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Original Article

Investigation of the interchromosomal effects in male carriers with structural chromosomal abnormalities using FISH

Özgür Balasar[1], Hasan Acar[2]

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ABSTRACT

Objective: The interchromosomal effect (ICE) refers to the uncertainty during meiosis where the rearrangement of the chromosomes affects the segregation of the chromosomes that are not involved in the structural chromosomal abnormalities. The aim of this study is to investigate the existence of ICE in the sperm nuclei of the males who have structural chromosomal abnormalities.

Material and methods: Nine male individuals who are the carriers of the structural chromosomal abnormalities (patient group) and 14 male individuals who did not have any chromosomal abnormalities (control group) were diagnosed by the classical cytogenetic analysis. The aneuploidy of chromosomes 2, 3, 12, 13, 17, 18, 21, X, and Y in the sperm nuclei was investigated using the fluorescence in situ hybridization (FISH) method in these individuals. The patient group included 5 Robertsonian translocation (ROB) carriers, 3 reciprocal translocation (RCP) carriers, and 1 inversion carrier.

Results: A total of 51921 sperm nuclei were analyzed (19484 from the patient group and 32437 from the control group). While ICE was determined in 4 of 5 patients who were the carriers of ROB and an inversion carrier patient, it was not determined in the patient carrier of RCP.

Conclusion: Our results suggest that there is ICE in the male carriers with a structural chromosomal abnormality, which appears to be translocation, breakpoint, chromosome, and patient dependent.

Keywords: Aneuploidy; chromosome segregation; genetic translocation; fluorescent in situ hybridization; spermatozoa.

ORCID IDs of the authors:
Ö.B. 0000-0002-2945-9355;
H.A. 0000-0002-7139-9261

[1]Department of Medical Genetics, Konya Training and Research Hospital, Konya, Turkey
[2]Department of Medical Genetics, Necmettin Erbakan University Meram School of Medicine, Konya, Turkey

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Corresponding Author:
Özgür Balasar
E-mail:
ozgbalasar@hotmail.com

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Introduction

The frequency of chromosomal abnormalities is about 0.6% during the newborn period and 7.5% in all conceptions.[1] The aneuploidies of all chromosomes in the human oocytes have been defined.[2] The chromosomes that are smaller in size are more prone to nondisjunction.[3] Structural abnormalities account for 41% of all chromosomal abnormalities[4] and are seen in one in every 375 newborns.[4] The frequency of the reciprocal translocations (RCP) in the general population is approximately 1/625.[5] Balanced translocations are seen more in couples with two or more spontaneous miscarriages and infertile males than the general population.[5] The translocation carriers are at a risk to have mentally and physically abnormal children because of the segmental imbalance of the translocation chromosomes. The frequency of the Robertsonian translocation (ROB) among males with oligozoospermia is higher than that in the normal population.[6]

The nondisjunction of any chromosome that is not involved in the translocation or rearrangement because of the translocation or structural rearrangement that occurs in an individual during meiosis I is called the "interchromosomal effect" (ICE). Lejeune argued that the chromosomes involved in the translocation and their homologs could abnormally affect the segregation of other chromosomes.[7] ICE remains a controversial issue in humans. Even though the mechanism of ICE is not known yet, it was shown that the frequency of aneuploidy was increased in the structural chromosomal abnormality carriers.[8,9] In the literature, other studies

have also reported that there was no such effect on the individuals who carried the structural chromosomal abnormalities.[10]

It has been postulated that the fundamental mechanism of ICE should be related to meiosis I[8] because the aberrations that might cause ICE occur during the meiosis I-prophase. Therefore, the existence of ICE in the sex chromosomes should be determined by displaying the frequency of XY disomies in the sperms. Since the disomy X and disomy Y occur in meiosis II, they should not be regarded as ICE.

The fluorescence in situ hybridization (FISH) method is being used widely in the detection of the chromosomal abnormalities in the sperms and in the interphase nuclei. The fact that FISH is a rapid and reliable method enables the analysis of the sample number of the sperms and ensures that the statistical data collected from these analyses are more reliable.

The aim of the present study is to investigate ICE on the sperm nuclei of the males with structural chromosomal abnormality. If there is an ICE, we aim to detect the type of chromosomal disorders and chromosomes that are affected, and the pre-data would be collected to reveal the mechanism of the chromosomal aneuploidy.

