



## Biochemical, Molecular and Genetic Studies on Human Male Fertility

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### Abstract

The present study was carried out at Elnada fertility centre (Almaadi, Cairo, Egypt) and in cooperation with the molecular genetics laboratory Faculty of Agriculture Ain Shams University, Shoubra El-Kheima, Egypt during the period from 2015 to 2018.

determining the nucleotide sequences responsible for human male fertility using specific PCR technique. detection of Y chromosome microdeletions in AZFa, AZFb regions performed by evaluation of fresh semen characteristics for sperm DNA fragmentation test and staining method. No correlation observed between sperm concentration, motility, vitality and tail midpiece defects. Negative correlation between sperm DNA fragmentation and acrosome defects and therefore a negative correlation with abnormal forms of sperm. 15 men were screened for submicroscopic Y-chromosome deletions. By using specific PCR technique Y chromosome microdeletion analysis revealed no microdeletion in AZFa, AZFb regions. PCR amplification produced an expected size for AZFa prox-2, SY 127 and AZFa dist-1 loci. So that, the cause of infertility is largely unknown and associated Y chromosome microdeletions cannot be ruled out.

**Keywords:** infertility, male infertility, DNA Fragmentation, SRY, microdeletions, AZF region, Y chromosome.

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### 1. Introduction

Infertility is a real extraordinary problem worldwide. Infertility is defined by the world health organization (WHO) as a failure of conception for a period of two years without reason [1]. Approximately 15 % of couples are infertile, and among these couples, male factor infertility accounts for about 50 % of causes [2]. Male infertility is a multifactorial syndrome involving a wide variety of disorders. In more than half of infertile men, the reason of their infertility is unknown (idiopathic) and could be congenital or acquired. Infertility in men can be diagnosed initially by semen analysis. Semen analysis of infertile men may reveal many abnormal conditions, which include azoospermia, oligozoospermia, teratozoospermia, asthenozoospermia, necrozoospermia and pyospermia. The current estimate is that about 30 percent of men seeking help at the infertility clinic are found to have oligozoospermia or azoospermia of unknown aetiology. Therefore, there is a need to find the cause of infertility. the clinical evaluation of the seminal analysis results do not give a sure evidences of whether a man is fertile or infertile [3]. The reasons are known in less than half of these cases, out of which genetic or inherited disease and specific abnormalities in the Y chromosome are major factors. The introduction of molecular techniques has provided great background into the genetics of infertility.

In current practice, male fertility case is detected indirectly based on the individual's semen parameters. Conventional semen analysis is the first step in the assessment of infertile men and it reflects the overall functioning of all male reproductive organs [4]. Abnormal

sperm quality has been linked to several infertility problems, *eg.* abnormal sperm morphology with an increased presence of tapered sperm heads has been associated with recurrent pregnancy loss [5]. In general, semen volume, pH, sperm concentration, motility, vitality, and morphology are determined according to the [6] parameters. Nearly 15% of infertile men have normal sperm parameters according to the WHO 2010 [7]. As a result of the high incidence of SDF (Sperm DNA Fragmentation) in the men with unknown infertility [8], recent research has focused more on determining the clinical value of evaluating SDF in male infertility and using SDF as an advanced sperm function test along with the conventional tests to evaluate the fertility status of the individual. The importance of the SDF assay has also been known in the latest AUA and European Association of Urology guidelines on male infertility [9].

The Y chromosome is essential not only for human sex determination but also for maintenance of sperm cells and their development. The regions of the Y chromosome responsible for male infertility are located on the long arm of the chromosome and are termed AZFa, AZFb and AZFc (AZF: azoospermia factor) [10]. Microdeletions in AZF are associated with male infertility [11]. As the severity of the spermatogenesis increases, the frequency of the microdeletions also increases.

Y chromosome microdeletions are common in about 10-15 percent of men with azoospermia or severe oligospermia. The Y chromosome contains over 60 million nucleotides, but least number of genes compared

to any other chromosome and acts as a genetic determinant of the male characteristic features.

## 2. Materials and Methods

Our study was carried out on 15 patients referred to the human assisted reproduction department in Elnada fertility centre (Almaadi, Cairo, Egypt). The centre established in 2012 to diagnose and treat infertility cases. All subjects were verbally informed and agreed that their samples would be examined, informed consent was written and approved from all samples.

