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RESEARCH ARTICLE

INTRODUCTION OF A ROUTINE SPERM DNA FRAGMENTATION TEST AS A SIGNIFICANT HELP IN EVALUATING MALE FERTILITY POTENTIAL

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ABSTRACT

Male fertility evaluation involves a semen analysis which normal values can also be found in infertile men. Sperm DNA fragmentation is highly associated with male infertility and presents obstacle in selecting healthy sperm for successful *in vitro* fertilization or intracytoplasmic sperm injection. The goal of this study was to examine how testing sperm for DNA fragmentation can help in diagnosis of patients who have normal semen parameters but problems with fertility. A 21 male patients in age from 24 to 55were examined by established standard semen parameters and using Halosperm® test kit to determine sperm DNA fragmentation (SDF). For every patient, the routine semen analysis included measurements of the amount of semen (volume), sperm count (concentration), sperm movement (motility) and sperm shape (morphology). The threshold value of DNA fragmentation index (DFI) in Halosperm® test was at 30%.Our results showed that in 50% of cases where patients had normal semen values, they had high sperm DNA fragmentation index, and in some cases even above 95%. These findings are suggesting that due to limitations of standard semen analysis an additional tests, such as test for sperm DNA integrity, can prevent selection of sperm with damaged DNA and increase pregnancy success rate. Testing for sperm DNA fragmentation could help with fertility diagnosis of male patients and could be used as a part of routine analysis of semen. It can give answers to male infertility in cases where standard semen analysis cannot.

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INTRODUCTION

Infertility is diagnosed in about 10 – 15% couples of reproductive age. In these cases couples are unable to become pregnant after one year of regular unprotected intercourse when women is younger than 35, and 6 months when women is older than 35. Male factor is responsible in almost 50% of cases with determined infertility, and 15% of these cases include men who have normal semen parameters (Evgeni, 2014). Conventional methods of semen analysis involve determining mobility, concentration and sperm morphology and they are a good indicator for evaluating male infertility, but insufficient to give reliable information about male fertility potential. They are not able to completely define male infertility status. It is necessary to understand male infertility at the molecular level (Cho, 2018). As a predictive factor for male infertility DNA fragmentation index can be useful. Numerous studies show correlation between sperm DNA damage and reduced fertility, improper embryo development, genetic malformations and the rate of miscarriages (López *et al*., 2013).

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Natural conception is difficult in case of sperm DNA damage and almost impossible if SDF is greater than 30%. The SDF testing is also relevant for process of counselling of couples struggling with infertility of unknown cause and helping them regarding the choice of method of becoming pregnant (Kim, 2018). DNA strand breaks that can be present in the male germ cells are critical for the quality of embryo, because of their inability to repair their own DNA (Fraczek, 2017). Sperm cells do not have ability to repair their DNA and this makes them more sensitive to these type of damages. Damages can be endogenous and exogenous. Among endogenous factors are abortive apoptosis and defective maturation, which can cause damages in testicular sperms, and oxidative stress (Cho, 2018; Fraczek *et al*., 2017). Mistakes in apoptosis during spermatogenesis and problems with change of histones with protamines are responsible for breaks in DNA chain. Apoptosis of testicular sperms happens during life and normally regulates over proliferation and selectively aborts abnormal sperms. Abortive apoptosis is unfinished program of cell death of precursor gamete (Grunewald, 2017). Sperm cells are prone to reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated damages because of

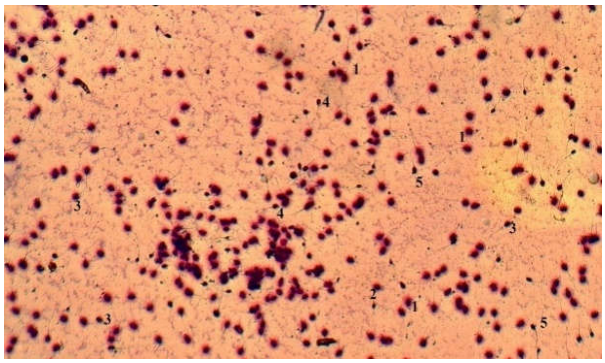

Figure 1.Sperm cells after Halosperm test (image taken under bright field microscope). (1) Sperm cells without DNA

abundant polyunsaturated fatty acids in cell membrane that are necessary for fertilization process. With peroxidation of the cell membrane fluidity and mobility are altered. Besides that, ROS and RNS are compromising mitochondrial functioning, and modify DNA, RNA and proteins (Lenzi *et al*., 2000). Some studies show that there is greater percentage of DNA fragmentation in epididymal and ejaculatory sperm, which indicates that exogenous factors are greater contributors to male infertility.

