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Journal of Bioscience and Applied Research
www.jbaar.org

Detection of DNA damage by SCD and Rate of Apoptosis DNA by Gel Electrophoresis among infertile males

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DOI : 10.21608/jbaar.2022.256197

Abstract:

Background: DNA damage as Fragmentation has adverse effects on fertilization and embryo development, so it is one of the main causes of a male factor for infertility. Several techniques have been mentioned to elevation this damage. In our study, we determine DNA damage in human spermatozoa by sperm chromatin dispersion (SCD) method and Apoptosis of DNA in human spermatozoa by Optical density in gel electrophoresis in male infertility. **Objects and Methods:** Semen samples were collected from 100 men and were analyzed by standard light microscopic according to the World Organization (5th edition) for diagnostic fertility. Furthermore, Sperm DNA damage was determined by using Halosperm Kit, then assessment apoptosis by optical density in Gel Electrophoresis. **Results**: The mean value of DNA by SCD method in infertile males increased with a value of 47.95±10.96 % when compared with the control value of 21.2 ±2.64 % with ($p< 0.00001$). On the other hand, the mean value of DNA by measurement of Optical density in Gel Electrophoresis in infertile males decreased with a value of 120.27±18.73 when compare with the control value of 144.4±45 with ($p =0.833$). **Conclusion:** The assessment of sperm DNA damage by SCD method and other methods for detection of DNA apoptosis by gel electrophoresis addition to routine semen analysis play important role in the diagnosis and management of male infertility.

Key Words: Apoptosis, DNA Fragmentation, Electrophoresis, Spermatozoa, SCD

Introduction

Infertility parents growing health and social problem, which affects about 15 % of couples, furthermore, the male factor infertility account for 50 % of infertile couples (Fleming Set al,1995). The causes of male factor infertility are varicocele, infection disease, infection of male sex glands, gene mutation, radiation, aneuploidy, lifestyle, etc. Normal sperm genetic material is required for successful fertilization, and embryo development and any damage to sperm DNA is acritical in Assisted Reproductive Techniques (ART), increasing rates of miscarriage.

Sperm DNA contributes half the offspring's genomic material and is a critical factor in male infertile men. These abnormalities in chromatin packaging and nuclear DNA damage are a strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters (Lopes

Received: June 15, 2022. Accepted : August 2, 2022. Published: August 25, 2022

et al., 1998; Irvine et al., 2000). Three hypotheses have been postulated to explain the source of DNA damage in sperm. First, it is believed that DNA damage is caused by improper packaging and ligation during sperm maturation (McPherson and Longo, 1992, 1993a, b; Gorczyca et al., 1993a, b; Sailer et al., 1995). Secondly, oxidative stress causes DNA damage (Agarwal and Saleh, 2002; Saleh et al., 2002a, b; Agarwal et al., 2003), and the increased levels of specific forms of oxidative damage such as 8-hydroxydeoxyguanosine in sperm DNA support such a theory (Lopes et al., 1998; Aitken, 1999; Shen and Ong, 2000). Thirdly, observed DNA fragmentation is caused by apoptosis (Sakkas et al., 1999, 2002).

DNA damage in sperms was significantly higher in infertile men who were abnormal in shape and decreased in count and motility and vitality when compared with control.

The routine analysis of male infertility includes a physical examination, seminal volume, PH, concentration, motility, Vitality, and morphology According to the World Health Organization (WHO) criteria 5th edition. Not sufficient to detect the cause of male infertility, so men with normal semen parameters may still be infertile, one of the reasons for unexplained infertile may be DNA fragmentation.

Apoptosis in the human spermatozoa is a result of DNA strand breaks induced by a cascade of regulatory mechanisms with infertility (Host et al., 2000). The degradation of DNA into fragments of approximately 185 base pairs and its multiples in size is one of the best characterized biochemical features of apoptotic cell death and is used as the basis for the commonly used labeling techniques for detecting apoptotic cells (Nagata et al., 2000).