### Semen analysis

All patients according to the guidelines of the World Health Organization 2010, Semen was collected at the laboratory after 3 to 5 days of sexual abstinence, samples were subjected to evaluation for total count, percent motility, and forward progression using haemocytometer chamber, and detect abnormal forms using spermac stain (Endomedix) (FP09 I21 R01 C.4 - Update: 21/01/2015) and examined under light microscope (100 x) using immersion oil to observe abnormal forms - acrosome = dark green - nucleus = stained red - equatorial region = pale green - midpiece and tail = green [12,13,14].

### Sperm Vitality

VitalScreen uses the eosin-nigrosin staining techniques to establishes the percentage of live sperm.

### DNA Fragmentation test

\*The degree of DNA fragmentation was evaluated using the sperm chromatin dispersion assay, reported by [15,16].

\*Sperm chromatin condensation was evaluated by aniline blue staining of semen samples fixed with 3%

glutaraldehyde according to halosperm test (Halotec DNA) [17]. This test is performed to cases with equal or more  $5 \times 10^6$  sperm /ml. DNA extracted from each patient was prepared at a concentration of 50-micron DNA (QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit 02-2003).

### Microdeletions of the Y chromosome

Genomic DNA was extracted from semen of each patient and three Y chromosome specific-sequence tagged site (STS) markers were used for the detection of Y chromosome microdeletions. Initial denaturation at 94°C for 4 minutes, followed by 30 cycles at 94°C for 1 minute, then primer annealing at 50°C for 1 minute, followed by final extension at 72°C for 1 minute and hold at 4 °c. The PCR products were analyzed on a 1% agarose gel containing ethidium bromide and visualized under UV exposure. As a length marker were used Gene Direx one MARK100 includes fragments ranging from 100-3000 base pairs with 12 band. In each PCR assay, sample from one normal fertile man, without Y chromosome microdeletions, were used as normal control. Each primer pair amplifies a specific region of the Y chromosome (i.e. an STS). The screening method for microdeletions was based on multiplex PCR technology using Y-chromosome specific STSs, published by [18], which corresponded to the AZFa, b and c regions, respectively. three previously published Yq STS were used: AZFa prox-2 and AZFa dist-1 (AZFa), sY127 (Table 1).

Table (1). Sequence tagged sites (STS, their product sizes length and their location.

| STS           | Sequence   | product (bp) | locus |
|---------------|--|--------------|-------|
| AZFa prox – 2 | F: GGT.TCC.TGA.ACA.GGG.GAC.T<br>R: GGC.AGC.AGA.AGG.GCC.TCT.C | 220 bp       | AZFa  |
| AZFa dist – 1 | F: GGT.TCC.TGA.ACA.GGG.GAC.T<br>R: GGC.AGC.AGA.AGG.GCC.TCT.C | 390 bp       | AZFa  |
| SY 127        | F:GGC.TCA.CAA.ACG.AAA.AGA.AA<br>R:CTG.CAG.GCA.GTA.ATA.AGG.GA | 274 bp       | AZFb  |

## 3. RESULTS AND DISCUSSION

### Fresh semen evaluation

Fifteen males participated in this study. According to WHO-2010, the lower reference limit for total motility (progressive and non-progressive) is 40%, the lower reference limit for vitality (membrane-intact spermatozoa) is 58%, the lower reference limit for sperm concentration is  $15 \times 10^6$  spermatozoa per ml and according to the Kruger's strict morphology criteria, the lower reference limit is  $\leq 4$  %. Two study participants (no.3,9) are normozoospermia have normal sperm parameters (control) one with previous offspring history and the other with unexplained infertility, one study participant with totally abnormal parameters (no.15), Nine study participants were teratozoospermia with abnormal forms 0 or  $< 4\%$  morphologically normal sperm (five of them with high teratozoospermia (no.1,6,8,12,14) and the others with

moderate teratozoospermia (no.2,7,10,13) and three others had more than one abnormal sperm parameters (no.4) was oligoteratozoospermia with count  $< 15 \times 10^6$  sperm/ ml and abnormal forms  $< 4\%$  normal sperm morphology, (no.5,11) were asthenoteratozoospermia with motility less than 40%. The dark boxes in the table show the highest value of defects while the red boxes show the lowest value of these defects. Table(2)

**DNA Fragmentation Index (%DFI: % sperm cells containing damaged DNA)** according to results of [19,20] that showing 4 statistical categories of fertility potential:  $\leq 15\%$  DFI = excellent to good sperm DNA integrity;  $> 15$  to  $< 25\%$  DFI = good to fair sperm DNA integrity;  $> 25$  to  $< 50\%$  DFI = fair to poor sperm DNA integrity;  $\geq 50\%$  DFI = very poor sperm DNA integrity. So, we have six cases with poor sperm DNA integrity and nine cases with good sperm DNA integrity (Table 2).

