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Assessment of chromosomal aneuploidies in sperm of infertile males by using FISH technique

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Abstract

Reproductive failure is one of the most important issues for the population at age of procreation and approximately 15% of the couples who try to conceive a baby encounter reproductive difficulties. 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 35 males with infertility and oligoasthenoteratozoospermia (OAT) and 20 males with normal fertility and normal semen characteristics for which the conventional cytogenetic investigation using peripheral blood revealed a normal karyotype. The overall chromosome disomy and nulisomy in OAT group was higher than the one identified in the control group. By comparing the incidence of the disomy in the OAT group, the highest incidence was the sex chromosome disomy, followed by the disomy of chromosomes 13, 21 (equal values) and then 18. The nulisomy incidence in the OAT group was higher for sex chromosomes, followed by the nulisomy of autosomes 13, then 21 and 18. As in these days, for patients with OAT, intracytoplasmic sperm injection (ICSI) is frequently used, it is important to inform the patients if they might have an increased risk of aneuploidies in embryos.

Keywords: hybridization *in situ*, oligoasthenoteratozoospermia, chromosomal aberrations, infertility.

Introduction

Reproductive failure is one of the most important issues for the population at age of procreation and approximately 15% of the couples who try to conceive a baby encounter reproductive difficulties. The causes of infertility are very complex, cytogenetic anomalies being one of the possible causes [1]. From the introduction of karyotyping in the clinical practice, it was demonstrated that constitutional chromosomal aberrations are involved in reproduction failure, aneuploidies affects one of 300 live births [2] and are responsible for an important percent of pregnancy loss.

In the last years was taken in consideration the hypothesis that not only somatic chromosomal anomalies but also germ cells chromosomal aberrations could lead to reproductive failure.

Recent studies [3] showed that the frequency of aneuploidy in meiosis II spermatocytes (14%) is similar to that observed in oocytes of young women (13–19%) [4].

These findings suggest that differences between paternal and maternal contribution to aneuploidy is not due to differences in the chromosome segregation errors, but rather more effective control point in spermatogenesis than oogenesis. Recent studies have showed that synaptic and recombination errors not only cause abnormal chromosome segregation but also lead to blocking meiosis. If a partial blockage, the result will be oligozoospermia, whereas a complete blockade affects all germ cells and lead to azoospermia [5, 6]. 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 the gametes chromosomes [7].

The difficulties related to chromosomal evaluation in germ cells were overcome by the development of molecular cytogenetic techniques use. There are several studies regarding the aneuploidy incidence in sperm cells evaluated by using fluorescence *in situ* hybridization (FISH) technique [8–12]. The existent studies using FISH to evaluate chromosomal aneuploidy in sperm show a great variability of the results, no consensus being reached yet.

FISH analysis for the evaluation of semen chromosome was done for patients that experience reproduction failure despite a normal spermogram but also for the males with abnormal parameters of the semen [13].

In this study, we used multicolor FISH probes for chromosomes 13, 18, 21, X and Y based on the evidence that these chromosomes are responsible for the most frequent found aneuploidies. We used strict scoring criteria and a minimum of 5000 sperm analyzed per chromosome for 35 patients with oligoasthenoteratozoospermia (OAT) and 20 individual with normal fertility.

Materials and Methods

The group of OAT patients included males referred to the Laboratory of Genetics from the "Victor Babeş" University of Medicine and Pharmacy, Timişoara, Romania. The control group included males having at least two children and no assisted reproductive techniques applied

for them. Our patients were included in the present study after having a clinical urological examination, a lymphocyte karyotype, a dosage of hormonal of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and testosterone. All the patients included in this study had normal results. The study group included 35 males with infertility for which the conventional cytogenetic investigation using peripheral blood revealed a normal karyotype. In this group were included males presenting less than 10 million sperm/mL, sperm mobility less than 40% and normal sperm morphology less than 4%. The control group included 20 males with normal fertility and normal sperm concentration, morphology and mobility. Information regarding exposure to teratogenic agents was collected and only patients that did not have exposure to this kind of environmental factors were included in the study.