Main Points:

- The frequency of chromosomal abnormalities is about 0.6% during the newborn period. The rate of chromosomal abnormalities among the infertile males is higher than in normal population.
- The nondisjunction of any chromosome, which is not involved in translocation or rearrangement because of translocation or structural rearrangement present in an individual, during meiosis I is called "interchromosomal effect" (ICE).
- It has been postulated that the fundamental mechanism of ICE should be in meiosis I because the aberrations that might cause ICE occur during the meiosis I-prophase. There are various theses about formation of ICE mechanism.
- In our study, ICE was investigated on the sperm nuclei of males with structural chromosomal abnormality. While ICE was detected in four of five patients carrier of a Robertsonian translocation and a inversion carrier patient, it was not detected in the patients carrier of a reciprocal translocation.
- Our results suggest that ICE appears to be translocation, breakpoint, chromosome, and patient dependent. The molecular genetics and synaptonemal complex studies should be conducted to understand the mechanism of ICE.

Material and methods

Subjects

The patient group consists of nine adult male individuals with chromosomal disorders. The control group consists of 14 individuals who had similar age characteristics as the patient group, but had no chromosomal rearrangements (including heteromorphism), no disease that could change the genetic structure, and did not receive radiotherapy, chemotherapy, and medication. The ethics committee approval was received from the Medical Ethics Committee of the Selçuk University (numbered 2008/035). Written informed consent was obtained from the patients and control individuals who participated in this study.

Peripheral blood culture for karyotyping

Karyotyping from the peripheral blood lymphocytes was performed according to the standard cytogenetic procedures using the GTG-banding technique[11] and the karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[12] The slides were analyzed using an imaging system (MacKtpye, California, USA). The patient and control groups were assessed for heteromorphism by applying C-banding and NOR-banding.

Sperm sample preparation

Sperm samples were obtained by masturbation after 3-5 days of sexual abstinence. The sperm morphology was assessed according to the Kruger's Strict criteria.[13] The semen samples for the FISH study were washed with phosphate-buffered saline (PBS) (Biological Industries, Haifa, Israel) 3 times, allowed to swell with hypotonic solution (0.056 M KCl; Amresco, Ohio, USA), and followed by fixation and 3-time rinsing with standard 3:1 methanol-acetic acid fixative (Serva, Heidelberg, Germany). The sperm-fix samples were stored at –20 °C until the FISH study.

Preparation of the sperm nuclei for FISH analysis and probes used

The sperm samples in the fixative solution were dropped onto the clean slides according to the intensity. The slides were kept in 2x standard saline citrate solution (SSC; Serva, Heidelberg, Germany) for 5 min at 37°C and were then incubated in 0.01 M Dithiothreitol (DTT; Sigma, St. Louis, USA)/PBS at room temperature. They were dehydrated in an ascending alcohol series (70%, 90%, and 96%) and then air-dried for subsequent sperm-FISH analysis.

Each sperm sample was analyzed in triple-color FISH X-Y-18 (homemade), with chromosome X (SpectrumRed), Y (SpectrumAqua), and 18 (SpectrumGreen) in dual-color FISH 13-21 (Kreatech, Bremerhaven, Germany); with chromosome 13 (SpectrumGreen) and 21 (SpectrumRed) in dual-color FISH 2-12 (homemade); with chromosome 2 (SpectrumGreen) and 12 (SpectrumRed) in dual-color FISH 3-17 (homemade), and with chromosome 3 (SpectrumRed) and 17 (SpectrumAqua) probes.

Following the addition of 10 μL of optimized labeled probe and hybridization buffer mix to the slides, the probe and sperm

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Table 1. Clinical history, age, and karyotype of the patients

Patient No	Karyotype	Age (Years)	Clinical History
P1	46,XY,t(9;14)(q21;q11)	39	Primary infertility, 5 failed IVFs and 1 miscarriage
P2	45,XY,rob(13;14)(q10;q10)	24	Primary infertility
P4	45,XY,rob(15;22)(q10;q10)	38	3 healthy live births
P5	45,XY,rob(14;22)(q10;q10)	31	Primary infertility, 2 failed IVFs, and 3 failed ICSIs
P10	45,XY,rob(13;14)(q10;q10)	50	Primary infertility, 6 failed IVFs, 4 failed ICSIs, and 2 miscarriages
P11	46,XY,t(6;15)(q23;q24)	35	5 miscarriages and 2 healthy live births
P12	46,XY,t(5;11)(q11.2;p11)	31	Secondary infertility, 1 failed IVF, 2 failed ICSIs, and 1 live birth
P20	46,XY,inv(6)(p22q13)	27	Primary infertility, 2 miscarriages
P22	45,XY,rob(13;15)(q10;q10)	45	Primary infertility, 1 failed IVF
		35.56±8.40	