Table (2): Sperm parameters and DNA fragmentation

| No | Count*10 <sup>6</sup> /m | Motility % | Vitality % | Abnormal forms% | Acrosome defects% | Head defects % | Tail defects % | Midpiece defects% | DNA Frag. % |
|----|--------------------------|------------|------------|-----------------|-------------------|----------------|----------------|-------------------|-------------|
| 1  | 15                       | 42         | 98         | 100             | 28                | 70.55          | 11.2           | 18.26             | 29.5        |
| 2  | 35                       | 65         | 92         | 97              | 4                 | 69.04          | 20.64          | 10.32             | 11.5        |
| 3  | 49                       | 55         | 97         | 95              | 3                 | 76.34          | 14.12          | 9.34              | 3.5         |
| 4  | 13                       | 45         | 65         | 100             | 30                | 69.03          | 19.03          | 11.94             | 32          |
| 5  | 26                       | 39         | 58         | 99              | 25                | 69.03          | 19.03          | 11.94             | 38.5        |
| 6  | 75                       | 75         | 100        | 100             | 3                 | 87.5           | 5              | 7.5               | 11.5        |
| 7  | 65                       | 65         | 98         | 98              | 7                 | 78             | 2.8            | 19.2              | 12          |
| 8  | 30                       | 70         | 90         | 100             | 4                 | 58.2           | 13.5           | 28.3              | 10          |
| 9  | 46                       | 60         | 100        | 94              | 4                 | 76.89          | 7.97           | 15.14             | 6           |
| 10 | 35                       | 60         | 90         | 99              | 2                 | 61.41          | 25.17          | 13.42             | 13          |
| 11 | 35                       | 35         | 75         | 100             | 46                | 72.73          | 16.23          | 11.04             | 28          |
| 12 | 15                       | 40         | 72         | 100             | 30                | 80             | 10             | 10                | 30          |
| 13 | 25                       | 60         | 89         | 98              | 6                 | 71.43          | 17.29          | 11.28             | 11          |
| 14 | 23                       | 55         | 90         | 100             | 23                | 81.25          | 7.64           | 11.11             | 13.5        |
| 15 | 5                        | 5          | 75         |                 | 39                | 78.83          | 6.2            | 14.96             | 42          |

No correlation was found between count, motility, vitality, tail defects and midpiece defects with DNA fragmentation as in no.1,5,11,12) the value of DF is high while that the count is in normal range and vice versa , in ( no.1,4,11) the value of DF is high while that the motility is in normal range and vice versa ,vitality was in normal range in all study cases and DNA of sperm located in the head so that it is not affected by tail and midpiece defects.

In 2014 Evgeni E, *et al.* [21] was agree with us and reported that high sperm DNA fragmentation is found in infertile men and while men with poor semen parameters are more likely to have high sperm DNA fragmentation, men with normal semen parameters also have high sperm DNA fragmentation that is may be diagnosed with unexplained infertility.

Also, a negative correlation between acrosome defects and therefore abnormal forms of sperm with DNA fragmentation.

Spermatozoa having no fragmentation were identified as: Sperm with large halos (thicknesses that were similar or larger than the length of the smallest diameter of the core) and sperm with medium sized halos (thickness greater than 1/3 of the smallest diameter of the core and less than the smallest diameter of the core). Spermatozoa with DF were identified as: Spermatozoa with a small halo (thickness similar or smaller than 1/3 of the smallest diameter of the core) and those with no halo. A significant negative correlation has been established particularly between the percentage of morphologically normal

spermatozoa and DNA fragmentation [22,23]. In addition, specific morphological shapes, such as tapered heads, have been linked to unexplained recurrent pregnancy loss in subjects that also increase DNA fragmentation (5). otherwise, sperm with abnormally small heads have shown poor diagnosis with IVF and that is related to a very high DNA fragmentation percentage [24].