Informed consent was obtained from each subject included in the study, in accordance with the Ethics Committee of the "Victor Babeş" University of Medicine and Pharmacy, Timişoara.

Sperm preparation and decondensation for FISH analysis

The sperm was collected after three days of abstinence and was washed three times with Dulbecco's phosphate-buffered saline, than centrifuged for five minutes at 280×g. The sediment was resuspended in fixative solution of methanol and glacial acetic acid (3:1 proportion). From this sediment, were prepared the slides that were deposit at -20^{0}C until next day. For sperm decondensation, it was used the protocol of Palermo *et al.*, with minor adaptation in our laboratory [14].

FISH protocol

For the beginning were used Vysis (Abbott) probes for chromosomes 18 and X/Y. The probes were prepared according to manufacturer protocol and denatured at 73±1^{0}C for five minutes. The probes were added to the slides, covered with a 22×22 mm cover slip, sealed with rubber cement and incubated at 37^{0}C for 12–16 hours. After removing the cover slip, the slides were washed and counterstained with DAPI II (4',6-diamidino-2-phenylindole dihydrochloride) solution. The same slides were used for the next step after the rehybridization. Vysis (Abbott) probes for chromosomes 13 and 21 were used. The preparation of probes, hybridization and washing steps were similar to those above described. Slides were analyzed on a Zeiss Axio Imager M 1 microscope using DAPI/Orange/Green/Aqua filters and MetaSystems Isis programme was used for capturing the images.

Statistical analysis

The MedCalc® software v.12.3.0 (Mariakerke, Belgium) was used for statistical analyses. The Student's *t*-test was used in order to establish if the average of results obtained differs significantly. Pearson's correlation coefficient *r* was used to asses the correlations between variable. Statistical significance was set at $p<0.05$.

Results

The age for the patients from the OAT group was between 31–42 years, with a mean of 37.2 years, while for the control group varied between 32–42 years and the mean age was 36.95 years. No correlation between paternal age and the rate of sperm aneuploidy was found.

The fluorescent signal for chromosomes 18, X and Y in the study group were analyzed in 5110 cells/patient, while in control group 5140 cells/patient. The hybridization efficiency was 99.75% for the study group and 99.9% for the control group. For chromosomes 13 and 21, were analyzed fluorescent signals of 5150 cells/patient from the study group and 5210 cells/patient from the control group.

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

Table 1 – ***Semen parameters in the study groups***

	Mean age [years]	Sperm concentration [×10^6/mL]	Progressive motility [%]	Normal morphology [%]
OAT group	37.2	3.29	15.71	5.8
Control group	36.95	61.75	62.8	78.5

OAT: Oligoasthenoteratozoospermia.

Chromosomes 18, X and Y

Incidence of disomy and nulisomy for chromosome 18 in OAT group (Table 2) was significantly higher than in the control group. The disomy of chromosome 18 varied between 0.09% and 1.92%; mean value was 0.42%. When compared with the disomy of chromomosome 18 in the control group, 0.42% *vs.* 0.04% was found that there is a significant statistical difference, $p=0.0003$.

Table 2 – ***Incidence of disomy and nulisomy for chromosomes 13, 18, 21 and sex chromosomes***

	Dis 18 %	Nul 18 %	XX %	YY %	XY %	Dis X/Y %	Nul X/Y %	Dis 13 %	Nul 13 %	Dis 21 %	Nul 21 %
OAT group	0.42	0.5	0.72	0.86	2.33	3.91	4.57	1.28	1.35	1.28	1.32
Control group	0.04	0.06	0.09	0.08	0.15	0.32	0.34	0.12	0.15	0.18	0.18

Dis: Disomy; Nul: Nulisomy; OAT: Oligoasthenoteratozoospermia.