They involve toxic environmental conditions, smoking, alcohol, genital temperature, variccocela and other (Cho, 2018; Fraczek *et al*., 2017). Urogenital tract infections are another risk factor for changes within the sperm DNA. These may be caused due to infections with microbes such as *Chlamydia trachomatis* or mycoplasma, since increased sperm DNA fragmentation levels were observed in infected males when compared to fertile control group (Gallegos *et al*., 2008). Early and comprehensive diagnostics of various sperm defects, especially DNA fragmentation detection, can open a novel possibilities in course of male infertility treatment and increase success rate for pregnancy achievement.

MATERIALS AND METHODS

Study design: All of the procedures were done in gynecological polyclinic ‘’Korak do života’’ in Tuzla, Bosnia and Herzegovina. 21 patients who visited the clinic in period of 2016 to 2017 were examined. Patient’s age was from 24 to 55 years old. For examination of DNA fragmentation Halosperm® test kit (Halotech) was implied. Results of the Halosperm® test were compared to the spermiogram results of the examined patients.

Standard semen analysis: Analysis of standard semen parameters (volume, concentration and mobility) was done according to WHO (World’s Health Organization) guidelines. Samples are collected by masturbation with 3-5 days of abstinence. After liquefaction (30 min, 37°C) samples were examined on Makler’s chamber with light microscope on 200X magnification. The volume values from 1.5 to 6 mL of the sperm sample were considered as normal. Concentrations values beyond 15% were taken as normal, whereas patients with concentration values below 15%were diagnosed as oligozoospermic. Sperm motility was evaluated based on its classification to progressive (a) motility, (b) slow motility, (c) motile *in situ* and (d) immotile. According to the sum (a) + (b), a total percentage was calculated. In case of value below 40%, an asthenozoospermia was diagnosed.

Sperm DNA fragmentation: The Halosperm® test kit was used for DNA fragmentation assay in response to SCD (Sperm chromatin dispersion) test that is based on denaturation of DNA molecule and protein separation. By acid treatment of sperm cells their membrane becomes semi permeable which allows exit of proteins. Sperm cells with normal DNA will have halo width, while DNA loops will exit on acrosome. Sperm cells with highly degraded DNA will no exhibit the halo effect. The procedure involves ACS (Agarose cell support) tubes which are incubated in water from 95°C to 100°C for 5 minutes. Solution 1 and solution 2 should be on room temperature (22°C) during entire procedure and the sperm concentration should be 20 million of spermatozoids per milliliter. 50µlofthe sperm should be mixed with a pipette, with 100µl of agar and kept at 37°C.

Thereafter, a drop of 8µl suspension must be applied in center of the sample well and covered with a cover glass. Slide has to be in horizontal position. Put slide for 5 min in fridge. Take out the slide and do the entire procedure on room temperature. Put slide horizontally on high position of petri dish, add solution 1 and cover with cover glass. Incubate 7 minutes. Put slide horizontally, add solution 2 and incubate for the next 20 minutes. Wash the slide for 5 minutes with distilled water, 2 minutes with 70% ethanol and 2 minutes with 100% ethanol. Add solution 3 and incubate 7 minutes. Add solution 4 and incubate 7 minutes. Inspect the slide under the microscope for halo effect. Percentage of sperm DNA fragmentation (SDF) was evaluated based on the sperm cell observation as described in Figure 1 and calculated as follows: SDF (%) = ((Fragmented + Degraded)/ Total cell counted) x 100.

RESULTS AND DISCUSSION

Halosperm test as a good predictor of disturbed sperm DNA integrity: Recent studies revealed that some abnormalities of sperm cells cannot be determined by conventional semen analysis techniques, since the sperm cells appear normal even if the DNA in the sperm head is fragmented, and it cannot be detected by the optical microscopy. The Halosperm test solves this problem and enables clear differentiation between healthy sperm and sperm with DNA damage. During our study, we were able to identify “big halo” around the sperm head if the normal DNA was present, and absence of halo if DNA within sperm head was fragmented (Figure 2A, 2B and 2C). Since all sperm samples were negative for microbiological analysis, a microbial infection as potential cause for DNA fragmentation can be exclude in this study.

Patient’s diagnosis associated with different levels of SDF: The examined patients were divided into two groups: patients with low DNA sperm fragmentation (LSDF) and patients with high sperm DNA fragmentation (HSDF). Limit value for SDF was 30%. The results showed that 57.14% of patients were associated with high SDF rate. Interestingly, in 50% patients with high SDF the diagnosis was *normozoospermia*. A lower values of sperm concentration were present in 16.6% of cases, in 16.6% lower values of sperm motility and in 16.6% lower values of both, concentration and motility (Figure 3).