P: patient; IVF: *in vitro* fertilization; t: individual with translocation; rob: individual with Robertsonian translocation; ICSI: intracytoplasmic sperm injection; inv: individual with inversion

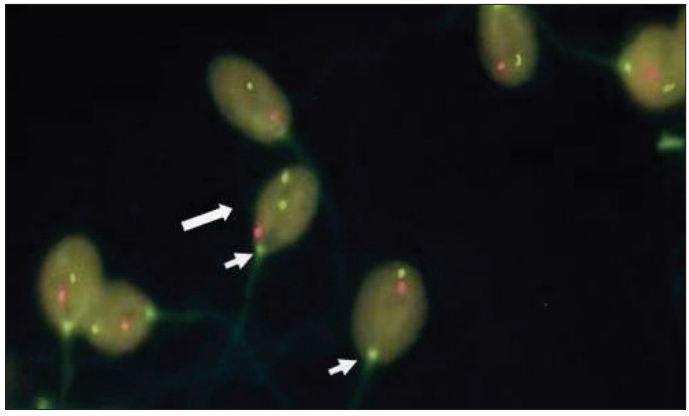

Figure 1. Fluorescence in situ hybridization (FISH) of the patient's sperm cells, chromosome 13 (green) and chromosome 21 (red). Large arrow indicates the cell with disomy. Mostly, the sperm head and neck junction have the green signal (shown by small arrows)

DNA were denatured together on the hot plate at 70°C for 5 min. The material was kept overnight at 37°C for hybridization. The slides were washed 2 times with 2xSSC and 2 times with 4xSSC-tween-20. Then, 10 μL DAPI (6-diamino-2-phenylindole; Sigma, St. Louis, USA) was added onto the slides and they were mounted with the coverslips and analyzed under the fluorescence microscope.

Scoring criteria

The slides were evaluated under the epifluorescence microscope (Nikon, Tokyo, Japan) equipped with DAPI, fluorescein isothiocyanate (FITC), aqua, rhodamine, and dual band filters. If the sperm nucleus showed two clear signals for one of the studied chromosomes while it showed one clear signal for the other chromosome, the sperm nucleus was evaluated to be disomic for the first chromosome. Since the expected value of the nullisomic sperms would be equal to the disomies and it is difficult to determine whether this situation arose from the nondisjunction or from a technical artifact, they were not taken into consideration.[14] Further, the sperm nuclei that overlapped, those whose borders could not be distinguished, those with widespread or indistinguishable signals, and the ones without tails were excluded from the study. The disomies detected in the sperm nuclei of the patients by FISH are shown in Figure 1.

Statistical analyses

ICE in individuals with structural chromosomal disorders, and the semen parameters obtained from the control and patient groups were statistically compared to the disomy rates. Statistical Package for the Social Sciences 10.01 (SPSS Inc., Chicago, USA) for Windows statistics package program was used. Categorical data were evaluated by the Chi-square test. Mann-Whitney U Test was used for the comparison of the age and semen parameters between the patient and control groups. The tables were prepared using the Microsoft Office Excel 2003 package program.

Results

A total of 51921 sperm nuclei, 19484 for patients and 32437 for controls, were analyzed. There was no significant difference in the ratio of the sperm with chromosome X to sperm with chromosome Y in the patient and the control groups. The

