Correlation between sperm oxidative stress and sperm DNA damage in subfertile men

[Samband mellan oxidativ stress och DNA-skada i spermier hos subfertila män]

LUNDS UNIVERSITET

Master thesis, LÄKM01, 30hp
Course dates: 2012-09-03 -- 2013-01-18

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ABSTRACT

Background: Approximately 15 % of all couples in the Western countries experience infertility. Up to half of these cases are thought to solely or partly depend upon the male factor. In most cases male infertility is unexplained. During the last decade there has been a growing focus on sperm DNA damage as a cause of male infertility. Sperm chromatin structure assay (SCSA) is increasingly used to evaluate male fertility status as it determines sperm DNA damage. This DNA damage can originate from several sources, the major being oxidative stress, i.e excess of reactive oxygen species (ROS). Increased ROS may be a result of different factors, for instance chronic diseases, infections or lifestyle factors such as smoking and high body mass index (BMI). Despite this, sperm ROS is not tested for in routine infertility assessment. A new promising analysis for assessing sperm ROS production is the nitro blue tetrazolium assay (NBT).

Objectives: The primary objective was to study the correlation between sperm oxidative stress assessed with NBT assay and sperm DNA fragmentation index (DFI) assessed with SCSA in subfertile men. Secondly, the objective was to correlate these parameters to BMI as well as to the level of serum testosterone.

Method: The prospective study was conducted on a cohort of forty-seven subfertile men. To assess sperm quality the NBT assay, SCSA and World Health Organization (WHO) sperm analysis were performed. For all men testosterone, lutenizing hormone (LH) and BMI were measured.

Result: There was a tendency to a correlation between NBT outcome and DFI ($n = 39$, $r = 0.281$, $P = 0.084$), however not statistically significant. Men with a BMI > 30 kg/m^2 had an increased risk for having a DFI > 30% (OR = 8.6, $P = 0.070$) compared to the normal weighted men, although not statistically significant.

Conclusion: There was a weak association between sperm oxidative stress and sperm DNA damage. A high BMI was correlated to an increased risk of DNA damage. Although non of these findings were statistically significant they show the same tendencies as previous reports. Sperm oxidative stress seems likely as a cause of male infertility and large scale clinical trials are warranted to further evaluate the NBT assay. The hope for the future is to be able to sort out the men with sperm oxidative stress caused infertility, who potentially would benefit from antioxidant strategies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Av alla par i västvärlden drabbas ungefär 15 % av ofrivillig barnlöshet. I upp till hälften av fallen är orsaken eller en bidragande orsak den manliga faktorn, som i många fall inte kan förklaras med dagens rutinspermaundersökningar. Standardmetoden för spermieanalys, utarbetad av World Health Organization (WHO), har i flertalet studier visat sig vara ett bristfälligt sätt att förutsäga förmågan att få barn. På senare år har teorier framkommit om att oxidativ stress, det vill säga ett överskott av fria syreradikaler, kan bidra till eller orsaka manlig infertilitet.

Fria syreradikaler finns naturligt i låg koncentration i sperma men när de ökar i mängd och överstiger antioxidationsförsvaret uppstår vad man kallar oxidativ stress. Den oxidativa stressen skadar bland annat spermiernas DNA och försvårar eller omöjliggör befruktningen av ägget och därmed graviditet. En ökning av fria radikaler har man bland annat sett vid fetma, större alkoholintag, rökning, efter intag av vissa mediciner, exponering av föroreningar och vid vissa sjukdomar.

Vårt huvudsakliga syfte var att studera sambandet mellan oxidativ stress i spermier, DNA-skada i spermier och fertilitet. Därför undersökte vi 47 stycken män med känd nedsatt fertilitet. Männen undersöktes med avseende hur mycket oxidativ stress deras spermier innehöll, mätt med nitro blue tetrazolium assay (NBT). Männen undersöktes även med avseende på hur skadat deras spermie-DNA var, mätt med sperm chromatin structure assay (SCSA). Könshormonsnivåer i blodet mättes och body mass index (BMI) beräknades. Vi fann ett svagt samband mellan oxidativ stress och DNA-skada i spermier. I tidigare studier har detta samband fastställts och varit betydligt starkare. Vi såg också, dock även detta osäkert, att grav övervikt ökar risken för att ha DNA-skada i spermierna.

Nitro blue tetrazolium assay är en ny, lovande metod för att mäta oxidativ stress i spermier, men fler studier krävs för att metoden ska kunna etablera sig i rutinmässig infertilitetsutredning. Förhoppningen är att man i framtiden ska kunna urskilja när oxidativ stress är en orsak till infertilitet. Då skulle man kunna avgöra vilka män som skulle kunna ha nytta av livsstilsförändringar och/eller behandling med antioxidanter, i syfte att normalisera spermiekvalitén. Vinsterna skulle då bli minskat lidande hos berörda och minskade ekonomiska kostnader.

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1 INTRODUCTION

Approximately 15 % of all couples in Western countries experience infertility, i.e. failure to conceive within a year of unprotected sexual intercourse, and up to half of all cases is believed to solely or partly depend upon the male factor (1, 2). Male infertility can have many possible causes, such as primary testicular dysfunction, genetic disorders and genital infections (3). However, due to limited diagnostic tools a significant number of men are diagnosed with unexplained infertility (2, 4, 5) and often treated symptomatically by assisted reproductive techniques (ART). This approach is invasive, costly and highly challenging for the couple as well as for the community.

The traditional World Health Organization (WHO) semen analysis evaluates three sperm parameters; concentration, motility and morphology (6). Although the WHO analysis is recognized as the golden standard, its role as a predictor of male fertility has been questioned (7-10). Despite this no other analysis that address sperm quality is commonly used in routine (11). However, during the last decade the search for better predictors of fertility has contributed to a growing focus on sperm DNA integrity (Reviewed in (9, 12)). Although still many questions remain to be answered, it is evident that sperm DNA integrity is a valuable marker of male fertility (4, 13, 14). Sperm DNA integrity can be used alone or in combination with the conventional semen parameters, in natural conception as well as in ART to predict male fertility potential (4, 13, 14).

