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# *Study of aneuploidies in gametes of infertile males by using FISH technique*

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***A R T I C L E I N F O***

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***ABSTRACT***

Male factors leading to infertility account for at least half of all cases of infertility worldwide. In this study, we used multicolor fluorescent in situ hybridization (FISH) probes for chromosomes 13, 18, 21, X and Y to evaluate the aneuploidy incidence in sperm cells. The study group included 12 males with infertility and oligoasthenoteratozoospermia (OAT). FISH revealed a significantly higher incidence of sperm aneuploidies compared with controls. By comparing the incidence of the disomy in the OAT group, the highest incidence was the sex chromosome disomy, followed by the disomy of the chromosomes 13, 21 and 18, respectively. The nulisomy incidence in the OAT group was higher for the sex chromosomes, followed by the nulisomy of the autosomes 13, and then 21 and 18. In conclusion, FISH may be considered as an additional assay for the evaluation of spermatozoa in addition to standard analysis, thus playing an important role during proper diagnosis and treatment of infertility. Since currently intracytoplasmic sperm injection (ICSI) is frequently used for patients with OAT, it is important to inform patients if they might have an increased risk of aneuploidies in embryos.

## Background

The infertility onus has been increasing over recent decades and it is estimated to affect 15% of couples worldwide (WHO, 2010). There is conspicuous evidence that males account for the etiology of half of infertility cases. The evaluation of male infertility is based on routine semen analysis, which measures both semen production and sperm quality. However, normal values of these parameters do not accurately reflect the fertilization capability of the sperm. Moreover, there are numerous known causes of male infertility (Sa et Sousa, 2015), which are not addressed in the current analysis.

Sperm aneuploidy is considered a major cause of pregnancy loss, aneuploid births, and

developmental defects (Hassold et Hun, 2001). Recent reports demonstrate a significant increase of the sperm aneuploidy rate in infertile men when compared with fertile counterparts, although this did not exceed 2% with regard to chromosomes X, Y, 18, and 21 **(**Tempest et Martin, 2009; Harton et Tempest, 2012).

These findings suggest that differences between paternal and maternal contribution to aneuploidy are not due to differences in chromosome segregation errors, but rather due to more effective control point in spermatogenesis than in oogenesis. Recent studies have shown that synaptic and recombination errors not only cause abnormal chromosome segregation, but also lead to blocking meiosis. A partial blockage results in oligozoospermia, whereas a complete blockade affects all germ cells and leads to azoospermia (Gonnsalves et al., 2004; Egozcue et al., 2005). As for many cases of spontaneous abortion and infertility, the causes are chromosomal aberrations of the embryo was suggested that the better estimation of the aneuploidy rate at conception can be done by assessing gametes chromosomes (Templado et al., 2013).

In this study, we used multicolor FISH probes for the chromosomes 13, 18, 21, X and Y based on the evidence that these chromosomes are responsible for the most frequently found aneuploidies. We used strict scoring criteria, and a minimum of 5000 sperms was analyzed per chromosome for 12 patients with oligoasthenoteratozoospermia (OAT) and 08 individuals with normal fertility.

## Materials and Methods

*Patients*

The group of OAT patients included males referred to medical laboratories. The control group included males having at least two children with no assisted reproductive techniques applied to them. The control group included 08 males with normal fertility, as well as with normal sperm concentration, morphology and mobility.

*Semen analysis*

Semen samples of the patients were collected by masturbation after 3 days of sexual abstinence and examined after liquefaction for 30 min at 37°C. Volume, pH, concentration, motility and morphology of the samples were evaluated according to the WHO guidelines (2010). Sperm morphology was evaluated by the May Grünwald-Giemsa (MGG) method.

After removal of seminal liquid, the spermatozoa were washed twice with sterile water (300g for 10 min), fixed with Carnoy's solution and then spread on a slide for FISH and TUNEL.

*FISH analysis*

The sperm samples were washed in phosphate-buffered saline. Subsequently, 20 mL of sperm was dropped on a slide and fixed with Carnoy's solution (methanol–acetic acid; 3:1, vol/vol). The sperm nuclei were partially decondensed for 3 min by using a solution of NaOH (1 mol/L) and then washed in 2XSSC for 10 min.