The nulisomy of chromosome 18, in the OAT group varied between 0.09% and 2.72%, the mean value was 0.5% *vs.* 0.06% the nulisomy 18 in the control group and it was also documented a significant statistical difference, $p=0.0001$.

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

Gonosomes aneuploidy rate presented large variations for the study group. Gonosomes nulisomy varied between 0.34% and 16.63%, with a mean value of 4.57%. Rate of nulisomy for sex chromosomes was significantly higher in the OAT compared to the control (4.57% *vs.* 0.34%, $p=0.003$).

For the disomy of chromosomes gonosomes larger

variations were registered, between 0.44% and 19.05%, the mean value was 3.91% *vs.* 0.32% for the control group. The differences between the sexual chromosome incidence of disomy for OAT patients and the controls were statistically significant (p=0.0021).

The incidence disomy due to meiosis I non-disjunction was 0.15% and disomy due to meiosis II non-disjunction was only 0.17% in the control group. For OAT group, the incidence of disomy due to meiosis I non-disjunction was 2.33% and disomy due to meiosis II non-disjunction was only 1.58%.

Chromosomes 13 and 21

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

The disomy of chromosome 13 varied between 0.14% and 3.88%, with a mean value of 1.28%. The statistical difference between the OAT group and the control group was significant, 1.28% compared to 0.12% (p<0.0001).

Chromosome 13 nulisomy in the OAT group varied between 0.19 and 8.46%, with a mean value of 1.35%. The incidence of chromosome 13 nulisomy was higher in the OAT group as compared with the control group: 1.35% to 0.15% (p<0.0001).

For the OAT group was found a large variation of the disomy, between 0.27 and 7.68%, the mean value was 1.28% (Table 2), while in the control group it was 0.18% (p=0.0064).

The variation of the chromosome 21 nulisomy ranged between 0.27 and 5.98% with a mean value of 1% while for the control group it was only 0.18% (p=0.0001).

By comparing the incidence of the disomy in the OAT group, the highest incidence was the sex chromosome disomy, followed by the disomy of chroosomes 13, 21 (equal values) and then 18. In the control group, the highest incidence was the sex chromosome disomy, followed by the disomy of chromosomes 21, 13 and then 18. The nulisomy incidence in the OAT group was higher for sex chromosomes, followed by the nulisomy of autosomes 13, then 21 and 18. Similar distribution was found in the control group.

Large interindividual variations were found in the group of OAT patients. The highest incidence of chromosome 13 disomy (Figure 1) was found in patient OAT 3 (3.88%), while patient OAT 4 had the lowest incidence of disomy 13 (0.14%). For chromosome 18, the incidence of disomy (Figure 2) varied between 1.92% (OAT 33) and 0.09% (OAT 18). The highest incidence of autosomal disomy was found for chromosome 21 (Figure 3), for this chromosome, the disomy incidence was 7.68% (OAT 33). The lowest incidence of chromosomes 21 disomy was 0.27% (OAT 4). The incidence of sexual chromosome disomy (Figure 4) was higher than the rate of autosome disomy and the interindividual variance for gonosome disomy was very large. Patient OAT 30 presented the highest incidence of gonosome disomy (19.05%), while patient OAT 18 showed the lowest incidence for sex chromosome disomy (0.44%). The overall incidence of disomy was the highest in patient OAT 30, and the lowest in patient OAT 18.