Table 2. The disomy rates in patients

Groups	Patients	No. of sperms	Frequency of disomy [f% (n)] XY	XX or YY	18	No. of sperms	2	12	No. of sperms	13	21	No. of sperms	3	17	No. of sperms	Frequency of disomy [f% (n)] pooled disomy **
Reciprocal translocation carrier group n=3	P1	594	0.50 (3)	0.17 (1)	0.17 (1)	608	0.16 (1)	0.33 (2)	505	0.20 (1)	0.20 (1)	503	0.20 (1)	0.40 (2)	2,210	0.54 (12)
	P11	516	0.19 (1)	0.39 (2)	0	692	0.14 (1)	0.14 (1)	503	0.40 (2)	0.20 (1)	585	0.51 (3)	0.34 (2)	2,296	0.48 (11)
	P12	607	0.16 (1)	0.16 (1)	0.33 (2)	727	0.14 (1)	0.14 (1)	504	0.40 (2)	0.40 (2)	529	0.38 (2)	0.38 (2)	2,367	0.51 (12)
	Total	1,717	0.29 (5)	0.23 (4)	0.18 (3)	2,027	0.15 (3)	0.20 (4)	1,512	0.33 (5)	0.27 (4)	1,617	0.37 (6)	0.37 (6)	6,873	0.51 (35)
Robertsonian translocation carrier group n=5	P2	540	0.37 (2)	0	1.48 (8)*	614	0.16 (1)	0.49 (3)	NC	NC	0.20 (1)	478	0.21 (1)	0.42 (2)	2,141	0.84 (18)*
	P10	462	1.08 (5)*	0.22 (1)	0.22 (1)	505	0.20 (1)	0.20 (1)	NC	NC	0.49 (2)	505	0.60 (3)	0.40 (2)	1,879	0.85 (16)*
	P22	513	0.19 (1)	0	0.19 (1)	507	0.20 (1)	0.20 (1)	NC	NC	0.58 (3)	504	0.40 (2)	0.40 (2)	2,044	0.54 (11)
	P5	559	0.36 (2)	0.18 (1)	0.18 (1)	725	0.14 (1)	0.14 (1)	503	0.40 (2)	0.20 (1)	507	0.79 (4)*	0.60 (3)	2,294	0.61 (14)
	P4	565	0.18 (1)	0	0	628	0.32 (2)	0.16 (1)	503	0.20 (1)	0.40 (2)	543	0.37 (2)	0.18 (1)	2,239	0.40 (9)
	Total	2,639	0.42 (11)	0.08 (2)	0.42 (11)*	2,979	0.20 (6)	0.24 (7)	1,006	0.30 (3)	0.38 (9)	2,537	0.48 (12)*	0.40 (10)	10,597	0.64 (68)*
	P20	252	1.19 (3)*	0	0.79 (2)*	583	0.17 (1)	0.34 (2)	545	0.55 (3)	0.37 (2)	634	0.31 (2)	0.31 (2)	2,014	0.70 (14)*

*p<0.05, **The term "pooled disomy rate" states the total rate of aneuploidies observed for the investigated chromosomes (2, 3, 12, 17, 18, 21, X, and Y) except chromosome13. P: patient; NC: not calculated

mean age of the patient group was 35.56±8.40 years while that for the control group was 32.86±6.98, and there was no statistical difference (p>0.05). The clinical history, age, and karyotype of the patients are detailed in Table 1. The patient group was divided into subgroups as the RCP carrier group and the ROB carrier group. Since there was only one individual with inversion (P20/inv(6)(p22q13)), the case was implicated in the patient group in the comparisons between the control and patient groups. Because the rates of the disomy X and disomy Y were low, they were categorized under disomy (X)+(Y). The results are presented in Table 2.

The semen parameters of the patients are shown in Table 3. When the semen parameters of the patient and control groups were compared, there were statistically significant differences among the viability, count, and morphology values (p<0.001, p<0.01, and p<0.05, respectively) while no differences were observed for the volume and motility (p=0.975 and p=0.301, respectively).

Aneuploidy in the patient carriers of a reciprocal translocation

No statistical difference in the disomy rates was observed for the patient carriers of RCP.

Aneuploidy in the patient carriers of a Robertsonian translocation and inversion

The rate of XY disomy was found to be statistically higher in cases P10 and P20. The rate of disomy 18 in cases P2 and P20, the rate of disomy 3 in P5, and the rate of pooled disomy in cases P2, P10, and P20 were found to be statistically higher (p<0.05). The disomy rates for chromosome 3 and 18 in the ROB carrier group were found to be statistically higher than that in the control group (p<0.05). The pooled disomy rates in the ROB carrier group were found to be statistically higher than in the control group (p=0.001 and p=0.001, respectively).

Pooled disomy and patient group

The pooled disomy rates observed in the sperm nuclei in the patient and control groups are given in Table 4. The term "pooled disomy rate" points to the total rate of aneuploidies observed in all the individuals for the investigated chromosomes (2, 3, 12, 17, 18, 21, X, and Y chromosomes). In the patient group, chromosome 3 disomy was the most common disomy (0.42%) and it was statistically higher than the control group (p=0.018). The most frequently observed

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Table 3. Semen profile of the patients according to Kruger's Strict criteria

Parameters/patients	P-1	P-2	P-4	P-5	P-10	P-11	P-12	P-20	P-22
Volume (mL)	4.5	4.8	1.7	4.0	3.1	2.8	3.2	1.5	1.9
Sperm conc. ($\times 10^6$/mL)	21	28	62	6	7	243	36	6	22
Progressive motility (%)	39	40	44	18	22	49	64	66	13
Morphology (% normal)	2	2	11	1	1	6	6	8	2
Viability (%)	42	43	65	44	22	73	67	94	58

