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Original scientific paper

INCIDENCE OF GENETIC CAUSES OF IDIOPATHIC MALE INFERTILITY IN SERBIA – TEN YEARS' EXPERIENCE OF SINGLE CENTRE

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The aim of the study was to estimate the type and the prevalence of chromosomal abnormalities and Y-chromosome microdeletions, analysed together for the first time in idiopathic infertile men in Serbia. During 10 years period among 823 couples with infertility problems, in 110 cases the cause of infertility was severe oligospermia or azoospermia in male partners. All of them underwent cytogenetic analysis, performed according to standard techniques. Testing for the presence of Y-chromosome microdeletions in AZF regions using multiplex PCR was done in all patients with normal karyotype (97) and in three cases with cytogenetically visible aberrations of Y chromosome, in order to specify the breakpoints. The overall prevalence of chromosomal abnormalities in the group of 110 infertile men was 11.82%. The most frequent aberration was Klinefelter syndrome (47, XXY), being found in 5.45%. Chromosomal aberrations

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were found in 13.89% in group of men with azoospermia, and in 7.89% in group of men with severe oligospermia. Among the infertile men with normal karyotype, the incidence of microdeletions of AZF regions was 7.22%. Two types of deletions were identified: AZFc and AZFbc, with frequencies of 6.19% and 1.03%, respectively. Y-chromosome microdeletions were found in 6.45% of azoospermic patients, and in 8.57% of severe oligospermia group of patients. Our findings demonstrate the presence of higher frequency of chromosome aberrations and Y-microdeletions in a group of infertile men with azoospermia/oligospermia in Serbia. Results confirmed importance of offering these tests as part of genetic counselling of infertile couples in our country.

Keywords: male infertility, azoospermia, oligospermia, karyotype, Y-chromosome microdeletions

INTRODUCTION

Infertility is defined as a failure to conceive over 12 months of unprotected sexual intercourse and affects approximately 10-15% of couples in reproductive ages. A male factor of infertility accounts for 30-50% of cases and represents a heterogeneous group of disorders with different etiologies. The congenital and acquired factors which are causes of male infertility can be described by immunological factors, infections, endocrine disorders or anatomical malformations (https://www.ncbi.nlm.nih.gov/books/NBK1339) (NG *et al*., 2009; CHOI *et al*., 2013). There is also an increasing frequency of cases with unknown etiology ("idiopathic infertility") where genetic factors were found to have an important role (OCAK *et al*., 2014).

Karyotype analysis is the basic genetic test that is performed in men with idiopathic infertility, since it was observed that in this group of patient's frequency of aberrant karyotype was multiple times higher than in the group of fertile men. Different studies showed reduction of sperm number and/or function and gametogenic impairment can be associated with chromosomal anomalies. The most common found genetic cause is numerical sex chromosomal abnormality, Klinefelter syndrome (47, XXY). Also it was shown that structural chromosome aberrations (both sex and autosomal chromosomes) such as reciprocal and Robertsonian translocations and inversions occur 5-10 times more frequently in infertile men with azoospermia and severe oligospermia (FORESTA *et al*., 2005; ARCHANA, 2009; MASSART *et al*., 2012).

Microdeletions of the azoospermia factor (AZF) regions located in the long arm of Y chromosome are also important causes of idiopathic male infertility. Frequency of Y-microdeletions in azoospermic and oligospermic men is significantly higher comparing incidence in general population (1:4000). Three spermatogenetic loci of azoospermia factors have been mapped (AZFa, AZFb and AZFc) and it is assumed that deletions within unstable amplicons clustered in this region directly influence genes responsible for normal spermatogenesis (CHOI *et al*., 2013; KRAUSZ *et al*., 2014).

At present, screening for possible genetic causes of infertility in men with azoospermia or severe oligospermia is the first step needed before offering assisted reproductive technique (ART). Cytogenetic and molecular genetic analyses have a significant impact on further treatment and genetic counselling of these patients (FORESTA *et al*., 2002; KRAUSZ *et al*., 2014).

