The effect of antioxidant therapy on DNA fragmentation of ejaculated sperm

Thesis

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AB	Aniline blue
ACL	Acetyl L-carnitine
AO	Acridine orange
ART	Assisted reproductive techniques
CMA3	Chromomycin A3
DSBs	DNA double strand breaks
DFI	DNA fragmentation index
ICSI	Intra cytoplasmic sperm injection
IUI	Intra uterine insemination
IVF	In-vitro fertilization
LC	L-carnitine
ROS	Reactive oxygen species
SCD	Sperm chromatin dispersion test
SCSA	Sperm chromatin structure assay
ssDNA	Single strand DNA
SSBs	Single strand breaks
TB	Toluidine blue
TUNEL	Terminal Deoxynucleotidyl nick end labeling assay

Abstract

***Keywords**: Infertility, Sperm DNA fragmentation, Sperm chromatin dispersion test, DNA fragmentation index (DFI), L-carnitine, Zinc.*

The aim of this work was to evaluate the effect of L-carnitine and zinc medication on the DNA fragmentation of ejaculated sperm using the sperm chromatin dispersion test.

Twenty five patients seeking fertility were recruited from KasrElEini andrology clinic. Their age ranged from 20 to 46 yrs old and infertility period ranging from a 4 months period up to 19 years. The first semen samples were collected, processed for the sperm chromatin dispersion test, then stained with DAPI & read by fluorescent microscope. Fifteen patients continued their follow-up and their second semen samples were collected and processed in the same manner after completing one month treatment with L-carnitine 3gm/day and Zinc gluconate 150 mg/d.

The mean for DNA fragmentation index before treatment was 39.17 %, (± SD 36.99%). The mean for DFI after treatment was 45.5% (± SD 28.47%). The Wilcoxon signed ranks test showed a very high p-value of 0.65 implying that there was no difference in the level of DFI before and after the treatment. By selecting the patients with an initial DFI above 30 we would find that their DFI improved after treatment from a mean of 71.01% (± SD 22.63%) to 48.14% (± SD 26.59%) , yet this trend in the improvement was also statistically insignificant according to the Wilcoxon signed ranks test.
No correlations were found between DFI and either sperm count, abnormal forms, motility and progressive motility.

Introduction

Infertility affects approximately 15 % of couples trying to conceive and a male cause is believed to be a sole or contributing factor in approximately half of these cases. (**Oehninger 2002)**. One of the most important routine investigations of the infertile male is semen analysis. However, In clinical practice , a definitive diagnosis of male fertility often cannot be made with the traditional, manual-visual light microscopic methods of basic semen analysis which consists of measuring seminal volume, pH, sperm concentration, motility, morphology and vitality, simply because this routine semen analysis does not identify defects in sperm chromatin structure. **(Centola and Ginsburg 1996)**

Sperm DNA integrity is essential for the accurate transmission of paternal genetic information, and is important for sperm fertilizing ability. Abnormalities in the male genome characterized by damaged sperm DNA may be indicative for male sub fertility regardless of routine semen parameters (**Lopes et al.,1998; Sakkas and Tomlinson 2000)**

There is a growing concern about transmission of genetic diseases, congenital malformations and developmental abnormalities in ICSI-born progeny because intracytoplasmic sperm injection (ICSI) bypasses the processes of natural selection during sperm-oocyte interaction- still present in conventional IVF. **(Hansen et al.,2002; Schieve et al.,2002; Moll et al.,2003; Orstavik et al.,2003)**

Thus the evaluation of sperm DNA damage appears to be a useful tool for assessing male fertility potential both *in vivo* and *in vitro and a*ccumulated

data allows it to be recommended among routine tests for infertility investigations

A recent study has demonstrated that sperm DNA fragmentation could be reduced with oral antioxidants such as vitamins C, Selenium and zinc. **(Ménézo et al. 2007)**

And since L-Carnitine & Zinc also have a protective role against reactive oxygen species (ROS) by exerting antioxidant properties **(Vicari and Calogero, 2001),** we tested their effect on sperm DNA fragmentation.