1.1 Male infertility and sperm DNA damage

Of the established causes of male infertility the most common is a primary testicular dysfunction that effects the sperm production and/or testosterone production (3). Other causes are genetic disorders, such as Klinefelters syndrome and microdeletion on the y-chromosome (3). Genital infections, for example chlamydia and gonorrhea, and endocrine disturbances, such as hypothalamus–pituitary–testis axis disturbances and diabetes, may also cause decreased fertility (15). Another acknowledged cause is when antibodies against sperm are formed for example following trauma, obstruction, infection and inflammation (15).

Despite the many well-known causes, most of infertile men are diagnosed with idiopathic infertility (3, 4). Although categorized as unexplained infertile, sperm DNA damage has been recognized as a possible explanation (4, 13, 16). Several studies has indicated that increased sperm DNA damage correspond well with reduced spontaneous conceptions (4, 16) and increases the risk of miscarriage (Reviewed in (17)).

The mechanisms behind DNA damage in the male germ line are not fully unraveled (18), however, four major causes are recognized: (a) DNA damage during spermatogenesis due to a defective checkpoint in the meiotic prophase. This may lead to continuation through meiosis although the DNA double strand breaks have not yet been fully repaired after the meiotic crossing-over. (b) DNA damage because of abnormal maturation of the spermatozoa (18). Two mechanisms have been proposed. Firstly, defective protamination may occur (9). In spermatogenesis histones are replaced by protamines which are essential for condensation and DNA stabilization in spermatozoa (19). Secondly, default ligation of transient DNA breaks may occur (9). Transient DNA breaks are required in the gamete´s evolvement, to relieve torsional stress and facilitate rearrangement of chromatin (9, 20). (c) Sperm DNA damage due to abortive apoptosis, which is when apoptosis is initiated but impeded. Apoptotic biomarkers have been recorded in spermatozoa, mainly in spermatozoa of infertile men (18), but the mechanisms of the failed apoptosis remains unsettled (12). (d) Sperm DNA damage on the basis of oxidative stress. As this appears to be the major mechanism of sperm DNA damage it is the main focus in this study and outlined henceforth (9, 18). However, it should be recognized that these mechanisms are presumably interrelating and sperm DNA damage most likely arises from combinations of the proposed factors (4, 17, 18).

1.2 Reactive oxygen species and oxidative stress

Oxidative stress has been suggested as the major cause of reduced sperm quality (Reviewed in (10)) and corresponds to decreased fertility (5, 21). It is proposed that oxidative stress contributes to sperm DNA damage in 30-80% of infertile men (Reviewed in (5)).

Oxidative stress is an excess of reactive oxygen species (ROS). As the name implies, ROS is a generic term for reactive molecules containing oxygen. The most important ROS in biology are free oxygen radicals. A free radical is a molecule that has gained an extra and consequently an unpaired electron. An example is when O_2 becomes the superoxide anion O_2^- with an extra unpaired electron. Free radicals are extremely reactive as they strive to get rid of their extra electron to other molecules. Examples of such reactions are oxidation of lipids in membranes, amino acids and carbohydrates in nucleic acids (22). In the normal energy metabolism of the cell ROS is generated. When energy is produced an enzymatic reduction of oxygen take place, and ROS are formed as byproducts (23). In homeostasis ROS play an important role, for example in destruction of infectious pathogens and intracellular signaling (23). Seminal ROS are desired at low levels to facilitate the capacitation in fertilization. If no fertilization takes place, ROS are built up over time and leads to sperm apoptosis.

The sperm is thereby easily cleared by the female immune system which prevents anti-sperm immunity (10).

The human body has a natural antioxidant defense to take care of redundant ROS and maintain balance. However, when ROS exceed the inbuilt antioxidant defense the cells are exposed to oxidative stress. In oxidative stress, excessive ROS with their reactive properties can have destructive effects on the cell (23).

1.3 Oxidative stress and spermatozoa

In semen both spermatozoa and seminal leukocytes produce ROS. Leukocytes are probably the dominant source of ROS in semen (5, 24, 25). Seminal plasma has the greatest antioxidant power of all body fluids (10), however, the spermatozoa itself lacks antioxidants (4). It has been observed that ROS produced by sperm have a significantly stronger correlation with decreased sperm DNA integrity than ROS produced by leukocytes (25). Hence, sperm produced ROS is presumed to be more significant in correlation to infertility (5).

Principally, oxidative stress may affect the spermatozoa in two ways. Firstly, oxidative stress impairs sperm motility, by ROS reacting with lipids in the sperm membrane through oxidation. The sperm membrane is especially vulnerable to ROS due to its high concentration of unsaturated fatty acids (10). A defect membrane causes loss of sperm motility and reduced ability to fuse with the oocyte (Reviewed in (5)). The mitochondria can also be directly damaged by ROS, which comprise energy production and leads to impaired motility (Reviewed in (5)). Secondly, oxidative stress directly damages sperm DNA. Protamination in spermatozoa of infertile men is often inadequate, i.e DNA is insufficiently stowed, and the DNA is therefore more vulnerable to oxidative stress (19). Oxidative stress can also induce caspase-mediated apoptosis, which degrades DNA (Reviewed in (5)).

Sperm oxidative stress has been suggested to arise due to many different factors (Figure 1). The field of research in this particular subject is dispersed and studies have been conducted focusing on different factors using different methods. Several parameters have also been used as a measure of oxidative stress, for example sperm ROS, leukocytal ROS, testicular ROS and systemic ROS.

One entity that summarizes several factors that might cause oxidative stress is lifestyle. Overweight has been linked with oxidative stress, it is proposed that adipose tissue increase the leukocyte ROS production and excessive adipose tissue in the groin region rises the temperature and thereby ROS

production (5, 26, 27). A high body mass index (BMI) has been linked to increased sperm DNA damage (27, 28) as well as to infertility (29-31). Hence, it is surprisingly that there is only one study , to the best of our knowledge, that has assessed the direct linkage between BMI and sperm oxidative stress (27). However, increased BMI has also been related to a decrease in testosterone levels (27, 30, 32). Decreased testosterone levels has shown to be more prevalent in infertile men than in fertile men (33, 34). The causality between these parameters and male infertility is not fully unraveled (35, 36).

Other examples of lifestyle factors causing oxidative stress are smoking and alcohol. Smoking has been correlated with just over a doubling in seminal ROS levels (37). Also smoking duration and quantity has shown to directly correspond to increased levels of ROS (38). Excessive alcohol consumption is known to increase systemic oxidative stress (39) and has been suggested to increase ROS levels within the testis (Reviewed in (5)).