The sperm samples were analyzed by using both dual FISH (the chromosomes 13 and 21) with a specific cocktail probe of 13q14 and 21q22 (Abbott, Rungis, France) and triple FISH (the chromosomes X, Y, and 18) with specific alphoid probes of the chromosomes X (probe DXZ1, spectrum green; Abbott), Y (probe DYZ3, spectrum orange; Abbott) and 18 (D18Z1, spectrum aqua; Abbott). Before hybridization, the sperm DNA slides were immersed in a jar of 2X SSC/0.4% NP40 solution for 30 min at 37°C and then passed through an ethanol series of increasing concentrations before being allowed to air dry. The denaturation was performed simultaneously on sperm nuclei and probes for 1 min at 72°C. The slides were incubated overnight at 37°C. Posthybridization washes included 45 seconds in 0.4X SSC/0.3% NP40 at 72°C, followed by 20 seconds in 2X SSC/0.1% NP40 at room temperature. The slides were counterstained with 4,6-diamino-2-phenylindole and observed by using a Zeiss Axioplan microscope (Zeiss, Le Pecq, France), with the appropriate set of filters. Subsequent image acquisition was performed by using a CCD camera with Isis (In Situ Imaging System; MetaSystems, Altlussheim, Germany).

*Statistical analysis*

Statistical analysis was performed using the Statistical Package for Social Sciences, version 22 (SPSS, Chicago, IL, USA). The comparisons between the controls and patients were calculated using Student's t-test. The Spearman's correlation coefficient *r* was used to assess the correlations between the variables. A significant statistical difference was accepted when $P < 0.05$.

## Results

*Semen analysis*

Sperm parameters and ages of the infertile patients are presented in Table 1 (values from the most recent semen analysis were used).

*Analysis of meiotic segregation*

All the OAT patients, as well as the individuals included in the control group, exhibited chromosomal aneuploidies of the semen; however, a large variability of the aneuploidy rates was found. For each individual included in the study, sperm chromosomal numerical aberrations involving all the evaluated chromosomes were found. The average sperm parameters in the OAT patients as well as in the subjects in the control group are presented in Table 1.

The incidence of the disomy and nulisomy for the chromosome 18 was significantly higher in the OAT group (Table 2) than in the control group. The disomy of the chromosome 18 varied between 0.19% and 0.43% with the mean value being 0.30%. When compared with the disomy of the chromosome 18 in the control group, 0.30% against 0.06% was found that there is a significant statistical difference, $p$=0.000.

The nulisomy of the chromosome 18 in the OAT group varied between 0.32% and 0.69%, the mean value was 0.45% *vs.* 0.06% the nulisomy 18 in the control group and it was also documented a significant statistical difference, $p$=0.000.

The overall sexual chromosome disomy and nulisomy in the OAT group (Table 2) were higher than those identified in the control group.

The gonosomes aneuploidy rate presented large variations for the study group. The gonosomes nulisomy varied between 2.99% and 6.57%, with a mean value of 4.51%. Rate of nulisomy for the sex chromosomes was significantly higher in the OAT compared to the control (4.51% *vs.* 0.34%, $p$=0.000).

For the disomy of the chromosomes (the gonosomes), larger variations were registered, between 0.84% and 2.63%, the mean value was 1.54% *vs.* 0.24% for the control group.

The differences in the sexual chromosome incidence of the disomy between the OAT patients and the controls were statistically significant ($p$=0.001).

The incidence of the disomy and nulisomy for the chromosome 13 was significantly higher in the OAT group than in the control group (Table 2).

The disomy of the chromosome 13 varied between 0.19% and 1.21%, with a mean value of 0.52%. The statistical difference between the OAT group and the control group was significant, being 0.52% compared to 0.14% ($p$=0.047).

The nulisomy of the chromosome 13 in the OAT group varied between 1.21 and 2.35%, with a mean value of 1.61%. The incidence of the nulisomy in the chromosome 13 was higher (1.61%) in the OAT group, as compared with the control group (0.15%)($p$=0.000).

For the OAT group, a large variation was found in the disomy of the chromosome 21 between 0.01 and 1.12% with the mean value being 0.62% (Table 2), while it was 0.15% in the control group ($p$=0.010).

The variation of the chromosome 21 nulisomy ranged between 1.12 and 2.37% with the mean value of 1.71%, while it was only 0.18% for the control group ($p$=0.000).

The variation of the chromosome 21 disomy ranged between 0.01 and 1.12% with the mean value of 0.62%, while it was only 0.15% for the control group ($p$=0.010).

Diploidy frequency in the OAT group varied between 0.35 and 0.93% with the mean value of 0.50%. The incidence of Diploidy was higher in the OAT group, as compared with the control group: 0.50% to 0.34% ($p$=0.030).

The incidence of the chromosome 13 disomy (Figure 1) was found the highest in the patient OAT 7 (1.21%), while it was the lowest in the patient OAT 8 (0.19%). For the chromosome 18, the incidence of the disomy varied between 0.43% (OAT 1) and 0.21% (OAT 6). The highest incidence of the autosomal disomy was found for the chromosome 21 (Figure 1). For this chromosome, the disomy incidence was 1.12% (OAT 12). The lowest incidence of the chromosome 21 disomy was 0.01% (OAT 7).