Aim of the work

This work aimed at testing the effect of a treatment regimen of L-carnitine and zinc on DNA fragmentation of ejaculated sperm using a novel test called the sperm chromatin dispersion test.

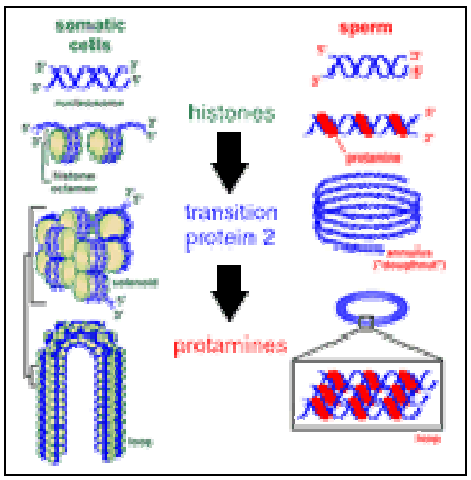
Chapter 1- Sperm chromatin susceptibility to damage in relation to its structure.

The fundamental packaging unit of mammalian sperm chromatin is a toroid containing 50-60 kb of DNA. Individual toroids represent the DNA loop-domains highly condensed by protamines and fixed at the nuclear matrix. Toroids are cross-1inked by disulfide bonds, formed by oxidation of sulphy-dryl groups of cysteine present in the protamines. **(Ward 1993; Bench et al., 1996; Sakkas et al., 1996; Fuentes-Mascorro et al., 2000; Irvine et al. 2000).**

Thus, each chromosome represents a garland of toroids, and all 23 chromosomes are clustered by centromeres into a compact chromocenter positioned well inside the nucleus with telomere ends united into dimers exposed to the nuclear periphery **(Zalensky et al., 1995; Soloveva et al., 2004).** To achieve this uniquely condensed state, sperm DNA must be organized in a specific manner, which differs substantially from that of somatic cells. **(Ward and Coffey 1991)**

This condensed, insoluble and highly organized nature of sperm chromatin acts to protect genetic integrity during transport of the paternal genome through the male and female reproductive tracts. It also ensures that the paternal DNA is delivered in the form that sterically allows the proper fusion of two gametic genomes and enables the developing embryo to correctly express the genetic information **(Ward and Zalensky 1996; DeJonge 2000; Soloveva et al., 2004).**

Mature sperm nuclei adopt a volume 40 times less than that of normal somatic nuclei. This very compact packaging of the primary sperm DNA filament is produced by DNA-protamine complexes, which, contrary to nucleosomal organization in somatic cells provided by histones, approach the physical limits of molecular compaction **(Fuentes-Mascorro et al., 2000; Ward and Coffey 2000)**



Mouse sperm nuclei contain more than 95 % protamines in their nucleoprotein component. Human sperm nuclei, however, contain considerably less protamines (approximately 85 %) . **(Gatewood et al. 1987; Bellve et al., 1988; Bench et al., 1996)**. The retention of 15 % histones, which are less basic than protamines, leads to the formation of a less compact chromatin structure. Human sperm chromatin, therefore, is less regularly compacted and frequently contains DNA strand breaks **(Sakkas et al. 1999; Irvine et al., 2000).**

Moreover, in contrast to the bull, cat, boar and ram, whose spermatozoa contain only one type of protamine (PI), human and mouse spermatozoa contain a second type of protamine (P2), which is deficient in cysteine residues **(Corzet et al., 2002).** Consequently, the disulfide cross-linking responsible for more stable packaging is diminished in human sperm as

compared to species containing PI alone. It is noteworthy that altered PI/P2 ratio and the absence of P2 are associated with human male fertility problems **(Yebra et al., 1993; Balhom et al., 1988; Bench et al. 1998; Carrell et al., 2001; Mengual et al., 2003;; Aoki et al., 2005).**

DNA fragmentation is characterized by both single and double strand breaks (SSBs, DSBs), and is particularly frequent in the ejaculates of subfertile men. In practice, in sperm DNA, contrary to somatic cell DNA, it is nearly impossible to distinguish single strand breaks from DSBs **(Manicardi et al. 1998, Irvine et al., 2000)**.

