Comparative Study of Sperm Chromatin Status in the First and Second Ejaculate Using Aniline Blue Test

Thesis

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ABSTRACT

The aim of this study is comparing the Sperm Chromatin condensation status between the first and second ejaculate using Aniline Blue Stain. Also each ejaculate was subjected to semen analysis including the semen volume , sperm count , and sperm motility .The results showed no statistically significant difference in the sperm chromatin condensation stained with Aniline Blue stain between the first and second ejaculates in all patients. Although the volume of the second ejaculate was decreased significantly than the first one, there was no significant difference in semen parameters (sperm concentration , percentage of total sperm motility , and motility grades (a+b)) between first and second ejaculates generally . however some patients showed improvement in semen parameters in the second ejaculate as compared with the first one .

key Words: Aniline Blue Stain - Chromatin condensation - first ejaculate- second ejaculate.

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Contents

List of abbreviations

8-OHdG	8-hydroxy-2-deoxyguanosine
AB	Aniline blue
AI	Acrosomal Index
AO	Acridine orange
ART	Assisted reproduction technology
ASMA	Automated sperm morphology analyzer
CASA	Computer-aided sperm analysis
CMA3	Chromomycin A3
CV	Coefficients of variation
DBD–FISH assay	DNA breakage detection– fluorescent *in situ* hybridization assay
Df	Degree of freedom
DFI	DNA fragmentation index
dUTP	Biotinylated deoxyuridine triphosphate
FISH analysis	Fluorescence in situ hybridization analysis
FP	Forward progression
HPLC	High-performance liquid chromatography
ICSI	Intracytoplasmic sperm injection
IL	Interleukin
IUI	Intrauterine insemination
IVF	*In-vitro* fertilization
IVF-E.T	*In-vitro* fertilization- embryo transfer
LSD test	Least significance difference test
MS	Mean squares
mtDNA	Mitochondrial DNA
NS	Non significant
NT	*In situ* nick translation
OTA syndrome	Oligo-terato-asthenospermic syndrome
PR	Pregnancy rate
ROS	Reactive oxygen species
SCD test	Sperm chromatin dispersion test
SCSA	Sperm chromatin structure assay

SDI	Sperm deformity index
SOD	Superoxide dismutase
SS	Sum of squares
ssDNA	Single stranded DNA
TB	Toluidine blue
TdT	Terminal deoxynucleotidyl transferase
TESE	Testicular sperm extraction
TNPs	Transition proteins
TUNEL assay	Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling assay
TZI	Teratozoospermia index
WHO	World Health Organization

Introduction

Most couples with severe male factor infertility can be treated with intracytoplasmic sperm injection (ICSI). In ICSI, a single spermatozoon is microinjected into the oocyte after the passage through the zona pellucida and the membrane of the oocyte (oolemma).

ICSI using ejaculated sperm can be applied in the presence of oligoasthenozoospermia, in cases of repeated fertilization failure after conventional in-vitro fertilization (IVF), in the presence of a high concentration of antisperm antibodies, in cancer patients in remission where sperm were cryopreserved prior to chemo-and radiotherapy, in patients with retrograde ejaculation and in patients where semen was banked prior to vasectomy. ICSI can also be applied with sperm from the epididymis and testis in case of obstruction of the seminal excretory duct. ICSI can be applied in cases of azoospermia caused by impaired spermatogenesis if sufficient sperms can be retrieved from testicular tissue (Devroey and Van Steirteghem, 2004).

The identification of sound, suitable semen and sperm characteristics enabling prediction of the chances of successful outcome after assisted reproduction technology (ART) is an area of active research. The presence of sperm with DNA fragmentation and chromatin abnormalities in human ejaculates is well documented in particular in men with poor semen quality. Injection of oocyte with spermatozoa with abnormal chromatin will probably result in failure of sperm decondensation and fertilization (Gandini et al., 2004).

Hammadeh et al., (2001) strongly suggests that chromatin condensation constitutes a valuable parameter in the assessment of male fertility, completely independent of conventional semen parameters, and should be recommended to routine laboratory investigations of semen prior to assisted reproduction. They demonstrated that chromatin condensation visualized by aniline blue staining is a good predictor for IVF outcome.

Normally, the sperm chromatin is a highly organized, compact structure consisting of DNA and heterogenous 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 (Agarwal and Said, 2003).

The degree of chromatin condensation can be assessed with the aid of acidic aniline blue staining, which discriminates between lysine-rich histones and arginine- and cystein-rich protamines.

This technique gives a specific positive reaction for lysine and reveals differences in basic nuclear protein composition of ejaculated spermatozoa.

Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain, On the other hand, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low lysine and will not be stained by aniline blue (Hammadeh et al., 2001).

Barash et al., (1995) compared the first and second ejaculation, they have noticed an improvement in ejaculate count and quality in second consecutive ejaculation collected 2 hours after the first one from infertile or subfertile men. During IVF, the fertilization rate, cleavage rate, and the pregnancy rates were all increased when oocytes were exposed to sperm from the second ejaculate compared with oocytes exposed to sperm from the first ejaculate. They suggested that in the group of infertile men with oligoasthenozoospermia, whose partners are scheduled for IVF-E.T., a second ejaculate may exhibits improvement in both semen parameters and reproductive potential.

Aim of the work

It is a comparative study using aniline blue staining, between the first and the second ejaculates collected from infertile men within 4 hours interval, to see if the second ejaculate sperm chromatin condensation can be better than the first one or not. If this will be true, aniline blue test may serves as a selective test for ICSI candidates helping the choice of better quality ejaculate for ICSI.

