RBM ONLINE

ELSEVIER

Article

Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress

Jaime Gosálvez [a], ***Lamberto Coppola*** [b], ***Jose Luis Fernández*** [c], ***Carmen López-Fernández*** [a], ***Alfredo Góngora*** [d], ***Ricardo Faundez*** [e], ***John Kim*** [f], ***Nabil Sayme*** [g], ***Moises de la Casa*** [h], ***Rebeca Santiso*** [c], ***Keith Harrison*** [i], ***Ashok Agarwal*** [j], ***Stephen Johnston*** [k,*], ***Sandro C Esteves*** [l]

[a] *Department of Biology, Universidad Autónoma de Madrid, Madrid, 28049, Spain*
[b] *Tecnomed, Centro Medico Biologico, Nardò, Lecce, Italy*
[c] *Genetics Unit, INIBIC-Complejo Hospitalario Universitario A Coruña (CHUAC), As Xubias, 84, 15006 A Coruña, Spain*
[d] *Centro de Fertilidad, Tuxpan 6. Col. Roma Sur. Mexico DF, Mexico*
[e] *Invimed Fertility Clinics, Embryology Laboratories, Center of Biomedical Research, WULS, 36 Rakowiecka Street, 02-532 Warsaw, Poland*
[f] *SJ Science Inc., 972-8, Shin Jung 4 Dong, Yang Chun Gu, Seoul, Republic of Korea*
[g] *Arzt, Frauenarzt (Gynäkologe), Weiterbildungen: Reproduktionsmedizin, Ambulante Operationen, Brühlstr. 19, 30169 Hannover, Germany*
[h] *Ginefiv, Madrid, Spain*
[i] *Queensland Fertility Group, 55 Little Edward Street, Spring Hill 4000, Australia*
[j] *Clinical Pathology, Cleveland Clinic Main Campus, Mail Code X11, 9500 Euclid Avenue, Cleveland, OH 44195, USA*
[k] *School of Agriculture and Food Sciences, The University of Queensland, Gatton 4343, Australia*
[l] *ANDROFERT, Andrology and Human Reproduction Clinic, Av. Dr. Heitor Penteado, 1464, Campinas, SP 13075-460, Brazil*

Jaime Gosálvez is Professor of Genetics at the Department of Biology, University Autónoma of Madrid. He has published 345 journal articles and co-edited two books. He has also been principal investigator in 21 national and international research projects and has participated in five patents.

KEY MESSAGE
Semen samples with high levels of oxidative stress as determined by a nitroblue tetrazolium assay, have diminished sperm DNA longevity after ejaculation. Given that seminal plasma was found to be the primary source of oxidative stress, rapid separation of this fraction may improve sperm DNA quality in these patients.

A B S T R A C T

The nitroblue tetrazolium (NBT) reaction as a tracer of oxidative stress was examined in 707 ejaculates from seven clinics. Semen was initially surveyed by classifying the NBT reaction using a pre-established rank for the Oxisperm® test based on three colourimetric levels: L1, low (n = 141 [20%]);

* Corresponding author.
E-mail address: s.johnston1@uq.edu.au (S Johnston).
http://dx.doi.org/10.1016/j.rbmo.2017.01.014

Please cite this article in press as: Jaime Gosálvez, Lamberto Coppola, Jose Luis Fernández, Carmen López-Fernández, Alfredo Góngora, Ricardo Faundez, John Kim, Nabil Sayme, Moises de la Casa, Rebeca Santiso, Keith Harrison, Ashok Agarwal, Stephen Johnston, Sandro C. Esteves, Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress, Reproductive BioMedicine Online (2017), doi: 10.1016/j.rbmo.2017.01.014

L2, medium (n = 538 [76%]) and L3, high (n = 28 [4%]). L3 was indicative of a high level of superoxide anions. Halosperm® chromatin dispersion assay was used to anlysise samples of ejaculates 30 min after ejaculation; no difference was found in DNA fragmentation of L1 or L3; L3 category semen samples incubated for 24 h at 37°C showed a significantly faster rate ($P < 0.001$) of DNA damage than those in L1. The NBT reaction was further characterized in the ejaculates of 100 patients to determine the relative contribution of seminal plasma, spermatozoa, or both. Seminal plasma was the most significant fraction of $•O_2^-$ localization, whereas sperm fractions generated detectable reactive oxygen species in only 32% of the ejaculates. Formazan precipitates were primarily associated with the sperm mid-piece and seminal leukocytes; however, not all spermatozoa stained positive to formazan and not all leukocytes presented with equivalent production of superoxide anions.