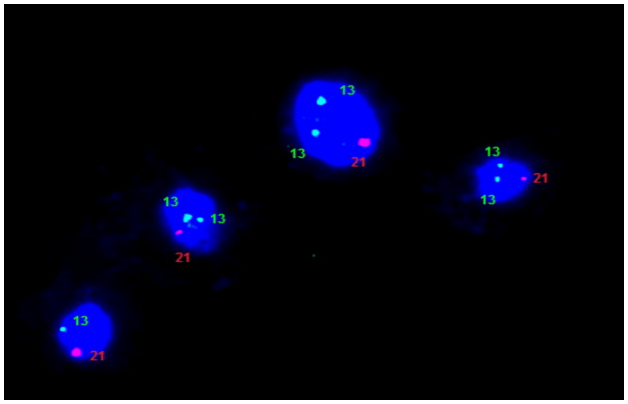

Figure 1 – ***Disomy of chromosome 13 – two green signals corresponding to chromosome 13 and one red signal corresponding to chromosome 21.***

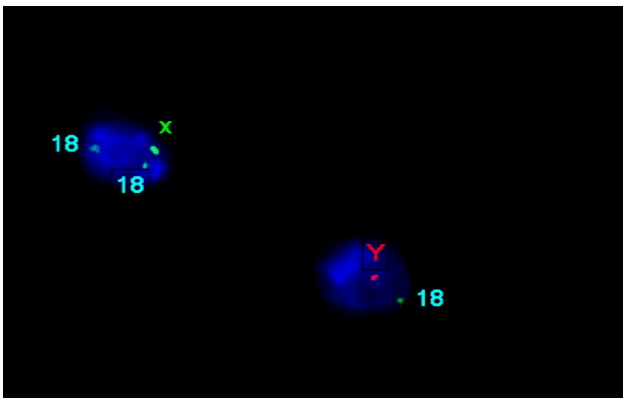

Figure 2 – ***Disomy of chromosome 18 – two blue signals corresponding to chromosome 18; red and green signals correspond to chromosomes X and Y.***

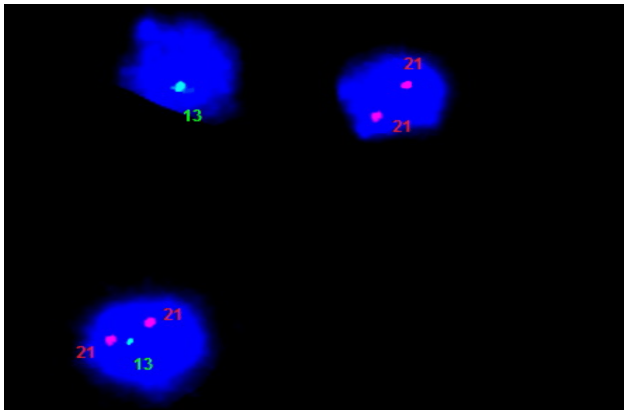

Figure 3 – ***Disomy of chromosome 21 – one green signal corresponding to chromosome 13 and two red signals corresponding to chromosome 21.***

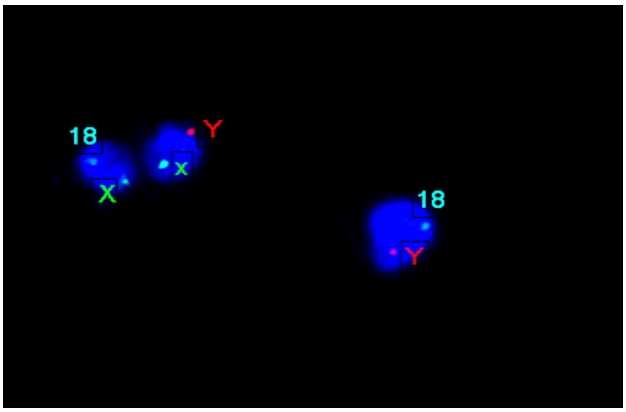

Figure 4 – ***Sexual chromosome disomy and chromosomes 18 nulisomy – one cell contains both gonosomes, X and Y, corresponding to red and green fluorescent signals and no blue signal corresponding to chromosomes 18.***

Discussion

The association of maternal advanced age with an increased risk for having an offspring with aneuploidy is well documented, while the effect of paternal advanced age is still unclear [15]. There are several reports [16, 17] showing that the incidence of sex chromosomes disomy is higher in cases of advanced paternal age.