In contrast, several studies could not to report a significant relation between the traditional seminal parameters, such as sperm concentration, motility, morphology and DNA fragmentation levels [25, 26, 27]. In contrast also, sperm chromosomal mutations have not always been accompanied by abnormal semen parameters in specific groups of patients, such as translocation carriers and cancer patients, *eg.* percentage of normal sperm morphology [28].

**Specific PCR for AZF regions**

A total of 15 men were screened for submicroscopic Y-chromosome deletions. Y chromosome microdeletion analysis revealed no microdeletion in AZFa, AZFb regions in the case and control group. PCR amplification produced a band of expected size for AZFa prox-2(220 bp) (figure 14) and SY 127 loci (274 bp) (figure 15) and AZFa dist-1 loci (390 bp) (figure 16). This may be due to polymorphisms or methodological mistakes. So that, the cause of infertility is largely unkwon and associated Y chromosome microdeletions cannot be ruled out.

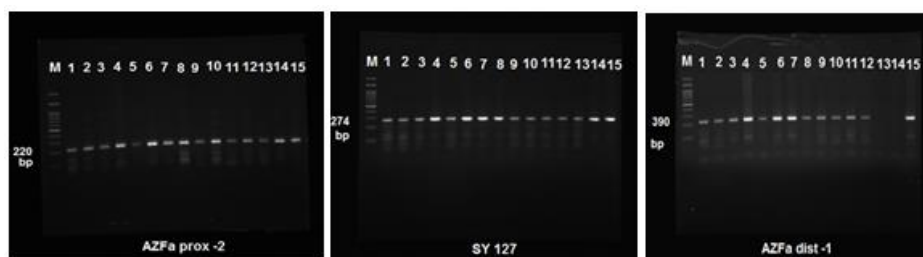


Fig (1): Specific PCR products for 15 severe infertile man., (M) marker (100-3000 base pair ladder), AZFa prox-2 (220 bp). SY 127 (274 bp) and AZFa dist-1

No Y chromosome microdeletions that can cause male infertility were found, although most Y linked mutations exert their effects on spermatogenesis as reported by [29,30].

Furthermore, Y chromosome microdeletions were detected in patients with suboptimal semen parameters (7.4%) [31].