In this study, we present and discuss the type and the frequency of both chromosomal abnormalities and Y-chromosome microdeletions, analysed together for the first time in idiopathic infertile men from Serbia with severe oligospermia or azoospermia

MATERIALS AND METHODS

Patients

During 10 years period (2006-2016), 823 couples with infertility problems visited the Genetic Counselling Service of the Institute for Mother and Child Health Care "Dr Vukan Cupic" in Belgrade, Serbia. In 110 couples the cause of infertility was the presence of azoospermia or severe oligospermia in male partners and these patients were included in this study. Group of patients in which the cause of sterility could be exposure to toxic agents or congenital absence of vas deference was excluded from this study. As control group we used already published data for fertile men (RISTANOVIC *et al*., 2007; HARTON and TEMPEST, 2012).

All patients underwent semen analysis during routine clinical practice management of infertile couples, since men who are candidates to ART are often being classified according to semen analysis. Diagnosis of azoospermia or severe oligospermia (<2-5x106 sperm/ml) was made according to World Health Organization (WHO) guidelines (WHO, 2010). Based on these results 110 patients were divided into two groups: 72 men with azoospermia (65.45%) and 38 men with severe oligospermia (34.55%). Peripheral blood samples of these patients were collected for cytogenetic analysis, and if needed, additional Y-chromosome microdeletions analysis in order to determine the cause of infertility. After receiving results from genetic testing, couples were offered genetic counselling.

Study was approved by the Ethical Committee of the Mother and Child Health Care Institute of Serbia “Dr Vukan Cupic” (No. 8/14; 2018).

Cytogenetic analysis

A karyotype analysis was performed for each patient following standard procedure for the cultivation of peripheral blood lymphocytes (GERSEN and KEAGLE, 2005). At least 11 metaphases were routinely analysed from phytohemagglutinin stimulated lymphocyte 72^{h} cultures of heparinised peripheral blood. Cytogenetic chromosome analysis was done at 550 to 700 band resolution according to standard G-banding technique (GTG) on a minimum 4 mitosis. When needed, constitutive heterochromatin staining, using C-bands by barium hydroxide using Giemsa (CBG) technique in addition was done. In cases with chromosome aberrations or suspected mosaicism number of analysed metaphases was increased up to 32-100. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2009).

Molecular genetic analysis

DNA analysis was performed in all patients with normal karyotype (97) and in three cases with cytogenetically visible aberrations of Y chromosome, in order to define the breakpoints. Genomic DNA was extracted from peripheral blood samples with EDTA using GenJet™ Genomic DNA Purification Kit (ThermoFisher Scientific Inc, USA) as instructed by the manufacturer. Analysis of AZF regions on Y chromosome was carried out by two multiplex PCR reactions, using sequence-tagged site (STS) mapped in this region. In both reactions simultaneously were multiplied all three AZF loci (AZFa, AZFb, AZFc) and two control fragment (*SRY* and *ZFY* gene), according to original method (SIMONI *et al*., 2004). As controls, normal female DNA, normal male DNA and negative control were run in parallel for each set of multiplex PCR tests. PCR reaction products were separated by electrophoresis on 2% agarose gel, and visualized by staining with ethidium bromide and exposure to ultraviolet light.

Interpretation of the results was based on the presence or absence of bands that characterize the products of specific AZF regions, and reports were written according to current recommendations (SIMONI *et al*., 2004; KRAUSZ *et al*., 2014)

RESULTS

Cytogenetic analysis

The overall prevalence of chromosomal abnormalities in 110 infertile men was 11.82% (13/110). Structural aberrations were detected in 6 patients (5.45%), while numerical chromosomal aberrations were detected in 7 patients mostly with diagnosis of azoospermia (6.36%) (Table 1). The most frequent aberration was Klinefelter syndrome (47, XXY), found in 6 patients (5.45%) (Table 2).

In a group of 72 patients with azoospermia the prevalence of abnormal karyotype was 13.89% (10/72), most of them having numerical aberrations (Table 1). In three patients Y structural aberrations were detected and they were precisely described with CBG technique and DNA analysis, used in addition (Table 2). In this group of azoospermic men, one patient had autosomal aberration (reciprocal translocation between chromosomes 8 and 22) (Table 1, Table 2).