P: patient

Table 4. The pooled disomy rates observed in the patient and control groups

Patient Group			Control Group		
Disomy	**Two signals**	**Total no. of sperm**	**Disomy**	**Two signals**	**Total no. of sperm**
XX+YY	6 0.13%	4,608	XX+YY	9 0.11%	8,046
2	10 0.18%	5,589	2	10 0.12%	8,616
18	16[a] 0.35%	4,608	18	10[a] 0.12%	8,046
12	13 0.23%	5,589	12	14 0.16%	8,616
3	20[a] 0.42%	4,788	3	16[a] 0.19%	8,293
XY	19[a] 0.41%	4,608	XY	16[a] 0.20%	8,046
17	18 0.38%	4,788	17	23 028%	8,293
13*	11 0.36%	3,063	13	22 0.29%	7,482
21	15 0.33%	4,499	21	28 0.37%	7,482
Pooled disomy **	117[a] 0.60%	19,484	Pooled disomy **	126[a] 0.39%	32,437

[a]p<0.05, * When calculating disomy 13 rate in patient group, chromosome 13 aneuploidies were not incorporated into P2, P10, and P22. ** The term "pooled disomy" states the total aneuploidies observed for the investigated chromosomes (2, 3, 12, 17, 18, 21, X, and Y chromosomes), except chromosome 13.

disomy was the disomy of chromosome 21 in the control group but there was no statistical difference between the control and patient groups (p=0.710). Disomy 3, disomy 18, XY disomy, and the pooled disomy rates of the patient group were found to be statistically higher in the patient group (p=0.018, p=0.008, p=0.028, and p=0.001, respectively). The least frequently seen disomies in both the patient and control groups were disomy (X)+(Y) and disomy 2.

Discussion

Lejeune doubted ICE when he observed an increased rate of balanced RCP carriers among the fathers of children with trisomy 21.[7] It was also reported that there was an increase in the sex chromosome aneuploidy and chromosome 21 in the fathers of children with Down syndrome, Turner syndrome, and Klinefelter syndrome.[15]

Literature does not offer any ICE studies in any case that has the same breakpoints belonging to the RCP carriers in our study. No ICE was found in the patient carriers with RCP. It has been reported that the type of translocation and chromosome, and the breakpoint position may affect ICE.[16] Although the rates of disomy for chromosome 21 in the control group were found to be higher than that in the RCP carrier group, there was no statistical difference ($p>0.05$). The increased incidence of RCP in the families with a Down syndrome child may determine if these parents would have their children at older ages because the problems the partners of the RCP carriers have at conceiving and maintaining pregnancy can lead to having children at older ages.

There are various theories about the mechanism of the occurrence of ICE. The studies on the synaptonemal complex demonstrated that the structural disorders could lead to aneuploidy by causing decreases in the number of chiasmata and pairing the anomalies at the pachytene stage.[8] It was also reported that the non-coupling areas led to disorders at the cell cycle checkpoints.[8] In balanced RCP carriers, the quadrivalent structure formed in the meiosis I-prophase pachytene can lead to unpaired areas and formation of unsynapsed segments. It has been argued that these unsynapsed segments could affect the other bivalents and increase the rate of nondisjunction in the chromosomes.[9] ICE can occur as a result of a series of events similar to the trivalent structure formation at the pachytene stage of the meiosis I-prophase in the ROB carriers. A relationship was observed between the sex vesicle in the meiosis I-prophase and the p-arm of acrocentric chromosomes.[8] ICE has also been explained by the formation of heterosynapsis between the sex vesicle (X-Y bivalent) and translocated chromosomes during meiosis I.[8] The electron microscopy studies have shown that the rates of heterosynapsis varied from translocation to translocation and the idea that ICE depended on the type of translocation was proposed, and that this rate was more consistent in the individuals with the same translocation.[14] However, there is no evidence that ICE can occur as a result of the formation of heterosynapsis that happens between the sex vesicle and translocated chromosomes.