Another entity that influences ROS production is environmental factors. Pollutions and toxins lead to antioxidant depletion and increased testicular ROS levels (40). Especially heavy metal exposure has shown to be correlated with sperm oxidative stress (Reviewed in (5)).

Chronic diseases, infections, inflammatory and autoimmune conditions can also interact with ROS production. It is proposed that in diabetes mellitus oxidative stress contributes to the well-known associated subfecundity (41). Infections also contribute to higher levels of ROS. Local genitourinary infections recruit leukocytes who produce ROS that interact with spermatozoa (42). In systemic infections systemic oxidative stress is generated and supposed to also strike the sperm while it afflicts the rest of the body (5). Varicocele has a well-established relation to infertility, and oxidative stress has lately been suggested to be the underlying mechanism (5). Varicocelectomy has shown to decrease seminal ROS levels (Reviewed in (43)). Another testicular event linked with oxidative stress is testis torsion which generates excessive ROS (44) contributing to the correlated decreased fertility (5).

1.4 Assessment of reactive oxygen species in spermatozoa

Despite the correlation between subfecundity and excessive ROS levels, infertile men are not routinely tested for sperm oxidative stress. There are different available analysis for sperm oxidative stress, for example a chemoluminescent procedure recommended by WHO (6). However, they are all costly, intricate and there is a lack of standardized measurements (5). If the prime obstacle, lack of standardized protocols, is solved, most consider oxidative stress testing beneficial in routine clinical practice (45). Recently, the nitro blue tetrazolium assay (NBT) traditionally used in diagnosis of

neutrophile produced ROS was applied in assessing sperm oxidative stress (21). It directly distinguishes between sperm and leukocyte ROS production and is relatively inexpensive and easy to set up (5, 21, 46). On the downside there is a shortage of published normal ranges (21). The first study to correlate sperm ROS production to sperm quality with NBT-assay was published in 2010, the results were promising, but due to the relatively small study group replications were suggested (21). A few replications have been made, showing similar results (27, 46).

The NBT assay quantifies sperm produced ROS as a measure of sperm oxidative stress. Sperm are separated from plasma to exclusively evaluate sperm ROS. NBT solution is taken into the sperm cytoplasm and there sperm ROS converts the NBT to formazan (Figure 2). The more ROS the more formazan. The amount of formazan is then quantified by measuring the absorbance with a spectrophotometer. The absorbance is correlated to a standard curve, presenting the absorbance values at given concentrations of formazan crystals, and expressed as microgram of formazan per 10^7 sperm. This as an indirect measure of sperm ROS production.

1.5 Assessing sperm DNA damage

Sperm DNA damage can be assessed with different methods, most commonly sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and single cell gel electrophoresis (COMET) assay. Sperm chromatin structure assay is so far the only sperm DNA integrity method that has demonstrated clear clinical cut-off levels in relation to fertility (9, 14, 16, 47). It quantifies spermatozoa with DNA strand breaks, is standardized (13), has a low intra- and inter-lab variety (9, 14) and can be used as an independent predictor of male fertility (47). Thus SCSA is starting to enter the field of routine sperm analyses.

The principles of SCSA are that a low pH buffer is added to the semen sample. Some spermatozoa are more vulnerable than others to acid, and consequently their DNA denaturates. This vulnerability is thought to be caused by impaired chromatin structure and/or DNA strand breaks (17). The denaturation is therefore used as an indirect measure of sperm DNA disturbance (4). Denaturated cells are susceptible for an added fluorescent staining, in SCSA acridine orange (AO) is used. Due to the metachromatic properties of the staining, intact double DNA strands stain green and denaturated single DNA strands stain red. The ratio of red to total fluorescence intensity is determined by flowcytometry. Hence the result is expressed as percentage of denaturated DNA (red) to total DNA (total fluorescence) and called DNA fragmentation index (DFI) (4, 14). Even if WHO semen standard parameters are normal an increased DFI between 20-30% correspond to extended time to pregnancy

(4, 48). With a DFI >30% the chances of spontaneous conception (13) and pregnancy by intra uterine insemination (IUI) are minimal (4, 14). If one of the WHO semen parameters is abnormal, fertility is decreased already at DFI >10% (47).

1.6 Objectives

The primary objective was to study the correlation between sperm oxidative stress assessed with NBT assay and sperm DNA fragmentation index (DFI) assessed with SCSA in subfertile men. Secondly, the objective was to correlate these parameters to BMI as well as to the level of serum testosterone.

2 METHOD

2.1 Study design

Prospective study.

2.2 Setting

The study took place at Reproductive Medicine Center (RMC), Skåne University Hospital, Lund University from September 2012 to November 2012.

2.3 Ethic approval

The study was approved by the Ethics Committee at Lund University 2012-05-22, EPN number Dnr. 2012/310, an amendment to Dnr. 2010/660. The men received no incentives, and participation was conditional based on written informed consent.

2.4 Study population

The study population consisted of subfertile men, who all had at least one previous sperm sample analysed at RMC. The cohort was based on an ongoing study at RMC (data to be published). According to the inclusion criteria, all participants were living in the Region Skåne and were aged between 18 and 50 years. They were diagnosed with male infertility according to International Criteria for Diseases (ICD-10) N46.9, between December 2006 and August 2012, and had all a sperm concentration below 20 million per millilitre ($< 20 \times 10^6$/mL). One hundred seventy-two participants were included in the study and obtainable 6th of September 2012. From those, one study subject was excluded due to abuse of anabolic steroids. Additionally 55 study subjects were excluded due to azoospermia. Consequently invitations were sent to 116 persons. Reminders were sent in case of no reply after a period of 8-11 days. One of the invited men was deceased. The total answering frequency was 83% (96 subjects). Out of these 43 % (49 subjects) participated, 34% (39 subjects) refrained and 7% (8 subjects) established contact but ended up not participating for different reasons. The rest, 17% (19 subjects), were non responders. Two participants were then excluded from the result due to testosterone treatment causing azoospermia (Figure 3).

2.5 Analyses of semen samples

Prior to semen collection participants were instructed to have an abstinence period, i.e. no ejaculation, of 2-4 days. Semen samples were produced at the clinic by masturbation, and liquefied in room temperature for 30 minutes (min). Within one hour post ejaculation, the conventional sperm

parameters were analysed and NBT assay was performed. Two hundred microliters (µL) of the semen sample were frozen for a later SCSA analyse. All analyses were performed at the RMC laboratory by experienced lab personnel.