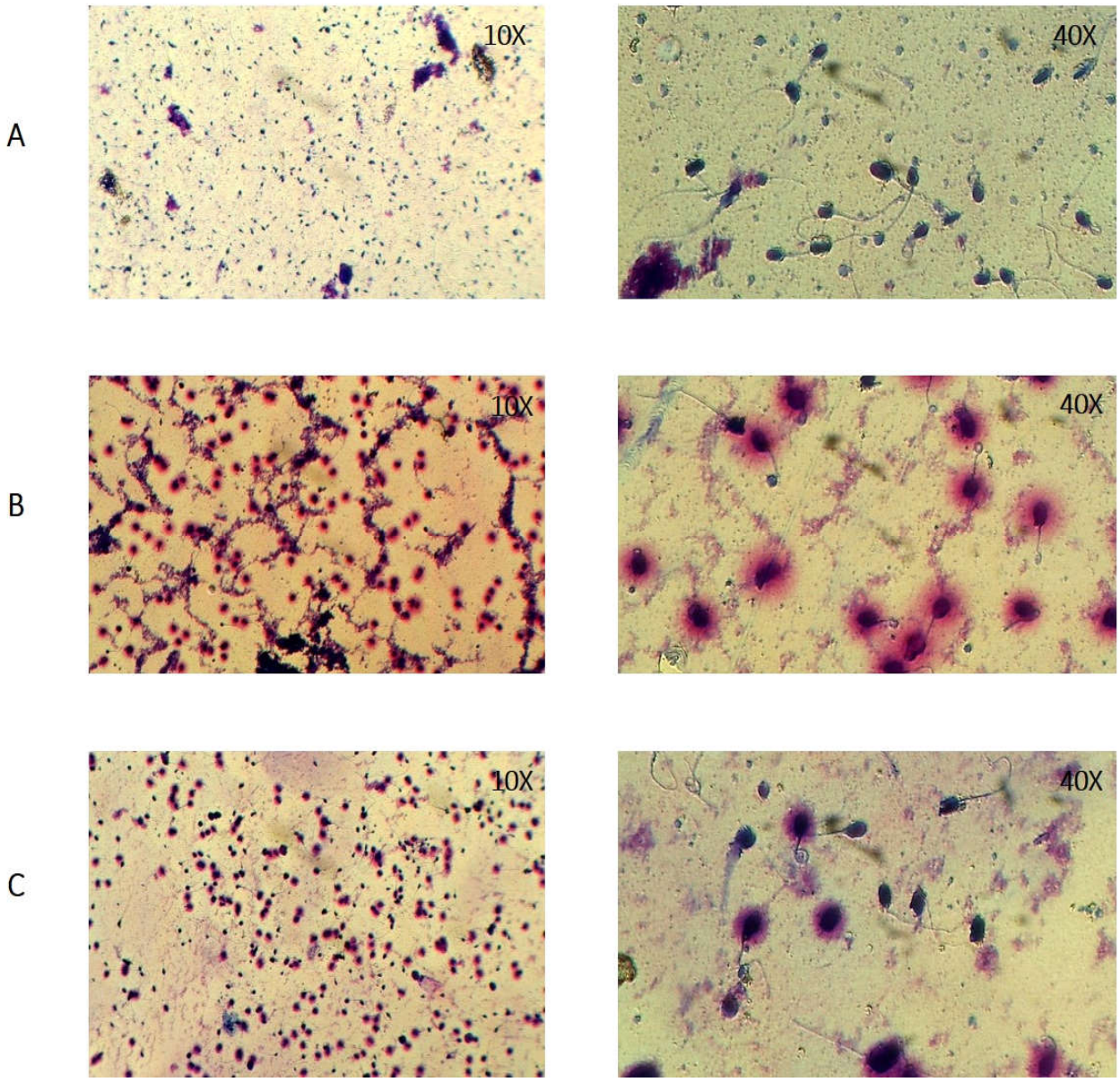

Figure 2.A: Halosperm test results with SDF value of 98%, showing sperm cells without "halo". B: Halosperm test results with SDF value of 13.37%, where "big halo" is noticeable around sperm head; C: Halosperm test results with SDF value of 57.88%. Results illustrate SDF percentage in sperm from three different patients, in 10X and 40X microscopic size magnification.