The incidence of the sexual chromosome disomy (Figure 4) was higher than the rate of the autosome disomy, and the interindividual variance for the gonosome disomy (Figure 2) was very large. The patient OAT 7 presented the highest incidence of the gonosome disomy (2.63%), while the patient OAT 2 showed the lowest incidence for the sex chromosome disomy (0.84%). The highest incidence of the diploidy (Figure 3) was found in the patient OAT 6 (0.93%), while the patients OAT 1 and 10 had the lowest incidence of the diploidy (0.35%).

**Table 1:** The semen parameters in the study groups

| Patient number | Mean age (years) | Sperm concentration (X $10^6$ mL) | Progressive motility (%) | Normal morphology (%) |
|---|---|---|---|---|
| OAT 1 | 31 | 4,60 | 25 | 16 |
| OAT 2 | 37 | 2,80 | 25 | 12 |
| OAT 3 | 27 | 0,18 | 00 | 2 |
| OAT 4 | 37 | 0,80 | 25 | 15 |
| OAT 5 | 35 | 8,80 | 20 | 14 |
| OAT 6 | 32 | 7,80 | 20 | 13 |
| OAT 7 | 45 | 0,38 | 00 | 00 |
| OAT 8 | 44 | 2,60 | 15 | 19 |
| OAT 9 | 38 | 1,70 | 30 | 17 |
| OAT 10 | 31 | 1,41 | 15 | 11 |
| OAT 11 | 37 | 1,40 | 10 | 8 |
| OAT 12 | 28 | 0,12 | 05 | 5 |
| Control group | 28-45 | 129±49,95 | 55,41±3,39 | 51,33±7,65 |

*OAT: Oligoasthenoteratozoospermia*

**Table 2:** Incidence of disomy, nulisomy and diploidies for the chromosomes 13, 18and 21, as well as for the sex chromosomes

| | Disomy frequency (%) | | | | | | Total rate of sex chromosomes disomy (%) | Diploidy frequency (%) | Nulisomy frequency (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1313 | 1818 | 2121 | XX | XY | YY | | | 18 | X/Y | 13 | 21 |
| **OAT 1** | 0.29 | 0.43 | 0.34 | 0.10 | 0.59 | 0.18 | 0.87 | 0.35 | 0.50 | 4.53 | 1.35 | 1.06 |
| **OAT 2** | 0.25 | 0.35 | 0.44 | 0.25 | 0.30 | 0.29 | 0.84 | 0.45 | 0.39 | 3.47 | 1.21 | 1.84 |
| **OAT 3** | 0.18 | 0.28 | 0.58 | 0.54 | 0.49 | 0.38 | 1.41 | 0.79 | 0.45 | 2.99 | 1.06 | 1.63 |
| **OAT 4** | 0.42 | 0.19 | 0.69 | 0.48 | 0.23 | 0.59 | 1.30 | 0.45 | 0.41 | 4.21 | 1.18 | 1.48 |
| **OAT 5** | 0.56 | 0.34 | 0.79 | 0.33 | 0.41 | 0.36 | 1.10 | 0.50 | 0.32 | 5.11 | 1.29 | 1.93 |
| **OAT 6** | 0.33 | 0.21 | 0.36 | 0.45 | 0.29 | 0.45 | 1.19 | 0.39 | 0.36 | 4.54 | 1.39 | 1.47 |
| **OAT 7** | 1.21 | 0.39 | 0.01 | 0.72 | 0.82 | 1.09 | 2.63 | 0.93 | 0.69 | 6.57 | 2.03 | 1.98 |
| **OAT 8** | 0.19 | 0.29 | 0.98 | 0.41 | 0.53 | 0.98 | 1.92 | 0.45 | 0.56 | 3.84 | 1.92 | 1.12 |
| **OAT 9** | 0.53 | 0.32 | 0.83 | 0.27 | 0.50 | 0.29 | 1.06 | 0.37 | 0.35 | 4.62 | 1.84 | 1.60 |
| **OAT 10** | 0.31 | 0.22 | 0.21 | 0.30 | 0.46 | 0.61 | 1.37 | 0.35 | 0.43 | 3.91 | 1.65 | 1.92 |
| **OAT 11** | 0.92 | 0.23 | 1.09 | 0.66 | 0.72 | 1.06 | 2.44 | 0.46 | 0.52 | 5.38 | 2.35 | 2.19 |
| **OAT 12** | 1.08 | 0.33 | 1.12 | 0.70 | 0.63 | 1.08 | 2.41 | 0.52 | 0.47 | 4.92 | 2.11 | 2.37 |
| **means** | 0.52 | 0.30 | 0.62 | 0.43 | 0.50 | 0.61 | 1.55 | 0.50 | 0.45 | 4.51 | 1.61 | 1.71 |
| **Control group** | 0.14 | 0.06 | 0.15 | 0.09 | 0.07 | 0.08 | 0.24 | 0.34 | 0.06 | 0.34 | 0.15 | 0.18 |