2.5.1 WHO sperm analysis

The sperm samples were analysed, ejaculate volume (milliliter) and sperm concentration (10^6/mL), according to the WHO guidelines (6). Sperm concentration > 15 x 10^6/mL was considered normal (6).

2.5.2 Nitro blue tetrazolium assay

The NBT assay protocol was previously established at RMC, based on a published article describing the method (21). Two hundred microliters of raw semen were placed in an Eppendorf tube (Eppendorf AG, Hamburg, Germany). The sample was centrifuged in Eppendorf minispin (Eppendorf AG, Hamburg, Germany), 10 min at 500g (3.5x1000rpm) and washed with Dulbeccos's phosphate buffered saline (PBS) (JRH Biosciences, Lenexa, KN, USA), in order to separate sperm from seminal plasma. Thereafter the sample was divided into two aliquots of 100 µL. NBT 0.01% stock was prepared dissolving one NBT tablet (Sigma Aldrich, St Louis, MO, USA) in MilliQ water (Millipore Corporation, Billerica, MA, USA) and then diluted 1:10 in PBS to create the working solution. An equal amount of the NBT working solution was added to each of the samples. The samples were incubated at 37ºC for 45 min, and thereafter centrifuged and washed twice to remove remaining NBT solution. The remaining product, a cell pellet with blue formazan crystals were released by 2 M potassium-hydroxide (KOH), KOH powder (Sigma Aldrich, St Louis, MO, USA). Dimethyl sulfoxide (Sigma Aldrich, St Louis, MO, USA) was added to the solvent. Subsequently 50 µL of the purple-black solution was quantified using a spectrophotometer, Microplate reader Model ELx800 (Bio-Tek Instruments Inc, Winooski, VT, USA), measuring the absorbance at 630 nanometres with the use of Gen5 2.0 software (Bio-Tek Instruments Inc, Winooski, VT, USA). The mean absorbance from the two aliquots was calculated and supposed to be correlated to a standard curve. The standard curve produced by NBT solution reacting with superoxide anions, from the xanthine/xanthine oxidase system, forming formazan. The amount of formazan was weighed and its absorbance measured, and formazan mass versus absorbance plotted into a standard curve. Consequently sperm ROS production would be expressed as formazan per ten million spermatozoa.

The standard curve was developed parallel to this study. However, no standard curve could be obtained in due time for our data analyses. Therefore, to enable intergroup comparison, we chose to

use the quote: absorbance divided with sperm concentration. This quote referred to as the NBT outcome.

2.5.3 Sperm chromatin structure assay

The SCSA was performed after a standard protocol (49) and analysed with dedicated software, SCSA-Soft (SCSA Diagnostics Inc., Brookings SD, USA). Following conventional sperm analysis 200 µL of the raw semen samples, were frozen at -80°C. At the time for the analysis samples were thawed and diluted with TNE buffer (0.01 molar (M) Tris–HCl, 0.15 M NaCl and 1 milllimolar (mM) EDTA, pH 7.4) to a sperm concentration of 6 x 10^6/mL in 5 mL round bottom Falcon tubes (Becton Dickinson Biosciences, San José, CA, USA). Four hundred microliters of acid detergent (0.1% Triton X-100, 0.15 M NaCl and 0.08 M HCl), pH 1.2, was then added to the samples. After exactly 30 seconds (sec), 1.2 mL of acridine orange staining , purified acridine orange powder (Polysciences Inc., Warrington, PA, USA) solved in Millipore water, was added. Thus the denaturated single-stranded DNA stained red and intact double-stranded DNA stained green. The fluorescence were measured in a flow cytometer, FACS-calibur (Becton Dickinson, Franklin Lakes, NJ, USA), by CELLQuest software (Becton Dickinson, San Jose, CA, USA), 10 000 cells per sample. The flow cytometer was calibrated with a reference sample from a sperm donor. Data was analysed, shown in a scatter plot and then translated so that the ratio of red to total fluorescence intensity was visualized in a histogram. The result was expressed as percentage of denaturated DNA (red) over the total DNA (total fluorescence intensity), so called DNA fragmentation index (DFI). In this study, a DFI > 30% was considered as severe sperm DNA damage.

2.6 Analyses of blood samples

Blood samples were obtained by venepuncture for analysis of testosterone and luteinizing hormone (LH) (50). Blood were drawn between 7.30 and 10.00 am, after an overnight fast (51, 52). The sample tubes were transported within one hour and analysed by accredited analyses at Labmedicin Skåne, the university and regional laboratories in Region Skåne. If the participants had previous analyse results from RMC within a period of six months, no new blood samples were taken.

Hypogonadism was defined biochemically. A testosterone below 12.0 nanomol per liter (nmol/L) (53) and/or lutenizing hormone above 10 international units per liter (IU/L) (54) were considered as hypogonadism. Clinical symptoms of male hypogonadism were not taken into account.

2.7 Body measurements

The participants´ weight (kilograms) was measured, without shoes, trousers and heavy material in pockets. The body height (meters), already measured at a previous study appointment, was used for calculating BMI, kilograms per square meter (kg/m^2). BMI < 25 kg/m^2 was considered normal weight, BMI 25-30 kg/m^2 considered overweight and BMI > 30 kg/m^2 was considered obese (55).

2.8 Additional data

The participants were asked for ongoing testosterone treatment (yes/no) and smoking habits (regular smoker/ non-smoker).

2.9 Statistical analysis

Firstly, the NBT outcome, i.e the ratio between absorbance and sperm concentration, was correlated to the level of DFI. Eight samples were excluded due to azoospermia, as no quote could be obtained for the NBT outcome. The bivariate correlation analysis was carried out using Spearman´s rho. The same procedure was repeated with exclusion of NBT outcome values above one (to eliminate outliners), as well as with a subgroup analysis. The subgroup consisted of samples with sperm concentration > 2 x 10^6/mL. The correlation between the NBT outcome and the level of DFI for the total study population was also visualized in a linear regression plot.

