The Effect of Varicocelectomy on Sperm DNA and Certain Enzymes: a Flow Cytometric Assay and Acridine Orange Staining

Thesis
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Dedicated to:

My Late Father

My Mother

My wife

My sons and daughters

Abstract

Sperm DNA damage can be caused by protamine deficiency, defective sperm chromatin packaging, Reactive Oxygen species (ROS), apoptosis, cancer, drugs, chemotherapy and radiation therapy, cigarette smoking, genital tract inflammation, environmental toxins, testicular hyperthermia, Iatrogenic sperm DNA damage, hormonal factors, varicoceles

Key words:
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CONTENTS

LIST OF ABBREVIATION

AAB	**Acidic aniline blue stain**
aCP	**Active caspase**
AGFPI	**α-glucosidase free of prostate isoform**
AMH	**Anti-mullerian hormone**
AO	**Acridine orange**
AOT	**Acridine orange test**
ARTs	**Assisted reproductive techniques**
AZF	**Azoospermia factor**
Bcl-2	**Anti apoptotic genes**
CASA	**Computer assisted semen analysis**
CK	**Creatine phosphokinase**
CK-M	**Creatine phosphokinase M isoform**
CP	**Caspase**
Cp3	**Caspase3**
CREM	**cAMP-responsive element modulator**
DD	**DNA denaturation**
DFI	**DNA fragmentation index**
DNA	**Deoxyribonucleic acid**
DS	**High DNA stainable**
dUTP	**Deoxyuridine triphosphate**
ELISA	**Enzyme-linked immunosorbent assay**
FasL	**Fas liand**
FCM	**Flow cytometer**
FISH	**Fluorescent in situ hybridization**
FSH	**Follicle stimulating hormone**
G6PD	**Glucose 6 phosphate dehydrogenase**
GnRH	**Gonadotrophin releasing hormone**
H1	**Histone 1**
HCG	**Human chorionic gonadotrophin**
HCG	**Human chorionic gonadotrphin**
HPLC	**High performance liquid chromatography**
HSP	**Heat shock protein**
HSP70-2	**Heat shock protein 70-2**
HspA2	**Creatine phosphokinase M isoform**
HZA	**Hemizona assay**
ICE	**Interlukin converting enzyme**
ICSI	**Intracytoplasmic sperm injection**
IUI	**Intrauterine insemination**
IVF	**In vitro fertilization**
LDH	**Lactate dehydrogenase**
LDH-X	**Lactate dehydrogenase X.**

LH	**Leutinizing hormone**
MDA	**Malondialdehyde**
mL	**Milliliter**
NADPH	**Nicotinamide adenine dinucleotide phosphate**
NAG	**Neutral α-glucosidase**
NO	**Nitric oxide**
NOS	**Nitric oxide synthase**
NT	**Nick translation**
8-OHdG	**8-hydroxy-2-deoxyguanoyine**
OS	**Oxidative stress**
P	**Potamines**
PCD	**Programmed cell death**
PCD	**Programmed cell death**
pCP	**Caspase inactive proenzyme**
PMNL	**Polymorphonuclear leukocytes**
ROS	**Reactive oxygen species**
SCD	**Sperm chromatin dispersion**
SCO	**Sertoli cell only syndrome**
SCSA	**Sperm chromatin structure assay**
SOD	**Super oxide dismutase**
ssDNA	**Single stranded DNA**
TAC	**Total antioxidant capacity**
TdT	**Terminal deoxynucleotidyl transferase**
TNFR	**Tumor necrosis factor receptor**
TP	**Transition protein**
TUNEL	**Terminal deoxynucleotidyl transferase-mediate deoxyuridine triphate-nick end labeling**
WBCs	**White blood cells**
WHO	**World health organization**
ZP	**Zona pellucid**
α- Gluc	**α- Glucosidase**

Introduction

Male factor infertility plays a role in approximately 50% of infertile couples (**World Health Organization, 1999**). A number of etiologies have been identified as potential causes of male infertility, which include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction (**Ollero et al., 2001**).

Varicocele, that is, dilatation of the pampiniform venous plexus above and around the testicle occurs in approximately 15% to 20% of the general male population, especially in adolescents. Varicocele occurs in 19% to 41% of men seeking infertility treatment and in around 80% of men with secondary infertility **(Baker et al., 1985; Comhaire et al., 1987).** The increased percentage of varicocele in secondary infertility may be due to patient selection bias **(Gorelick and Goldstein, 1993)**. Recently, a higher frequency of sperm cells with fragmented DNA has been reported in the ejaculate of subjects with varicocele, in comparison with fertile donors **(Saleh et al., 2003; Chen et al., 2004)**.