On the molecular level, apoptosis is organized DNA damage. DNA double-strand cleavage occurs in the linker region between nucleosomes and produces DNA fragments that are multiples of 180 base pairs (Wyllie 1995). These fragments can readily be demonstrated by agarose gel electrophoresis as DNA ladders and this method has been widely used to detect apoptosis.

Several techniques have been used to detect sperm DNA fragmentation, such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), sperm chromatin structure assay (SCSA), a single-cell DNA gel electrophoresis –based method (comet), and sperm chromatin dispersion (SCD).

The SCD assays were performed with a Halosperm kit, Halosperm is an improved, economical, and simple that measures the susceptibility of sperm DNA to acid denaturation. The sperm with fragmented DNA fails to produce halos of dispersed DNA, which are characteristics of sperm with intact DNA.

Another method for estimation of apoptosis is agarose gel electrophoresis, this method doesn't require expensive and environmental hazards reagent

Subjects and method:

Semen samples were collected from 20 healthy males and 80 infertile males by masturbation after 3 to 5 days of sexual abstinence, we evaluated basic parameters for Semen analysis concentration, motility, vitality, morphology. then assessment DNA fragmentation by SCD technique with Halosperm Kit, following determine the rate of apoptosis by electrophoresis.

Analysis of Concentration, Motility, Vitality, and Morphology of Human Spermatozoa

After liquefaction of semen at 37°for 30 min the samples are examined for concentration, motility was evaluated in a total of 200 sperm by using, determine the percentage of viable sperm by [Olympus Co., BH-2 (BHTU), Japan] with an objective optical magnification (40 X). 10 µl of semen was mixed with 10 µl of 0.5 % Eosin stain (Sigma –Aldrich) on glass microscopic slide under light microscopy, live sperm were visible as white where dead sperm stained red, the assessment of morphology was stained by diff quick stain kit and classified using Kruger's stain criteria.

Detection of DNA fragmentation in Human Spermatozoa by SCD assay with a Halosperm Kit

Semen samples were diluted in an appropriate human sperm extender or PBS to a maximum of 20 million sperm per milliliter. The provided gel–filled Eppendorf tubes were heated by microwave for 5 minutes to melt the agarose, and then they were placed in an incubator at 37° for temperature equilibration. Then 50 µl of a diluted semen sample to the Eppendorf tube and mixed gently with a micropipette, 8 µl of the mixture was placed onto a super coated slide and covered with a 22 x 22 mm coverslip. Slides must be held in a horizontal position. slides were kept for 5 min at 4°in the refrigerator to create a micro gel with an implanted sperm and solidify the agarose coverslips were carefully removed by sliding it off gently then immersed in solution 1 (DA) Denaturant Agent for 7 min, after that, slides were transferred to the tray with a lysing solution incubated for 20 min. Rinsing with distilled water for 5 min was followed by dehydration for 2 min in increasing the concentration of Ethanol (70 %, 100 %). After drying, slides were stained with Eosin staining (Solution A) incubated for 7 min then apply second stain thiazine staining (Solution B) incubated for 7 min, rinsed under tap water, and allowed to dry at room temperature. The slide was examined under a bright field and 200 sperm were scored.

DNA Extraction and apoptosis detection:

Semen samples 700 µl in Eppendorf, centrifugation at 900 r.p.m for 2 min, the pellet was lysed with 600 microliters lysing buffer (50 mM NaCl, 1mM Na2EDTA, 0.5 % SDS. pH 8.3) and gently shacked, the mixture was incubated overnight at 37°, 200 microliters of saturated NaCl was added to the samples, shacked gently, and centrifuged at 12,000 rpm for 10 min. the supernatant was transferred to a new Eppendorf tube and then DNA precipitated by 600 microliters of cold isopropanol. The mix was inverted several times till fine fibers appear. Then centrifuged for 5 min at 12,000 r.p.m. The supernatant is removed, and the pellets were washed with 500 microliters of 70 % Ethyl alcohol and centrifuged at 12,000rpm for 5 min. After centrifugation, the alcohol was decanted or tipped out and the tubes blotted on Whitman paper or clean tissue, till the pellets appeared to be dry. The pellets were resuspended in a 50-microliter appropriate volume of TE buffer (10 mM tris, 1 mM EDTA, pH 8). They're suspended DNA was incubated for 30 – 60 min loading mix (Ranse + loading buffer) and then loaded directly into the gel- wells.