In the literature, there are studies that evaluated ICE in the ROB carriers. ICE was detected in 4 of 5 patient carriers of ROB. The rates of disomy and the affected chromosomes might prove to be different in the patients carrying the same translocation. This finding indicates that ICE involving different chromosomes in these patients refers to the existence of individual differences in ICE. The pooled disomy rates of both ROB (13;14) carrier patients were found to be statistically higher than that in the control group ($p<0.05$). In the literature, the rates of disomy 18 and XY disomy were higher in the ROB carrier patients.[17] Similarly, in the present study, the rates of disomy 18 and XY disomy were higher in the ROB carrier group. In the literature, the studies also reported that the sex chromosome disomy rates in the ROB carriers were higher.[18] In another study, it was observed that the ROB carriers had higher sex chromosome disomy rates than that in the controls.[16] However, it was not classified as observed aneuploidy types, such as disomy X, disomy Y, and disomy XY. Chromosome XY disomy is related to an error in meiosis I, whereas disomy X and disomy Y are related to an error in meiosis II. It is necessary to have the error in meiosis I to indicate ICE.[8] Some studies had categorized disomy X, disomy Y, and disomy XY data under a single category.[8] These reports show that there are no standard evaluation criteria for the effect of chromosomal rearrangement on ICE.

The trivalent structure formed at the meiosis I-prophase pachytene in the ROB carriers may conduce to ICE by causing coupling errors and/or decreases in the number of chiasma. The studies on the synaptonemal complex demonstrated that there was a close relationship between the synaptonemal complexes belonging to chromosomes 15 and chromosome 21, and the sex vesicle.[8] It was stated that this condition might be taking its roots from the sequence homology in the q arm of the Y chromosome and the short arms of chromosomes 15 and 21.[8]

In our study, in the inv(6) carrier, who was not included in any subgroups, the rates of disomy 18, disomy XY, and the pooled disomy were found to be statistically higher than that in the control group ($p<0.05$). In the literature, the inversion carriers are less prone to show ICE.[8] This might be based on the dissimilarity between the breakpoints of the inversion carriers and in our patient. In addition, it was reported that there is an inverse correlation between the rates of disomy and chromosome size[16], and the small chromosomes and sex chromosomes are more prone to aneuploidy.[15] Similarly, our results showed that the aneuploidy rates of the small chromosomes and sex chromosomes in both the control and patient groups were higher.

In the literature, some studies emphasized that there was no ICE by analyzing a limited number of chromosomes through FISH but ICE involving different chromosomes in carriers with the same arrangement can be seen. If more chromosomes are analyzed in the studies, more dependable results can be obtained.

In the literature, there were individual differences between the aneuploidy rates of the sperms in the normal individuals.[19] Furthermore, it was observed that there was a difference even in the aneuploidy rate of the sperm samples obtained from the same individual at different times.[19] Great differences were seen between the individuals and chromosomes when ICE was evaluated in the patients carrying the same translocation.[13] The reason for these differences may be related to the dissimilarity of the criteria in patient selection, patients from different geographic areas related to the environment, differences in sexual abstinence before the collection of the semen samples, differences in the

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application of the FISH technique and the probes used, and the lack of using the same scoring criteria at all times.[13]

There was a statistical difference among the viability, count, and morphology values of the patient group as compared to that in the control group ($p<0.05$). It is known that the frequency of aneuploidy in the sperm nuclei is correlated with the poor semen parameters.[15] The aneuploidy rate in our patient group was also found to be higher than that in the control group. Therefore, it is not clear whether the detected aneuploidy is because of ICE or poor semen parameters. It was observed that the infertile carriers of the structural chromosomal abnormalities may generate lower counts of sperm aneuploidy than the chromosomally normal men with similarly impaired semen parameters.[20] It can be caused by different reasons of male infertility. Limitation of our study includes the relatively small number of patient population.

Consequently, while ICE was detected in the patient and ROB carrier groups, it was not detected in the RCP carriers. These results should not be evaluated as general results because there are differences regarding ICE even in cases showing the same anomaly. Therefore, the studies that can demonstrate the mechanism of these differences should be planned. The molecular genetics and synaptonemal complex studies should be conducted to understand the mechanism of ICE. In addition, the extent of the effectiveness of the factors affecting the semen parameters in the genome of the sperm should be determined. When all these data are evaluated together, there may be a chance to predict the individual risks for the couples with clinical risks of having a healthy baby.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Selçuk University (numbered 2008/035).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - H.A.; Design - H.A., Ö.B.; Supervision - H.A.; Resources - H.A., Ö.B.; Materials - H.A., Ö.B.; Data Collection and/or Processing - Ö.B.; Analysis and/or Interpretation - Ö.B., H.A.; Literature Search - Ö.B., H.A.; Writing Manuscript - Ö.B.; Critical Review - H.A., Ö.B.; Other - Ö.B., H.A.

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