One area of research that has been studied intensely during the past decade as a cause for male infertility is the integrity of DNA in the nucleus of mature ejaculated spermatozoa (**Sakkas et al., 1999a**). This focus on the genomic integrity of the male gamete has been further intensified by the growing concern about transmission of genetic diseases through ICSI (**Barroso et al., 2000**).

Normally, the sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. It is condensed and insoluble in nature, features that protect genetic integrity and facilitate transport of the paternal genome through the male and female reproductive tracts (**Manicardi et al., 1998**). For a spermatozoon to be fertile, it must be capable of undergoing decondensation at an appropriate time in the fertilization process (**Amann, 1989**). Infertile men manifest various nuclear alterations, including an abnormal chromatin structure, chromosomes with microdeletions, aneuploidies and DNA strand breaks (**Hofman and Hilscher, 1991**).

Accumulating evidence suggests that disturbances in the organization of the genomic material in sperm nuclei are negatively correlated with the fertility potential of spermatozoa, either in vivo or in vitro (**Sun et al., 1997; Spano et al., 2000**). Some recent reports have indicated that when >30% of sperm DNA is damaged, natural pregnancy is not possible (**Evenson et al., 1999, 2002, 2005**). Also, it has been suggested that sperm DNA integrity may be a more objective marker of sperm function as opposed to the standard semen analysis. This was attributed to the fact that conventional semen analysis does not adequately represent the diverse array of biological properties that the spermatozoon, as a highly specialized cell, expresses (**Zini et al., 2001a; Evenson et al., 2002**). In addition, the results of semen analyses can be very subjective and prone to both intra- and inter-observer variability (**Keel and Webster, 1990**).

The epididymis plays a crucial role in the maturation of spermatozoa and their acquisition of progressive motility and fertilizing capacity (**Cooper, 1996; Haidl and Schill, 1997**). Several markers of

epididymal function are available including L-carnitine, glycerylphosphocholine and α-glucosidase (α-gluc) (**Cooper et al., 1988; Alzanaty et al., 2005**). The determination of α-Gluc in semen, particularly of its neutral isoenzyme, has been claimed a rapid, sensitive and non-invasive method to differentiate secretory azoospermia from the excretory type, to localize the site of obstruction in the male genital tract, and to identify partial obstruction at the epididymal level (**Guerin et al., 1986; Cooper et al., 1988; Garcia Diez et al., 1992**). Low levels of α-GLUC in semen may be related to epididymitis (**von der Kammer et al., 1991**) and have been associated with defective sperm maturation in the epididymis (**Haidl et al., 1993**). A high level of α-gluc was correlated with strong binding capacity of the spermatozoa to the human zona pellucida (**Ben Ali et al., 1994**), and with a high probability of success following intrauterine insemination (**Milingos et al., 1996**).

Creatine kinase (creatine phosphokinase, CK) in human sperm is a marker of cytoplasmic retention and thus, diminished sperm maturity (**Huszar and Vigue, 1990, 1993**). Functional evidence for sperm membrane remodelling originates in studies of CK-immunostained sperm-hemizona (halved unfertilized human oocytes) complexes (**Hallak et al., 2001**). Immature sperm with cytoplasmic retention were not able to bind to the zona pellucida, suggesting that the formation of the zona-binding site(s) is part of the membrane remodelling process (**Yesilli et al., 2005**).

Aim of the work

- To measure DNA damage in spermatozoa in infertile men with varicocele before and six months after varicocelectomy by the meaning of two techniques, the first is sperm DNA staining with Propidium Iodide (PI), using flow cytometric technique (FCM) (**Molina et al., 2001**). The second technique involving sperm staining slides with Acridine Orange stain and examined by fluorescent microscopy (**Hoshi et al., 1996**).

- Also to measure two seminal enzymes, total creatinine Kinase (CK) as a marker for spermatozoal maturity (**Jorge et al., 2001**) and neutral α-Glucosidse as a marker of epididymal function (**Mahmoud et al., 1998**).

Human sperm DNA and chromatin structure

The formation of mature spermatozoa is a unique process involving a series of mitoses and meioses, changes in cytoplasmic architecture, replacement of somatic cell-like nuclear histones with transition proteins and the final addition of protamines leading to a highly packaged chromatin (**Poccia, 1986**).