Results:

Data were coded and entered using the statistical package of social science (IMB SPSS) version 20 (Chicago, USA) for statistical analysis Clinical data were compared between the two groups by one-way ANOVA, mean ± SD, and student's t-test. A P-value of less than $p<0.05\%$ was considered statistically significant.

The samples were divided into two groups, group one with Normal as the control and group two with infertile males, the mean and distribution of basic sperm parameters are shown in (table 1) The average concentration of semen samples for Normal (n=20) was 40.02±17.87 mill/ml; an average of movement A (Progressive Motility) Was 21.00 ±4.89 while an average of movement B (Non-Progressive Motility) Was 40.5 ±6.10.

The average concentration of semen samples for infertile males (n = 80) was 20.7±18.94 mill/ml; the average of movement A (Progressive Motility) Was 3.52 ±4.95 % while the average of movement B (Non-Progressive Motility) Was 21.25 ±17.09 %.

Then evaluate DNA damage using two different techniques. We used Haosperm Kit for detection of sperm DNA fragmentation by SCD assay and used Eosin staining (Solution A) and second stain thiazine staining (Solution B), the sperm of unfragmented DNA (has large to medium halo around the head), while the

sperm fragmented DNA (has small, without halo or degraded head) fig 1, 2

We counted at least 200 and then took the percentage of Sperm DNA fragmentation Index (SDFI %) which was divided into 3 Categories: Low SDFI (< 25%), Moderate SDFI (25–50%), High SDFI (> 50%). The Standard division of the Infertility group (80 patients) of SDFI (47.95± 10.96) while The Standard division of the Fertile group (20 Control) of SDFI (21.2± 2.64). table 1

To determine DNA Fragmentation by Gel Electrophoresis could be easily visualized on agarose gel figure 3, The intensity of apoptotic bands could be measured by software Gel program as maximum optical density values.

The optical density of infertile males shows High DNA damage in the 3,4,5,6 lanes where migration for DNA content appears at 500 bp, while in fertile males no migration of intact DNA and Low DNA damage in lane 1,2,7 at 1200 bp.

The Standard division in infertile males decreased with a value of 120.27±18.73 when compared with the control(fertile) value of 144.4±4.45.

	Infertility group (80 patients) Average/Std	Fertile group (20 patients) Average/Std	t-test	Significant P < 0.05
Concentration 1 million sperm	20.7± 18.94	40.02±17.87	-2.887	P=0.0056
Progressive (PR)	3.52± 4.95	21.00 ± 4.89	-9.916	P<0.00001
Nonprogressive (NP)	21.25± 17.09	40.5± 6.10	-3.445	P= 0.00057
Sperm DNA-fragmentation index (SDFI %)	47.95± 10.96	21.2± 2.64	7.525	P<0.00001
Optical density (OD)	120.27±18.73	144.4±4.45	0.671	P=0.833

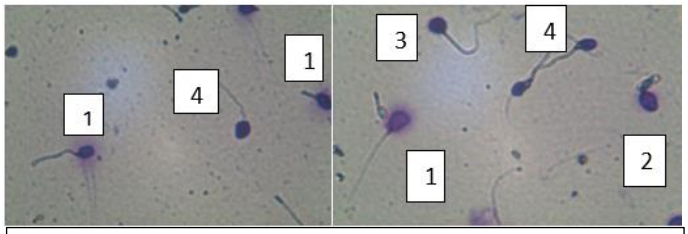

Figure 2
Sperm DNA fragmentation by Halosperm test (image taken from the light microscope)
Sperm Without fragmentation: sperm with big halo (1), medium halo (2)
Sperm with fragmentation: sperm with small halo (3), without halo (4)