Secondly, the NBT outcome was related to the level of BMI. Here BMI was treated as a continuous variable as well as categorized into three groups; BMI < 25 kg/m^2, BMI 25-30 kg/m^2 and BMI > 30 kg/m^2. An univariate linear model was used for the comparison, with age, abstinence time and smoking as confounders in the model. Thereafter, the same analysis where performed for DFI in relation to BMI. Identical analysis, however with testosterone as a continuous variable and hypogonadism (yes/no) as categorized variable was also performed.

Finally binary logistic regression was used to calculate odds ratios (OR) for the risk of having a DFI >30%; a) for the two higher BMI categories, i.e. BMI 25-30 kg/m^2 and BMI > 30 kg/m^2, in reference to the lowest BMI category, i.e. BMI < 25 kg/m^2 and b) for the men with hypogonadism compared with the non-hypogonadistic men. These elaborations adjusted for age, abstinence time and smoking.

Data were analyzed using the statistical software programme IBM SPSS Statistics version 21 (SPSS Inc., Chicago, IL, USA). Demographical and outcome data expressed as mean (± SD) or median

(range). Confidence intervals were set to 95% and a P-value < 0.05 was considered statistically significant.

3 RESULTS

3.1 Descriptive and outcome data

The cohort consisted of 47 men with a mean age of 34.8 years (SD±6.2). The mean abstinence time was 4.1 days (SD± 3.2). Twenty-five participants were overweight (BMI > 25 kg/m^2). Two of the men were regular smokers. Concerning sperm quality the median sperm concentration was 1.1 x 10^6/mL (range 0-18.2 x 10^6/mL). The median DFI was 19% (range 7-48 %). Ten men had a DFI level above 30%. The NBT outcome had a median of 0.036 (range 0.035-1.800). Sixteen of the men had a biochemically defined hypogonadism (Table I).

3.2 Correlation between sperm oxidative stress and sperm DNA damage

The outcome of the NBT and SCSA analyses showed a vague correlation which was not statistically significant (n = 39, r = 0.281, P = 0.084) (Figure 4). By exclusion of two outliners with extreme NBT outcome, the correlation was shown to be identical (n = 37, r = 0.288, P = 0.084) to the entire study group.

The same analysis for correlating NBT outcome to DFI was also performed for a subgroup of men having a sperm concentration > 2 x 10^6/mL due to a proposed cut off level for reliable NBT results (21). This correlation was, however, even weaker compared to the analysis of the entire group (n = 17, r = 0.171, P = 0.511).

3.3 Sperm oxidative stress and sperm DNA damage in relation to BMI and testosterone/hypogonadism

No relation was seen between NBT outcome and BMI, continuous as well as categorized. However, while there appears to be the possibility of a positive relation between DFI and categorized BMI this relation was not statistically significant (regression coefficient = 3.237, P = 0.119) (Table II).

Neither testosterone level nor hypogonadism were related to NBT outcome or to level of DFI (Table III). These analysis were adjusted for age, abstinence time and smoking.

3.4 Risk for sperm DNA damage on the basis of BMI as well as on the basis of hypogonadism

Compared to the normal weighted men (BMI < 25 kg/m^2) the overweighed (BMI > 25-30 kg/m^2) had a fivefold increased risk for having a DFI > 30% (OR = 5.4, P = 0.131). For the obese group (BMI >

30 kg/m^2) the risk compared to the normal weighted men was nine fold (OR = 8.6, P = 0.070). However, none of these odds ratios were statistically significant (Table IV, Figure 5).

Hypogonadism was related to a doubled increased risk of having a DFI exceeding 30% compared to the non-hypogonadistic group (OR = 2.2, P = 0.331) (Table IV), although not statistically significant. Male age, abstinence time and smoking habits were used as potential confounders in this model.

4 DISCUSSION

The main finding of this study was that, although not statistically significant, levels of sperm oxidative stress were slightly associated with levels of sperm DNA damage in subfertile men. To the best of our knowledge, there is only one previous study that has assessed sperm oxidative stress with the NBT-assay in relation to sperm DNA damage (21) and they demonstrated a statistically significant positive correlation between these parameters. The study of Tunc and colleges consisted of 36 infertile and 21 fertile men, almost the same number of infertile as included in the present study. The weaker association in our study compared to Tunc´s, may be influenced by the method for evaluating sperm DNA damage (21). While Tunc used the TUNEL assay, the present study used SCSA and the two methods have been proposed to measure different aspects of sperm DNA damage (4). Although not shown, it cannot be neglected that one of the methods is more prone to detect sperm oxidative stress caused DNA damage compared to the other.

Tunc´s study also supported the presumption that sperm oxidative stress can cause infertility. They showed a fourfold increase in sperm oxidative stress in infertile compared to fertile men, this positive correlation also reported by others (46). The study of Amarasekara and colleges had an infertile group double the size of our cohort and a fertile group of 30 men. Both studies also proposed two different NBT assay cut off levels for determination of fertile-infertile status. If a cut off level is defined, NBT assay can be used as a tool to sort out the men suffering from sperm oxidative stress caused infertility, which could be of important value in clinical practice.

In the absence of a standard curve for the NBT assay, the comparison to other laboratory data could only be made regarding trends and not specific NBT assay outcome values. This unfortunate as we could not correlate our NBT outcome to the studies mentioned. Hence, the proposed NBT assay cut off levels for determination of fertile-infertile status could not be related to (21, 46). Due to the lack of a fertile control group nor could we create our own cut off level.

The weak association and the lack of a statistically significant result in this study may have several explanations. We acknowledge that this is a low power study and it is possible that a larger sample size had resulted in stronger correlations as well as statistic significance. In the NBT analysis the number of men was further diminished as the absorbance was divided with the sperm concentration and therefore eight azoospermic samples could not be included in the statistical analysis.

Another major contributing factor might be that the cohort had low median sperm concentration (range 0-18.2 x 10^6/mL). This as one inclusion criteria was sperm concentration < 20 x 10^6/mL. This was considered the lower limit for normal sperm concentration before the new guidelines of 15 x 10^6/mL (56). The Tunc-study suggested that the outcome of the NBT assay might be uncertain at low sperm concentration and proposed a lower limit of 2 x 10^6/mL for analyses. This lower limit used in the Tunc-study but in the Amarasekara-study infertile men were included regardless of their sperm concentration (21, 46). As of this, we performed a subgroup analysis excluding all men with sperm concentrations < 2 x 10^6/mL (64 % excluded). However, this analysis between sperm oxidative stress and sperm DNA damage ended up with a very limited number of men and presented even weaker results. Hence, no further analysis on the subgroup was executed.