Introduction

Reactive oxygen species (ROS) are commonly known to negatively affect somatic or germ cell lines and are a major cause of cellular damage. Examples of ROS include the superoxide anion ($•O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and the peroxyl radical ($•HO_2^-$); the former being the most abundant ROS in human semen and the precursor of other ROS types. The superoxide anion is primarily produced from mitochondrial electron transport chain complexes I and III (Chen et al., 2009), and there has been significant interest in using it as a marker of oxidative damage given its ubiquity and strong chemical reactivity. All types of ROS can induce cellular damage in biological molecules such as proteins, lipids and DNA (Agarwal et al., 2014; Du Plessis et al., 2015; Kohen and Nyska, 2002). Excessive ROS production in the male reproductive tract is of concern because it leads to oxidative stress, which has potential toxic effects on sperm quality and function. In fact, between 25 and 50% of patients attending infertility clinics have high concentrations of ROS that may be associated with abnormal sperm motility, membrane integrity and DNA quality (Aitken, 1995; Ferramosca et al., 2007, 2013). For example, patients with a varicocele, typically have ROS concentrations higher than those of other infertile patients; in these patients, the affect of ROS is likely to be associated with heat stress affecting developing germ cells (Smith et al., 2006). Nevertheless, the exact prevalence of ROS in the subfertile male population remains poorly understood (Gharagozloo and Aitken, 2011). Part of the problem stems from the complexity of some techniques that are used to measure ROS, which limits the widespread utilization of ROS measurements as a routine procedure in the andrology clinic. Additionally, the plethora of different ROS radicals being assessed and how they are being assessed makes it challenging to draw firm conclusions on the real prevalence and significance of ROS in male infertility.

Various strategies have been used to assess the presence and effects of ROS (Agarwal et al., 2014; Kohen and Nyska, 2002). The NBT assay is a technique that has traditionally been used to determine the production of $•O_2^-$ in somatic and germ cells (Choi et al., 2006; Dimitrova et al., 2013; Sharma and Agarwal, 1996). The yellow NBT molecule is water-soluble, membrane permeable and reduced by $•O_2^-$ to a blue formazan deposit (Halliwell and Gutteridge, 1985). The specificity of this reaction has been demonstrated by the inhibitory effect of superoxide dismutase (SOD) as this enzyme catalyzes the dismutation of two molecules of $•O_2^-$ to form one molecule of oxygen and one molecule of H_2O_2 (Baehner et al., 1975). Therefore, the levels of $•O_2^-$, H_2O_2 and •OH are in constant conversion until equilibrium is established. Although $•O_2^-$ in semen samples has previously been evaluated using NBT (Amarasekara et al., 2014; Esfandiari et al., 2003; Tunc et al., 2010), a clear understanding of the association between positive reactions to NBT and the different fractions of the ejaculate (sperm versus seminal plasma), or of the prevalence of positive reactions in the neat ejaculate within a large cohort of men seeking fertility assistance, is lacking. Moreover, the effect of ROS on sperm DNA quality deserves more detailed attention, especially in establishing direct relationships between both concepts.

We conducted a multinational and multicentre cross-sectional study to determine the prevalence of positive responses to NBT in the ejaculates of a large number of infertile men attending infertility clinics around the world. The following were studied: the relationship of high and low NBT reacting ejaculates on initial sperm DNA quality and after incubation at 37°C for up to 24 h; the relative contribution of seminal plasma, spermatozoa, or both, to the strength of NBT reaction; and which primary region of the sperm cell the signal is being generated from.

Material and methods

Study design

This prospective design was a cross-sectional study that incorporated fresh ejaculates obtained from 707 men seeking fertility evaluation in 2014 and 2015 at seven participating clinics in Australia, Brazil, Germany, Mexico, Poland, South Korea and Spain. All participants provided informed consent to use their semen samples for the analysis. The data obtained by individual centres were compiled and subsequently analysed at the Genetics Unit of the Autónoma University of Madrid. This study was approved by the research committee or internal clinical board of each participant institution and complied with the standards for the reporting of cross-sectional studies (STROBE statement, http://strobe-statement.org). Patients were offered the assay in addition to the standard seminogram and informed that their semen sample would be analysed as part of larger worldwide multicentre study on seminal oxidative stress. No charged was levied for the assay and signed consent for participation was requested.

Participants

Men were included if they were between the ages of 18 and 45 years, seeking fertility evaluation and consented to donate a semen specimen for research. Participants were asked to abstain from ejaculation for 2–3 days before collection. All participants used a collection room located in the same facility as the andrology laboratory. Ejaculates were collected by masturbation into sterile cups. A single ejaculate was obtained from each individual and all specimens were discarded after assessments. Men were excluded if they reported semen spillage or loss during collection, had a history of the following, or

Please cite this article in press as: Jaime Gosálvez, Lamberto Coppola, Jose Luis Fernández, Carmen López-Fernández, Alfredo Góngora, Ricardo Faundez, John Kim, Nabil Sayme, Moises de la Casa, Rebeca Santiso, Keith Harrison, Ashok Agarwal, Stephen Johnston, Sandro C. Esteves, Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress, Reproductive BioMedicine Online (2017), doi: 10.1016/j.rbmo.2017.01.014