$ time is seen after the addition of the oxidative trigger. Therefore, the concentration $H_2O_2$ used might not be enough to affect whole semen.

The strength of this study is that we demonstrated relaxometry for the first time in human (whole and separated) semen [24]. We concluded that measurement of semen fluid is not favourable for free radical measurements regarding the nanoscale resolution of $T_1$ relaxometry. To the best of our knowledge, measuring free radical generation in human semen using relaxometry has never been done before and this report describes the pioneering steps in the use of $T_1$ relaxometry measuring free radical levels as a surrogate measure for local OS state in the male genital tract. While we here do not make use of the largest advantage that high spatial resolution provides, there are still a few distinctive arguments in favour of our technology. 1. It is sensitive to different types of molecules (namely free radicals with a free electron) than most organic dyes and since background tends to bleach, it is less affected by background issues. 2. Since the fluorescence is stable it is possible to do before and after measurements and 3. Conventional methods reveal the history of the sample while we see what is present during the time of the measurement. We have added these arguments to the manuscript.

It also should be mentioned that T1 is sensitive to molecules with a free electron and to whatever is present during the time of the measurement. This means that if a substance is short lived there will be less of it present at a given time.

A limiting factor of this study is that our measurements are very local and the FNDs might not be in nanometres proximity of the sperm cells where the free radicals are produced and diffuse to afterwards. To improve the diagnostic value of our study it might be required to attach the FNDs to the sperm surface, as done in previous studies in boar sperm [24]. Another limiting factor in this study is the small sample size collected for the experiments with the oxidative trigger and antioxidant addition as a positive control. Nevertheless, we do see effects on the $T_1$ time after the addition of the different compounds, but the changes are subtle.

While relaxometry is already a well-established method in physics, the technique is entirely new in the biomedical field. Translating this technique in a clinical setting could result in more knowledge about the pathophysiological processes of unexplained male infertility, which in turn could be essential to implementing suitable management and treatments to increase the probability of successfully conceiving. Future research perspectives should focus on optimization of the relaxometry technique to be able to measure at single-cell and subcellular levels in human spermatozoa. Existing literature on boar sperm shows promising results enabling the detection of biological variability between different sperm cells within one sample or the identification of specific sources of free radical production within spermatozoa for example, the acrosome of the sperm cells. Next to this, relaxometry in whole and separated semen should be tested in larger trials using OS-inducing compounds in semen, finding the right concentrations balancing OS stimulation and cell viability preservation. Lastly, the antioxidant composition and concentration in semen can be studied to see to what extent the semen is able to balance its own redox balance and counteract the OS.

In the future, $T_1$ relaxometry could be used to determine OS state in human semen, leading to a better understanding in the aetiology of idiopathic male infertility. This could result in shifting the focus of treatment for male infertility from assisted reproductive technology (ART) to the reduction or prevention of OS in semen, by for example focussing on OS-enhancing lifestyle-related factors with proper counselling and coaching or the development of OS-decreasing therapeutics. Therefore, measuring OS and identifying its exogenous and endogenous sources could be essential to implement suitable management and treatments to improve sperm quality and increase the probability of successfully conceiving. Further research needs to focus on establishing the potential role of OS in the links between lifestyle factors and sperm quality.

## 5. Conclusions

We showed that measurement of semen, rather than at the sperm cell, is not favourable for free radical measurements in relation to the nanoscale resolution of $T_1$ relaxometry. Future studies with larger sample sizes should focus on the identification of specific sources of free radical production at the human sperm cell, and specify the composition and concentration of the local antioxidant defence system. This could reveal potential links between patients' lifestyle and OS. Nevertheless, this study opens new perspectives for further development of relaxometry within its ability to measure OS state in unexplained male infertility.

## CRediT authorship contribution statement

Conceptualization: H.T.L., A.M., A.E.P.C., R.S.; methodology: H.T.L., A.M.; software H.T.L., A.M., R.S.; validation: all authors; formal analysis: H.T.L.; investigation: H.T.L., A.M., J.E.; resources: A.M., J.E., R.S.; data curation: H.T.L., A.M., J.E., A.E.P.C.; writing- original draft preparation: H.T.L.; writing – reviewing and editing: all authors; visualization: H.T.L.; supervision: A.M., A.H., A.E.P.C., R.S.; project administration; H.T.L., A.E.P.C.; funding acquisition: A.M., A.H., A.E.P.C., R.S. All authors have read and agreed to the published version of the manuscript.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Romana Schirhagl reports financial support was provided by NWO.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

The SI contains Fig. S1 which represents a distribution of absolute T1 values measured for dry FNDs, whole and separated semen and Fig. S2 showing the results of the CellROX Green assay with the accessory materials and methods. Fig. S3 gives an example of the raw data of T1 measurements.

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