An unexpected finding, supporting a lower sperm concentration limit for reliable NBT results was that the azoospermic samples all produced absorbance results in the mid/lower range. Possible explanations to this mechanism could be sperm in the sample producing ROS, contamination of non-sperm produced ROS and methodological errors. One possible error could be that with a small amount of spermatozoa there might be difficulties in removing all residual NBT-solution in the washing procedure. If the proposed lower limit is accurate, it is a limitation of the NBT-assay. However, set in the context of the chemoluminiescent assay, WHOs recommendation for assessing sperm oxidative stress, which needs at least a sperm concentration of 6-7 x 10^6/mL (i.e. excluding 81-85% of the men in this study) this limitation seems acceptable.

Concerning the SCSA measurements, the DFI results were in the higher range, as expected in a subfertile cohort and suitable when assessing a potential cause of sperm DNA damage. Nevertheless, our study showed a weak correlation between sperm oxidative stress and sperm DNA damage. Since there are several possible etiologies of sperm DNA damage, it cannot be neglected that our results could owe to a predominance of sperm DNA damage originating from other mechanisms than sperm oxidative stress.

Due to the relatively low rate of participation (43 %) selection bias has to be considered. The reasons for not participating could be a recent involvement in other studies and/or ongoing treatment at RMC. As travel expenses and participation were not compensated, reason for not participating might be economical. It is also possible that the men with successful infertility treatment or spontaneous conception are more prone to participate than the men who are in for prolonged unsuccessful infertility treatment. This might have eliminated the men with severest infertility. On the other hand, it

is possible that the men with severe infertility are eager to try anything that might bring new insight into their situation.

In regard to our secondary objective we found no association between sperm oxidative stress and BMI. To the best of our knowledge there is only one study that has assessed the correlation between sperm oxidative stress as measured with the NBT-assay and BMI (27). In contrast to our finding, this study including twice the number of men compared to our study showed a weak statistically significant positive correlation between sperm oxidative stress and BMI.

In our study BMI was related to sperm DNA damage although not statistically significant. The obese men had about a nine fold increased risk for severe sperm DNA damage, although not statistically significant, compared to their normal weight counterparts. This was in line with previous published studies (27, 28). It seems evident that BMI and testosterone are interrelated (30, 32). The indifferent association between testosterone and sperm oxidative stress as well as sperm DNA damage seen in the present study was therefore not unexpected.

The relation between BMI or testosterone and NBT outcome or DFI, may be influenced by additional factors than limited study population, low sperm concentrations, selection bias and methodological errors mentioned above.

There is a risk of measurement bias, although minimization strategies were applied. For weight and length no self reporting was accepted. Blood samples were taken according to present clinical routine (50) and analysed by a accredited laboratory. Three study subjects had not been fasting for the testosterone sampling. This might reduce their testosterone levels, although there is only limited evidence and the present routine does not prescribe mandatory fasting (51, 52, 57). As testosterone treatment was self-reported there is always a risk for recollection bias.

The potential confounders age, abstinence time and smoking were adjusted for. The cohort seems representative for the reproductive age, the mean age of the participants is close to the mean age for fathering a child in Sweden, reducing male age as a confounder (58). Abstinence time and smoking habits were self-reported, hence there is a risk of over/underestimations. There may also be other confounders, such as medications, infections, inflammations and lifestyle factors, however, these were not taken into consideration.

In conclusion, the weak association seen between sperm oxidative stress and sperm DNA damage, although not statistically significant, is in line with previous reports. Large scale clinical trials are warranted to further evaluate the NBT assay. In these trials, as opposed to this study, we recommend to include subjects independent of their sperm concentration and to also have a fertile control group.

If sperm oxidative stress is established as a cause of male infertility and easily assessed, it could be included in routine infertility assessment and likely reduce the number of men diagnosed with idiopathic infertility. The hope for the future is to be able to sort out the men with sperm oxidative stress caused infertility, who would benefit from causative treatments, such as lifestyle changes and antioxidant therapy. This would lead to reduced suffering for the afflicted and reduced economic costs.

ACKNOWLEDGEMENTS

We wish to thank all participants and staff of RMC who contributed to this study. We would especially like to acknowledge the help of Irené Leijonhufvud and Johannes Bobjer for assistance in participant recruitment and Sandy Christiansen and Niclas Forsell for the sperm analysis.

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TABLES

Table I. Demographic and outcome data, total study population.

Parameters	
Patients, n	47
Age, years, mean ± SD	34.8 ± 6.2
Abstinence time, days, mean ± SD*	4.1 ± 3.2
BMI, kg/m^2, mean ± SD	26.1 ± 3.8
BMI <25 kg/m^2, n	22
BMI 25-30 kg/m^2, n	16
BMI >30 kg/m^2, n	9
Smokers, n	2
Sperm concentration, 10^6/mL, median (range)	1.1 (0 -18.2)
DFI, %, median (range)	19 (7 – 48)
DFI >30 %, n	10
NBT outcome, absorbance/sperm conc, median (range)**	0.036 (0.035 – 1.800)
Testosterone, nmol/L, mean ± SD	16.3 ± 7.2
LH, IU/L, mean ± SD	6.0 ± 2.6
Hypogonadism , n	16

* Three men excluded due to unknown abstinence time
** Eight men excluded due to azoospermia

Table II. Relation between BMI (kg/m^2) and NBT outcome (absorbance/sperm concentration), n = 38. Relation between BMI and DFI (%), n = 44. Adjusted for age, abstinence time and smoking.

Parameters	Regression coefficient	P-value	CI (95%)
BMI and NBT outcome	-0.005	0.699	-0.033 – 0.022
BMI (<25, 25-30, >30) and NBT outcome	-0.020	0.767	-0.157 – 0.117
BMI and DFI	0.435	0.306	-0.414 – 1.283
BMI (<25, 25-30, >30) and DFI	3.237	0.119	-0.868 – 7.343

Table III. Relation between testosterone (nmol/L) and NBT outcome (absorbance/sperm concentration), n = 38. Relation between testosterone (nmol/L) and DFI (%), n = 44. Adjusted for age, abstinence time and smoking.