## Discussion

The association of maternal advanced age with an increased risk for having an offspring with aneuploidy has been well documented, while the effect of paternal advanced age is still unknown (Erikson et al., 1978). There are several reports (Griffin et al., 1995; Lähdetie at al., 1996) showing that the incidence of the sex chromosomes disomy is higher in cases of advanced paternal age.

Further studies are required to find correlation between the quality of semen and the incidence of chromosomal aneuploidies in sperm. This hypothesis was raised after observing a higher incidence of chromosomal abnormalities in cases where intra-cytoplasmic sperm injection (ICSI) was performed due to low concentration/motility/morphology of semen (Bonduelle et al., 2002).

For the control group, the rates of aneuploidy were similar with those reported by Templado et al. (2013). For the OAT group, the overall rate of the chromosomal aneuploidy was 11.78%, which is comparable with the reports of Andreescu et al. in Romania (14.63%) (Andreescu et al., 2016), Pylyp et al. in Ukraine (Pylyp et al., 2013) and

Kumtepe et al. in Turkey (12%) (Kumtepe et al., 2009). Lower rates of chromosomal aneuploidy were reported by Wang et al. in China (8.5%) (Wang et al., 2010), Rao et al. in India (7.9%) (Rao et al., 2005) and Gekas et al. in France (6.9%) (Gekas et al., 2001).

In this study, we also recorded the incidence of the nulisomy, which is not often reported. There is a debate regarding the correct assessment of nulisomy and its distinction to a failure of hybridization. Taking into consideration chromosomal non-disjunction during meiosis as the mechanism underlying the occurrence of disomy/nulisomy, the incidence of nulisomy should be similar to the rate of disomy. We believed that our results regarding the incidence of the nulisomy were not due to artifacts during the procedure; this is because in both the groups, for the studied autosomal chromosomes, the rate of disomy/nulisomy was close to 1:1 (0.52% *vs.* 1.61%, 0.30% *vs.* 0.45%, 0.62% *vs.*1.72%, 0.14% *vs.* 0.15%, 0.06% vs. 0.06%, and 0.15% *vs.* 0.18%). In the OAT group, the rate of the nulisomy for the sex chromosomes compared with the disomy rate was 2.90 (4.51% *vs.* 1.55%), which could be attributed to the high levels of the sex chromosomes nulisomy. These findings can be explained by the anaphase lag that can occur in spermatogenesis (Cimini et al., 2003; Cupiste et al., 2003).

We studied the hypothesis of a possible correlation between sperm parameters and incidence of aneuploidy. The results revealed slight negative correlations between the semen parameters and the aneuploidy of the studied chromosomes . The correlation coefficients were -0.30 for the sperm concentration and aneuploidy rate, -0.24 for the morphology and chromosomal aberrations and -0.40 for the sperm motility and aneuploidy. The overall incidence of the disomy in the OAT group showed a weak to moderate correlation with the semen parameters. Previous studies reported negative correlation between the rate of chromosome aneuploidy and oligospermia (Durak Aras et al., 2012; Mougou-Zerelli et al., 2011).

In this study, we found a weak negative correlation between the disomy incidence and the sperm concentration ($r$=-0.51). By comparing the disomy incidence and the progressive motility and the normal morphology, we found a moderate to weak negative correlation, with the coefficients being $r$=-0.54 and $r$=-0.26, respectively.

Different results were found with regard with the correlation between low motility and rate of aneuploidy. Some researchers reported modest correlation between these two parameters (Aran et al., 1999; Vegetti et al., 2000), while in other cases, no correlation was found (Serrate et al., 2010). However, several reports indicated a positive correlation between the high incidence of teratozoospermia and the rate of aneuploidy(Tang et al., 2010; Brahem et al., 2011), while in other cases no correlation was documented (Serrate et al., 2010).

## Conclusion

The results of our study sustained the importance of sperm FISH analysis for patients with OAT, who usually undergo assisted reproductive techniques. The molecular cytogenetic analysis allows the evaluation of sperm aneuploidy rates and should be recommended before the application of any assisted reproductive procedure. These investigations allow the identification of patients with an increased risk for reproduction failure and facilitate an appropriate counseling in order to inform patients about their reproductive options, genetic preimplantation testing and prenatal genetic tests that are available.

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