## References

- [1] Dohle, G., Weidner, W., Jungwirth, A., Colpi, G., Papp, G., et al. Guidelines on male infertility. European Association of Urology. (2004).
- [2] Moore FL and Reijo-Pera RA. Male sperm motility dictated by mother's mtDNA. *Am. J. Hum. Genet.* 67 (2000)543-548.
- [3] Nallela KP, Sharma RK, Aziz N, Agarwal A. Significance of sperm characteristics in the evaluation of male infertility. *Fertil Steril*; 85 (2006)629-34.
- [4] Agarwal A, Gupta S, Sharma R. Basic Semen Analysis. In: Agarwal A, Gupta S, Sharma R, editors. *Andrological Evaluation of Male Infertility A Laboratory Guide*. New York: Springer. p(2016) 39–46.
- [5] Carrell DT, Wilcox AL, Lowy L, Peterson CM, Jones KP, Erickson L, et al. Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. *Obstet Gynecol.* 101 (2003)1229–35.
- [6] World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. fifth edn. Geneva, Switzerland: WHO press (2010).
- [7] Agarwal A, Allamaneni SS. Sperm DNA damage assessment: a test whose time has come. *Fertil Steril* .84(2005) 850–3.
- [8] Oleszczuk K, Augustinsson L, Bayat N, Giwercman A, Bungum M. Prevalence of high DNA fragmentation index in male partners of unexplained infertile couples. *Andrology*.1(2013)357–60.
- [9] Jarow J, Sigman M, Kolettis P. Optimal evaluation of the Infertile Male: Best Practice Statement reviewed, and validity confirmed. Available at: [http://www.auanet.org/guidelines/male-infertility-optimal-evaluation-\(reviewed-and-validity-confirmed-2011\), 2016](http://www.auanet.org/guidelines/male-infertility-optimal-evaluation-(reviewed-and-validity-confirmed-2011), 2016). Accessed December 2017(2011).
- [10] Burgoyne PS. The mammalian Y chromosome: a new perspective. *Bioessays* 20 (1998)363-366.
- [11] Vogt PH, Edelmann A, Kirsch, S, et al. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Ygl. *Hum Mol Genet.* 5(1996)933-943.
- [12] Menkveld, R., Franken, D. R., Kruger, T. F., Oehninger, S., & Hodgen, G. D. Sperm selection capacity of the human zona pellucida. *Molecular reproduction and development*, 30(4) (1991)346-352.
- [13] Menkveld, R., Stander, F. S., Kotze, T. J. V., Kruger, T. F., & Zyl, J. A. V. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Human Reproduction*, 5(5) (1990)586-592.
- [14] Oettlé, E. E. Changes in acrosome morphology during cooling and freezing of dog semen. *Animal Reproduction Science*, 12(2) (1986)145-150.
- [15] Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R & Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl* 24 (2003) 59–66.
- [16] Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, LaFromboise M & De Jonge C. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 84(2005) 833–842.
- [17] Sergerie, M., Mieusset, R., Croute, F., Daudin, M., & Bujan, L. High risk of temporary alteration of semen parameters after recent acute febrile illness. *Fertility and sterility*, 88(4) (2007) 970-e1.
- [18] Vollrath, D., Foote, S., Hilton, A., Brown, L. G., Beer-Romero, P., Bogan, J. S., & Page, D. C. The human Y chromosome: a 43-interval map based on naturally occurring deletions. *Science*, 258(5079) (1992) 52-59. (19)
- [19] Evenson DP. Sperm chromatin structure assay (SCSA(R)). *Methods Mol Biol* 927 (2013) 147–164.
- [20] Evenson DP. The Sperm Chromatin Structure Assay (SCSA((R))) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Anim Reprod Sci* 169 (2016)56–75.
- [21] Evgeni, E., Charalabopoulos, K., & Asimakopoulos, B. Human sperm DNA fragmentation and its correlation with conventional semen parameters. *Journal of reproduction & infertility*, 15 (1) (2014) 2.
- [22] Younglai, E. V., et al. "Sperm swim-up techniques and DNA fragmentation." *Human reproduction* 16.9 (2001): 1950-1953.
- [23] Tang SS, Gao H, Zhao Y, Ma S. Aneuploidy and DNA fragmentation in morphologically abnormal sperm. *Int J Androl.* 33(1)(2010)e163–79. [PubMed]
- [24] Menkveld R, Holleboom CA, Rhemrev JP. Measurement and significance of sperm morphology. *Asian J Androl.*13(1)(2011)59–68. [PMC free article] [PubMed]
- [25] Lin, M. H., Lee, R. K. K., Li, S. H., Lu, C. H., Sun, F. J., & Hwu, Y. M. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection but might be related to spontaneous abortion rates. *Fertility and sterility*, 90(2) (2008) 352-359.
- [26] Xia Y, Cheng S, Bian Q, Xu L, Collins MD, Chang HC, et al. Genotoxic effects on spermatozoa of carbaryl-exposed workers. *Toxicol Sci.* 85(1) (2005)615–23. [PubMed]
- [27] Karydis S, Asimakopoulos B, Papadopoulos N, Vakalopoulos I, Al-Hasani S, Nikolettos N. ICSI outcome

is not associated with the incidence of spermatozoa with abnormal chromatin condensation.

In Vivo.19(5) (2005)921–5. [PubMed]

[28] Sun F, Ko E, Martin RH. Is there a relationship between sperm chromosome abnormalities and sperm morphology? *Reprod Biol Endocrinol.* 4(2006)1. PubMed, CrossRef., Google Scholar

[29] Hargreaves, A. Four ages of professionalism and professional learning. *Teachers and Teaching: Theory and*

*Practice,* 6 (2) (2000) 151-182, doi:10.1080/713698714, <http://dx.doi.org/10.1080/713698714>

[30] Singh AR, Vrtel R, Vodicka R and Dhaifalah I. Genetic factors in male infertility and their implications. *Int. J. Hum. Genet.* 6 (2006)163-169.

[31] Erasmuson T, Sin IL and Sin FY. Absence of association of androgen receptor trinucleotide expansion and poor semen quality. *Int. J. Androl.* 26 (2003)46-51.