Chapter 1:

Sperm structure and function

The primary function of the spermatozoon is to provide male pronucleus for the fertilized egg. Creation of a new diploid individual critically requires the contribution of both the male and the female haploid pronuclei. The success of this primary function is dependent upon the outcome of a series of secondary processes, the spermatozoon must conserve its DNA, transport it to the site of fertilization and be able to recognize and fuse the receptive egg (Curry and Watson, 1995).

The spermatozoon has developed a highly specialized morphology with its various structural components elegantly tailored to specific aspects of function. It can be divided into two major parts, the head containing all important DNA and the mechanisms for zona recognition and for sperm-egg fusion, and the flagellum concerned with energy production and the initiation and maintenance of motility. Both these two major regions can be subdivided into a number of cellular compartments each with their own functional correlates (Yanagimachi, 1988).

1- Head

The function of the sperm head is to contain and conserve the cell DNA and to deliver it at the time of fertilization by fusing with the egg.

The sperm head contains a very limited range of structures with which to perform these functions; apart form the nucleus the only major organelles

in the head region are the acrosome and the post acrosomal sheath (Curry and Watson, 1995).

1-1- Acrosome

The acrosome is a membrane-bond vesicle forming a cap-like covering over the anterior part of the nucleus. It is relatively small, covering approximately two-thirds of the nucleus (Curry and Watson, 1995).

The outer acrosomal membrane lies directly beneath the plasma membrane and is continuous at the posterior margins of the cap with the inner acrosomal membrane which overlays the nuclear envelope. The two membranes are broadly parallel enclosing a narrow space filled by the acrosomal matrix.

The matrix consists of a number of different hydrolytic enzymes. The two major and best characterized enzymes are hyaluronidase and acrosin, a trypsin-like proteinase present in an inactive zymogen form as proacrosin. Spermatozoa also contain a second zymogen called sperminogen (Siegel et al.; 1987).

Other enzymes present include acid phosphatase, phospholipases, N-acetylglucosaminidase and collaginase. The matrix is also rich in carbohydrates (Holt, 1979).

It has been generally accepted that the hydrolytic enzymes released serve to assist the spermatozoon in its passage through the extensive

cumulus mass and in its penetration of the formidable zona pellucida (Harrison, 1982).

A unique feature of the spermatozoa, is the presence of a stable region situated at the posterior border of the acrosomal cap, termed the equatorial segment, which is crescent shaped and extends at its widest point to approximately one-quarter of the total acrosome length. It can be identified in cross-section by an abrupt narrowing of the acrosomal space to 40-45 nm in width compared to 70-80 nm in the anterior region. The enzyme-rich matrix present in the anterior acrosome is absent from the equatorial region (Virtanen et al; 1984).

1-2- Perinuclear material

Directly beneath the acrosome and separating it form the nucleus is a thin layer of perinuclear material. The perinuclear material forms a continuous layer coating the nucleus and appears to act as a cement-like substance between the acrosome and the nucleus (Olson et al; 1976).

Posteriorly to the acrosome, this material forms the post-acrosomal sheath. Longitudinal sections through the sperm head show the post-acrosomal sheath to be composed of two distinct regions separated by a shallow groove in the plasma membrane:

a. The anterior region of the sheath consists of a homogenous layer of electron dense material lying parallel to the plasma membrane, and separated form it by approximately 20 nm. Along this layer are a series of rounded

projections extending towards the plasma membrane with a separation of 12 nm between them (Pedersen. 1972b).

b. The posterior region of the sheath is composed of granular material with no particular features seen in longitudinal section but with a series of obliquely oriented cordlike structures visible in freeze-etches preparations (Koehler, 1972).

1-3- Nucleus

The nuclear status of sperm cells is determined by two major events that occur during spermiogenesis: acquisition of the final nuclear shape and the replacement of somatic-type histones by protamines (sperm-specific basic nuclear proteins) leading to highly packaged chromatin. Sperm DNA is organized in a specific manner to keep the chromatin in the nucleus compact and stable. It is packed into a tight, almost crystalline status that is at least six times more condensed than mitotic chromosomes. It occupies nearly the entire nucleus volume, whereas somatic cell DNA only partly fills the nucleus (Fuentes-Mascorro et al; 2000).

This DNA organization not only permits the very tightly packaged genetic information to be transferred to the egg, but also ensures that the DNA is delivered in a physical and chemical form that allows the developing embryo to access the genetic information (Poccia, 1986).

Sperm nuclei do not have the volume required for the type of packaging present in somatic cells, because packing the DNA in even a

single, closely packed nucleosome would require 9.9 μm^3, which is more than twice the volume of an average sperm nucleus. Thus, a completely different type of DNA packaging must be present in mammalian sperm nuclei (Ward and Coffey, 1991).

Organization of chromatin for packaging in the spermatozoon takes place at four different levels: chromosomal anchoring, which refers to the attachment of the DNA to the nuclear annulus; formation of DNA loop domains as the DNA attaches to the newly added nuclear matrix; replacement of histones by protamines, which condense the DNA into compact doughnuts; and chromosomal positioning (Ward and Coffey, 1991).

The histones are first displaced by transition proteins (TNPs), which are removed from the condensing chromatin at later stages and replaced by protamines. It is of interest to note that the condensation of chromatin begins in the posterior pole and proceeds apically, which is a unique feature in humans that is not present in other mammalian species (Dadoune, 1995).

Sperm epididymal maturation implies a final stage of chromatin organization involving protamine cross-linking by disulfide bond formation—a step that is supported by the fact that protamines contain a significant number of cysteine residues that participate in sperm chromatin compaction by forming multiple inter- and intraprotamine disulfide cross-links. All of these interactions make mammalian DNA the most condensed eukaryotic DNA (Ward et al; 1994).