Further studies try 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 [18]. The OAT patients included in this study were considered for ICSI in order to conceive a child and the semen testing revealed information useful for the management of the couple.

FISH analysis was introduced in 1990 for assessment of chromosomal aneuploidy in sperm. Initially, it was used a single hybridization probe for a single chromosome in a cell, and then it was the two-color FISH and three-color FISH.

Initial studies had obvious limitations, which are reduced accuracy of the results using the FISH technique. These limitations include patient selection, small number of spermatozoa evaluated for each patient, protocols for FISH technique and fluorescent signal quantification criteria.

Since 2000, several studies using FISH analysis for detection of aneuploidy in sperm was done [7, 10, 12, 14, 19–22], but still there is a great variability of the size of the groups, the methodology, the chromosomes evaluated and especially the numbers of scored spermatozoa, so different results were reported as regard of the incidence of chromosomal imbalanced found. Many of the reports found an increased rate of disomy, especially for the sex chromosomes, and in some cases, positive/negative correlations were found between patients' characteristics and severity of the aneuploidy [8, 9, 11, 12, 22].

For the control group, the rates of aneuploidy were similar with those reported by Templado *et al.* [7]. For the OAT group, the overall rate of chromosomal aneuploidy was 14.63%, which is comparable with the reports of Kleiman *et al.* [23] in Israel (16.6%), Pylyp *et al.* [24] in Ukraine, Kumtepe *et al.* [25], in Turkey (12%). Lower rates of chromosomal aneuploidy were reported by Wang *et al.* [26], in China (8.5%), Rao *et al.* [27], in India (7.9%), Gekas *et al.* [28], in France (6.9%).

In this study, we have recorded also the incidence of 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 in 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 consider that our results regarding the incidence of nulisomy are not due to artifacts during the procedure so because in both groups, for the autosomal chromosomes studied, the rate of disomy/nulisomy is close to 1:1 (1.28% *vs.* 1.35%, 0.42% *vs.* 0.5%, 1.28% *vs.* 1.32%, 0.12% *vs.* 0.15%, 0.18% *vs.* 0.18%). In the OAT group, the rate of nulisomy for sex chromosomes compared with the disomy is 1.16 (4.57% *vs.* 3.91%) explained by the high levels of sex chromosomes nulisomy. These findings can be explained by the anaphase lag that can occur in spermatogenesis [29, 30].

We have studied the hypothesis of a possible correlation between sperm parameters and the incidence of aneuploidy. Between the semen parameters and aneuploidy of the studied chromosomes were found weak negative correlations. The correlation coefficients were: -0.49 for sperm concentration and aneuploidy rate, -0.53 for morphology and chromosomal aberrations, -0.62 for sperm motility and aneuploidy. The overall incidence of disomy in the OAT group showed a weak to moderate correlation with the semen parameters. Previous studies have reported negative correlation between the rate of chromosome aneuploidy and oligospermia [9, 31].

In this study, we found a weak negative correlation between the disomy incidence and the sperm concentration (r=-0.45). By comparing the disomy incidence and the progressive motility and the normal morphology, we found a moderate to weak negative correlation, the correlation coefficients were r=-0.57 and r=-0.49 respectively.

Different results in regards with the correlation between low motility and rate of aneuploidy were found. There are researchers that reported modest correlation between those two parameters [32, 33], while in other cases, no correlation was found [34]. In regards with the correlation between the high incidence of teratozoospermia and the rate of aneuploidy, several reports indicated a positive correlation [35, 36], while in other cases no correlation was documented [34].

Conclusions

The results of our study sustain the importance of sperm FISH analysis for the patients with OAT, which usually undergoes 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 the patients about their reproductive options, the genetic preimplantation testing and the prenatal genetic tests that are available. We consider that this study bring a contribution to characterization of OAT patients and to our knowledge is the first study on OAT patients in Romanian population.

Conflict of interests

The authors declare that they have no conflict of interests.

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