Parameters	Regression coefficient	P-value	CI (95%)
Testosterone and NBT outcome	0.006	0.410	-0.009 – 0.021
Hypogonadism (yes/no) and NBT outcome	0.044	0.712	-0.197 – 0.285
Testosterone and DFI	-0.112	0.607	-0.549 – 0.324
Hypogonadism (yes/no) and DFI	4.193	0.207	-2.421 – 10.806

Table IV. The risk of having a DFI >30 %. Categorized DFI (%); DFI <30 (n= 37), DFI >30 (n=10). Categorized BMI (kg/m^2); BMI <25 (n=22), BMI 25-30 (n=16), BMI >30 (n=9). Hypogonadism; yes (n=16), no (n=31).

The risk of a DFI >30	OR	P-value	CI (95%)
BMI 25-30 compared to BMI <25	5.4	0.131	0.607 – 47.95
BMI >30 compared to BMI <25	8.6	0.070	0.837 – 88.839
Hypogonadism compared to non-hypogonadism	2.2	0.331	0.456 – 10.299

FIGURES

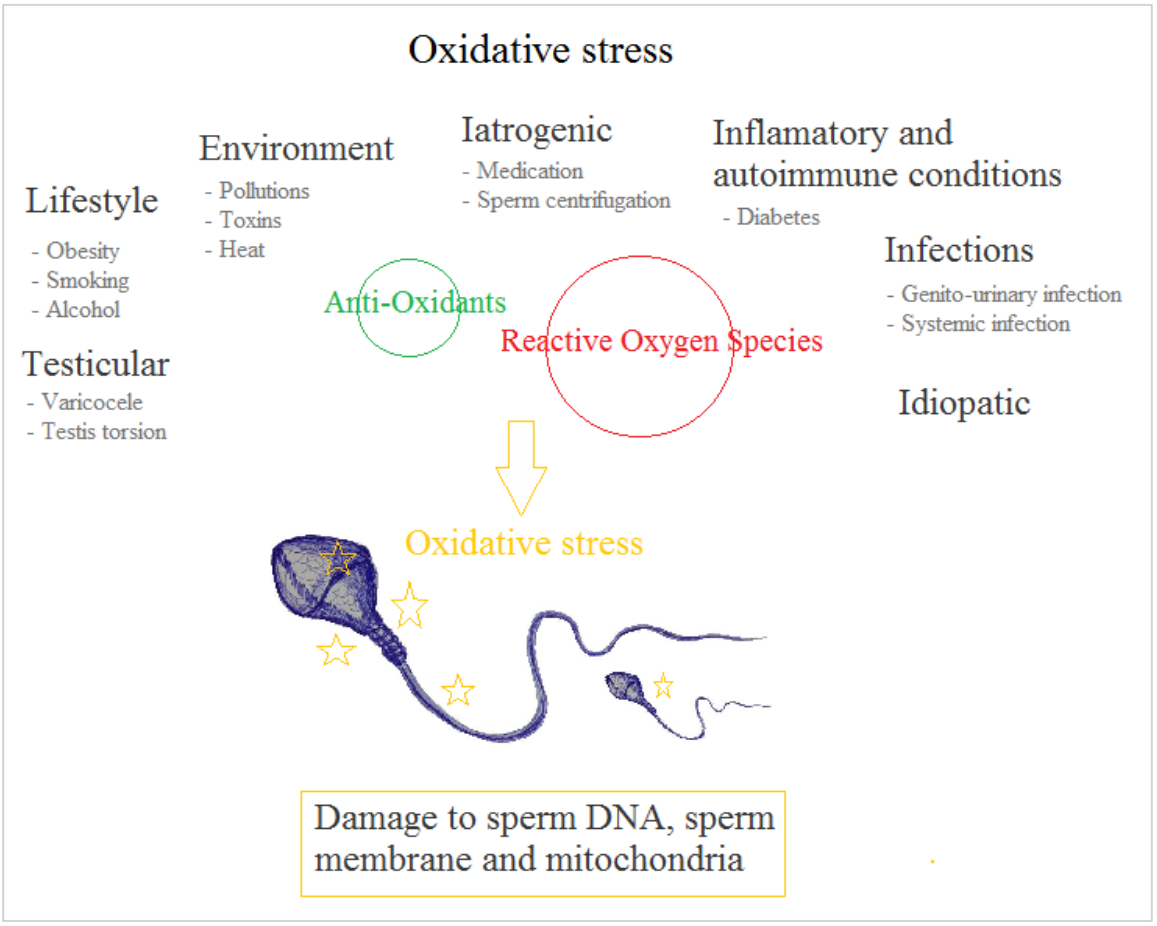

Figure 1.

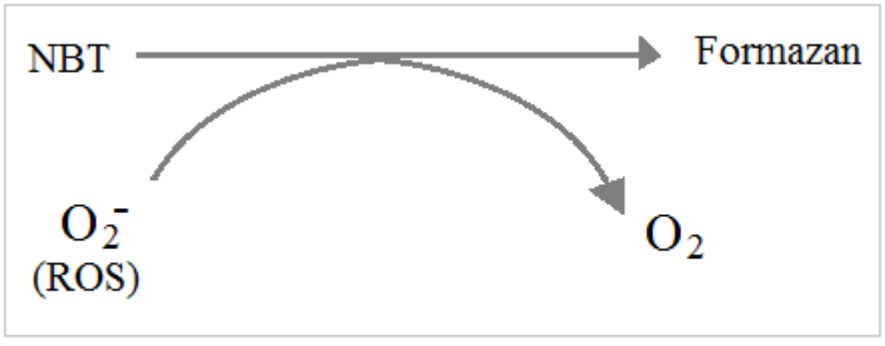

Figure 2.

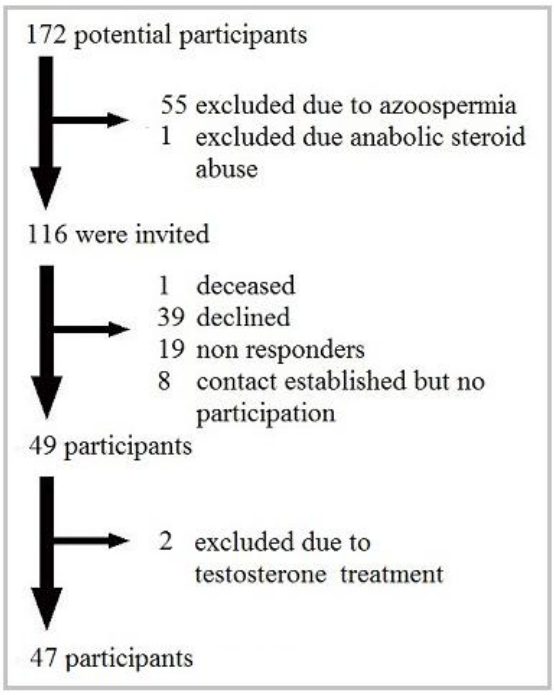

Figure 3.