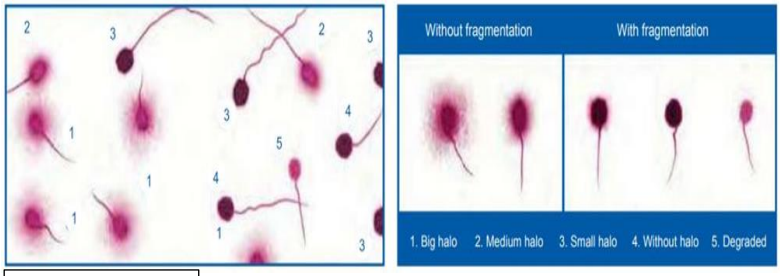

Figure 1

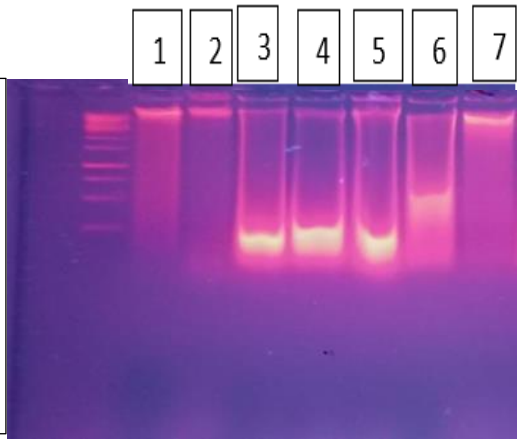

Figure 3
Electrophoretic pattern of DNA damage:
lane 3,4,5 ,6 High DNA damage
lane 1, 2 ,7 Low DNA damage .

Discussion:

DNA Fragmentation has adverse effects on fertility and embryo development and effects on all living Cs, it has been studied using different detection techniques (HorioT et al. ,2007). In the Field of ART, Sperm DNA has been defined as a parameter of semen quality.

Several techniques have been used to detect DNA damage (TUNEL, COMET, SCD, and SCSA), The ideal, must be Simple, not expensive, and not complex. assessment of SCD test with men has abnormal semen parameters are characterized by higher levels of DNA strand breaks, which can indicate apoptosis (Irvine et al., 2000).

The SCD test is based on a controlled DNA denaturation process to remove the proteins contained in each spermatozoa. In this way, normal spermatozoa create halos formed by loops of DNA at the head of the sperm (No damage). Furthermore, detection of the rate of apoptosis, and measuring of optical density of intact DNA increasing compared with infertile were decreasing in optical density indicator for DNA damage.

The detection rate of Apoptosis by optical density in electrophoresis is another tool for the detection of DNA fragmentation in human semen alone or combined with SCD assay.

So, other research should examine the usefulness of this technique in clinical applications such as IVF and compare it with other tests (TUNEL, Comet, SCSA)

Conclusion:

Sperm DNA Fragmentation test plays important role in ART where physicians and researchers make efforts to obtain healthy sperm with nuclear DNA integrity to minimize effects on offspring and decrease miscarriage, so sperm DNA fragmentation has been defined as a parameter of semen quality next to routine laboratory evaluation of Concentration, Motility, Vitality, and morphology.

In addition, Apoptotic DNA in infertile which assessment by Gel Electrophoresis may help to recognize High Sperm DNA damage which assessment by SCD assays, especially with poor sperm indices.

So, the method of Apoptotic DNA Fragmentation by gel electrophoresis which is simple and not complex or expensive can help in the detection of DNA Fragmentation by SCD assay.

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