Sperm DNA is organized in a specific manner to keep the chromatin in the nucleus compact and stable (**Sakkas et al., 1999a**). DNA in mammalian sperm is packaged differently than in somatic cells. Sperm DNA occupies nearly the entire nuclear volume, whereas somatic cells DNA only partly fill the nucleus. The DNA in somatic cell nuclei is first packaged into nucleosomes, in which the DNA is wrapped twice around an octamer of histones, every 200 base pairs. The nucleosomes are further coiled into a solenoid (**Ward and Coffey, 1991**). This kind of DNA packaging increases the volume of the chromatin (**Pienta and Coffey, 1984**). Sperm nuclei, however, do not have the volume required for this type of packaging (**Ward and Coffey, 1991**). The sperm's entire haploid genome is organized into DNA loop domains that have an average length of 27 kilobytes. These loops, which can be visualized by using fluorescent *in situ* hybridization, are attached at their bases to a structural element within the sperm nucleus known as the nuclear matrix. The sperm DNA loop domains are approximately half the size of the loop domains in somatic cells (**Jerome et al., 2004**).

The major protein constituents of the chromatin of eukaryotic cell nuclei are histones, which can be divided into five classes, termed H1, H2A, H2B, H3 and H4. The most divergent class of histones is the H1

protein family, which consists of seven subtypes, termed H1.1- H1.5, H1o, and H1t **(Doenecke et al., 1994, 1995)**. During spermiogenesis, histones are gradually displaced by transition proteins, followed by the replacement by protamines **(Kistler et al., 1996; Oko et al., 1996; Brewer et al., 1999)**.

Transition proteins are thought to promote the histone-protamine exchange and to cease transcription in the spermatid elongation phase **(Meistrich et al., 1977; Balhom et al., 1984; Heidaran et al., 1988)**. Although the number of transition proteins varies with the species, the two transition proteins TP1 and TP2, which have been identified in man seem ubiquitous among mammals **(Luerssen et al., 1988; Schlüter et al., 1992).**

Protamines are the most abundant of the nuclear proteins in the sperm nucleus that package the human male genome. There are 2 types of protamines: the P1 type, which is present in all mammalian species studied so far, and the P2 family, composed of the P2, P3, and P4 components, which have been isolated in sperm nuclei of only a few mammalian species, including humans. The components of the P2 family differ only by an aminoterminal extension of the 1-4 residues **(Gusse et al., 1986; Oliva and Dixon, 1991; Bianehi et al., 1992; Querait et al., 1995)**. Protamine P1 is synthesized as a mature protein, whereas protamine P2 is synthesized as a precursor. The protamine P2 content in the nucleus of normal sperm cells is similar to that of protamine P1 (P1/P2 = 1) **(Balborn et al., 1988; De Yebra et al., 1993)**, and the P1/P2 ratio has been taken as evidence of nuclear maturity **(Belokopylova et al., 1993; Colleu et al., 1996)**. **Balhorn (1982)** proposed that protamines are bound to the DNA lengthwise inside the minor groove of the DNA molecule. The protamine-DNA complex fits into the major groove of a neighboring DNA strand so that the DNA strands of the

sperm nucleus are arranged side by side in linear arrays. Covalent disulfide bonds between the protamines stabilize the chromatin. In this manner, all DNA can be tightly packaged in a very small volume (**Bedford and Calvin, 1974; Marushige and Marushige, 1975**). The idea that DNA strands in sperm chromatin are positioned side by side is supported by the observation that DNA in sperm nuclei is not super-coiled (**Ward et al., 1989**).

Inter- and intramolecular disulfide cross-links between the cysteine-rich protamines are responsible for the compaction and stabilization of the sperm nucleus. The arrest of transcription in the spermatid elongation phase is concomitant with the loss of the DNase-I hypersensitive regions, which are normally located in transcriptionally active or potentially active genes **(McPherson and Longo, 1992).** However, major modifications in both nuclear and cytoplasmic structures continue throughout spermiogenesis, assuming transcriptional and translational control mechanisms in elongating spermatids.

Correct histone-to-protamine exchange in haploid spermatids is known to play a vital role in the production of fertile sperm. Only up to 15% of the DNA remains packaged by histones at specific DNA sequences **(Tanphaichitr et al., 1978; Gatewood et al., 1987)** and histones are partially retained in the nuclei of mature spermatids and spermatozoa and occur together with protamines which first appear in nuclei of step 4 spermatids and persist in all sperm nuclei **(LeLannic et al., 1993; Lescoat et al., 1993; Prigent et al., 1996).** The histone-bound DNA sequences are less tightly compacted, and it is thought that these DNA sequences or genes may be involved in fertilization and early embryo development **(Gatewood et al., 1987).**