Table 1: SDF values in comparison with patients diagnosis

LSDF	DIAGNOSIS	HSDF	DIAGNOSIS
22%	NORMOZOOSPERMIA	39.6%	ASTHENOZOOSPERMIA, TERATOZOOSPERMIA
19.15%	ASTHENOZOOSPERMIA	39.06%	OLIGOZOOSPERMIA
16.18%	NORMOZOOSPERMIA	39.06%	NORMOZOOSPERMIA, ASTHENOZOOSPERMIA
27.93%	NORMOZOOSPERMIA, TERATOZOOSPERMIA	88.78%	NORMOZOOSPERMIA, TERATOZOOSPERMIA
24.59%	ASTHENOZOOSPERMIA	30.11%	NORMOZOOSPERMIA
11.24	NORMOZOOSPERMIA	95.54%	OLIGOASTHENOZOOSPERMIA
13.37%	NORMOZOOSPERMIA	89.02%	NORMOZOOSPERMIA
27.16%	OLIGOZOOSPERMIA, TERATOZOOSPERMIA	97.14%	NORMOZOOSPERMIA
14.97%	NORMOZOOSPERMIA	48.42%	NORMOZOOSPERMIA, TERATOZOOSPERMIA
		65.71%	OLIGOZOOSPERMIA
		65.71%	OLIGOASTHENOZOOSPERMIA
		57.88%	ASTHENOZOOSPERMIA

Patients diagnosed with reduced sperm motility (*asthenozospermia*) together with low spermatozoon count (*oligozoospermia*) also had HSDF rate of 95.54%. Besides spermatozoon count and motility, the results clearly showed that HSDF also negatively affects morphology of spermatozoa (*teratozoospermia*), which was diagnosed in combination with *asthenozoospermia* or with *normozoospermia*. Diagnosed *normozoospermia* with *teratozoospermia* was observable in only one patient with LSDF (Table 1). There are still no concrete and exact values in sperm concentration, motility or morphology above which pregnancy is certain or below which it will be impossible, except in case of absolute azoospermia,

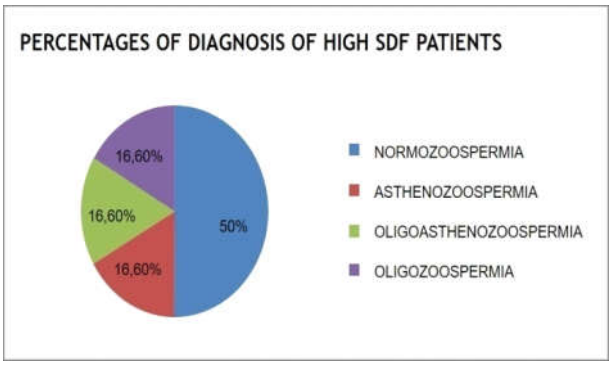

Figure 3. Percentages of diagnosis among patients with high SDF 50% of them was diagnosed as normozoospermic

total *athenozoospermia*, total *necrozoospermia* and total *teratozoospermia* (Natarajan, 2017; Lin, 2008).

Conclusion

Based on our results we could see that sometimes standard semen analysis does not give enough information to exactly diagnose male fertility. In 50% cases where patients had high SDF (even extreme cases with over 95% SDF) standard analysis showed normal semen parameters (Table 1). Our findings confirm some previous studies where significant percentage of men with normal conventional semen analysis profiles still suffered from infertility, which might be associated with increased rate of SDF (El-Sakk, 2017). This implies that Halosperm test can indicate reason for infertility in cases where standard analysis could not. Great number of research shows correlation between sperm DNA fragmentation and infertility in men. It affects the rate of miscarriages and success rate of medically assisted reproduction (MAR). Including the test for sperm DNA fragmentation into routine analysis of sperm would help to improve outcomes of MAR. Some studies proved that by selecting a non-fragmented sperm the chances for successful outcome, as well as the embryo quality, are increasing (López, 2013). DNA integrity represents individual parameter of fertility and it is complement to the standard semen analysis, by which more precise possibility of diagnosis in male patients is achieved. The test is easy, available and profitable, which makes it possible to be included in routine semen analysis (Tandara *et al*., 2014). Used reference value for SDF nowadays is 30%, and in case of lower SDF values, the quality of an embryo and pregnancy rate become noticeably lower. Nevertheless, further analysis is necessary in order to establish exact reference values for this test (Fortunato *et al*., 2013). Semen analysis as conventional tool for male infertility evaluation was first introduced in the last century and is based on The WHO Laboratory Manual for the Examination and Processing of Human Semen, with the first attempt to standardize semen variables (WHO, 1980; WHO, 2010). Selection of spermatozoids with minimal damage in MAR techniques can be achieved by testing the sperm for DNA fragmentation. This would improve the outcome of the procedure. In cases where standard semen analysis fails to give answers to male infertility, the Halosperm test could give. DNA is the most important component of spermatozoa and disruption of its integrity can be used as indicator of male infertility. However we still do not know what is the minimum number of non-fragmented spermatozoa for successful *in vivo* conception (Natarajan, 2017). Due to establishment of exact referent values for this test, it would be desirable to include it in the routine semen analysis, which would increase the rate of positive pregnancy outcomes, also with great potential to reduce incidence of idiopathic infertility diagnosis for some couples as a results of limitations of available testing (Lewis, 2015).

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