Oocytes and early embryos have been shown to repair sperm DNA damage. Consequently, the biological effect of abnormal sperm chromatin structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it **(Matsuda and Tobari I 1988; Genesca et al., 1992).**

Abnormal sperm chromatin/DNA structure is thought to arise from four potential sources. Deficiencies in recombination during spermatogenesis, abnormal spermatid maturation (protamination disturbances); abortive apoptosis; and finally oxidative stress **(Sakkas et al., 1999; Agarwal and Said 2003).**

1- Deficiencies in recombination

Meiotic crossing-over is associated with the genetically programmed introduction of DNA double strand breaks (DSBs) by specific nucleases. These DNA DSBs should be ligated until the end of meiosis I. Normally the recombination checkpoint in the meiotic prophase does not allow meiotic division I to proceed until the DNA is fully repaired. A defective checkpoint

may lead to persistent sperm DNA fragmentation in ejaculated spermatozoa. However, direct data for this hypothesis in humans is lacking. **(Page and Weaver 1997; Bannister and Schimenti 2004).**

2- Abnormal spermatid maturation

Stage-specific introduction of transient DNA strand breaks during spermiogenesis has been described in round and elongating spermatids **(Mc Pherson and Longo 1993; Marcon and Boissonneault 2004)**. DNA breaks are necessary for transient relief of torsional stress, favoring casting off the nucleosome histone cores, and aiding their replacement with transitional proteins and protamines during maturation in elongating spermatids. However, if these temporary breaks are not repaired, DNA fragmentation in ejaculated spermatozoa may occur **(Kierszenbaum 2001; Laberge, and Boissonneault 2005)**.

DNase l-hypersensitive sites were found to be localized throughout the maturing spermatid nuclei. DNase I-sensitive sites are formed in pachytene in the chromatin domain containing protamine 1 (PI) and protamine 1 (P2) and the transition protein *Tnp2* genes. Chromatin re-packaging includes a sensitive step necessitating endogenous nuclease activity, which is fulfilled by coordinated loosening of the chromatin by histone hyper-acetylation and introduction of breaks by topoisomerase II (topo II), capable of both creating and ligating breaks .Chromatin packaging around the new protamine cores is completed and DNA integrity restored during epididymal transit **(Erenpreiss et al., 2001; Wykes and Krawetz 2003).**

A huge radiation dosage of 30 Gy or more is necessary to produce detectable levels of X-ray-induced damage in elongated spermatids . This is probably due to the uniquely tight chromatin packaging produced by

protamines **(Sakkas et al., 1995; Fernandez et al. 1998; Manicardi et al., 1998).**

The link between disturbances in chromatin packaging and the consequent occurrence of DNA strand breaks is also confirmed in knock-out mouse models defective in the expression of transition proteins and protamines**(Yu Ye et al.,2000;Clio et al., 2003;Miestrich et al.,2003; Zhao et al., 2004).** Also, human sperm DNA, compared to leukocytes, is enriched-in segments of partially denatured DNA, which can also be considered alkali-sensitive sites. These sites represent potential DNA breaks if induced by any factors **(Muriel et al. 2004)**.

Although protected by proper chromatin packaging the relative spermatid chromatin fragility may be responsible for the presence of higher levels of spontaneous DNA damage in sperm than in somatic cells. In addition, elongating chromatids have a lower repair capacity for strand breaks **(Fernandez et al., 1998).**

The presence of DNase I in acrosome vesicles, from their initial formation in early spermatids to their presence in mature sperm, was shown in rats The ability of spermatozoa to use it and to digest their own DNA, if exposed to stressful conditions, has been suggested **(Stephan et al., 1996 ; Ward and Ward 2004).**

3- Abortive apoptosis

An alternative etiology for the DNA DSBs in the spermatozoa of infertile patients can arise through an abortive apoptotic pathway. Apoptosis