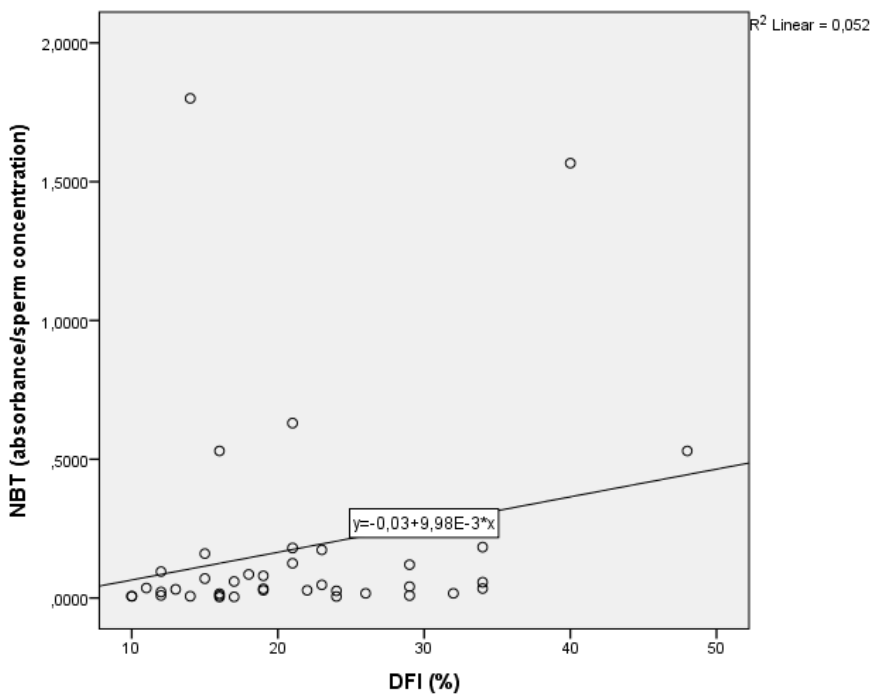

Figure 4.

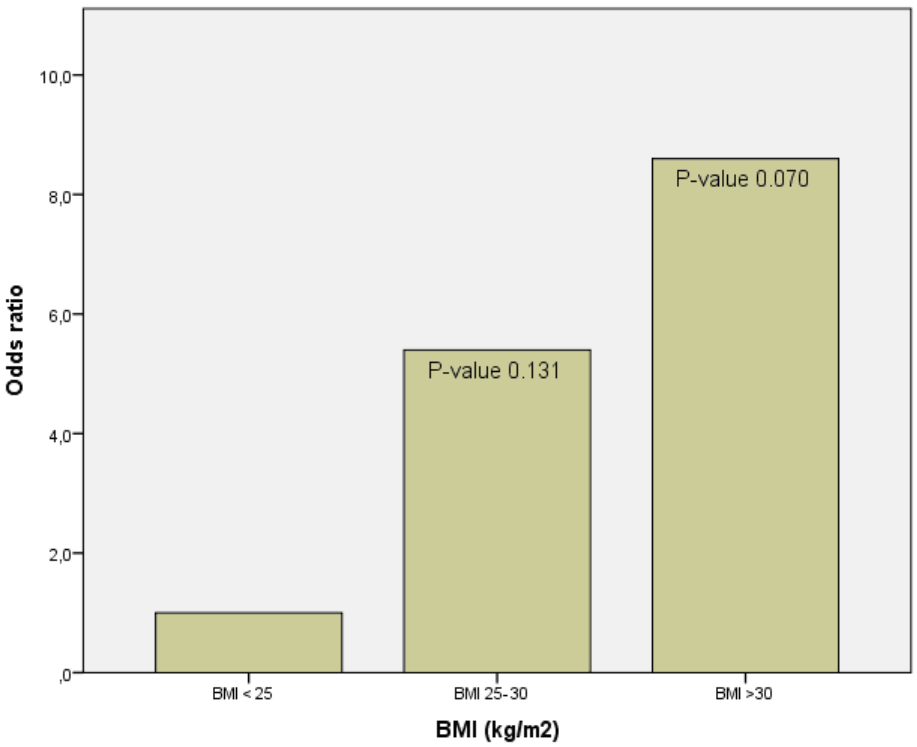
10,0
8,0
6,0
4,0
2,0
,0
Odds ratio
P-value 0.070
P-value 0.131
BMI < 25
BMI 25-30
BMI >30
BMI (kg/m2)

Figure 5.

FIGURE TEXTS

Figure 1. Causes of sperm oxidative stress. Processed by the authors after Kelton Tremellens Figure 1 (5).

Figure 2. Primary chemical reaction in the NBT-assay.

Figure 3. Participants, the inclusion process.

Figure 4. Linear regression with a trend line. DFI in correlation to NBT outcome. N=39.

Figure 5. Staple graph. The risk of a DFI >30 %. Categorized DFI (%); DFI <30 (n= 37), DFI >30 (n=10). Categorized BMI (kg/m^2); BMI <25 (n=22), BMI 25-30 (n=16). P-values in correlation to BMI <25.

ABBREVATIONS

ART	Assisted reproductive techniques
BMI	Body mass index
CI	Confidence interval
COMET	Single cell gel electrophoresis
DFI	DNA fragmentation index
g	Acceleration: Relative centrifugal force
ICD-10	International criteria for diseases – 10
IU	International unit
KOH	Potassium hydroxide
LH	Luteinizing hormone
NBT	Nitro blue tetrazolium assay
OR	Odds ratio
PBS	Phosphate buffered saline
r	Correlation coefficient
RMC	Reproductive Medicine Center
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCSA	Sperm chromatin structure assay
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay
WHO	World Health Organization