In the group of 38 patients with oligospermia total number of chromosomal aberrations found was 7.89% (3/38). Three patients had abnormal karyotypes, one with Klinefelter syndrome and the other two had autosomal reciprocal translocations (Table 1, Table 2).

Table 1. Chromosomal abnormalities in the group of 110 patients with primary infertility

Diagnosis / number (N)	Abnormal karyotype N (%)		Normal karyotype N (%)
	Structural aberrations N (%)	Numerical aberrations N (%)	
Azoospermia (72)	4/72 (5.55)	6/72 (8.33)	62/72 (86.11)
Oligospermia (38)	2/38 (5.26)	1/38 (2.63)	35/38 (92.10)
Total (110)	6/110 (5.45)	7/110 (6.36)	97/110 (88.18)
	13/110 (11.82)		

Table 2. Aberrant karyotypes found in the group of 110 patients with primary infertility

Karyotype	Number of patients	
	Azoospermia	Oligospermia
46,X,der(Y)t(Y;Y)(p11.3;q11.1)dn	1	/
46,X,delY(q11.22)	2	/
46,XY,t(4;6)(q31;q21)	/	1
46,XY,t(2;8)(q37;p11.2)	/	1
46,XY,t(8;22)(q24.3;q13.1)	1	/
47,XXY	5	1
47,XYY	1	/
Total	13	

Molecular genetic analysis

DNA analysis of Y-chromosome microdeletions was done in 97 patients with normal karyotype, 62 having azoospermia and 35 with severe oligospermia. In total, 7/97 patients (7.22%) had microdeletions of AZF regions. Two types of deletions were identified: AZFc found in both group with frequency of 6.19% (6/97) and AZFbc, found in only one patient with azoospermia (1.03%) (Table 3) (Figure 1, Figure 2). Microdeletions of AZFa and AZFb regions separately were not detected.

Table 3. Microdeletions of Y chromosome in the group of 97 patients with primary infertility

Diagnosis / number (N)	Deletion of AZFc region N (%)	Deletion of AZFbc region N (%)	No deletion of AZF regions N (%)
Azoospermia (62)	3/62 (4.84)	1/62 (1.61)	58/62 (93.55)
Oligospermia (35)	3/35 (8.57)	0 (0)	32/35 (91.43)
Total (97)	6/97 (6.19)	1/97 (1.03)	90/97 (92.78)
	7/97 (7.22)		

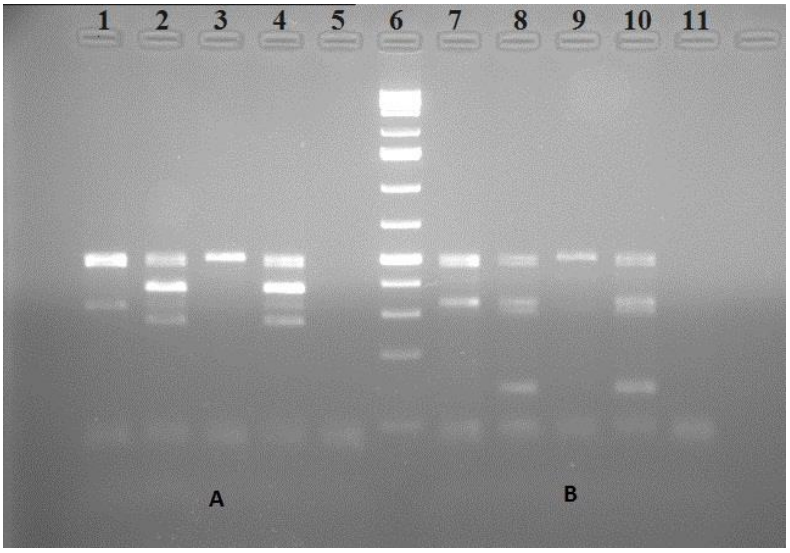

Figure 1. PCR-multiplex products for detection microdeletions in AZF region, 2% agarose gel, 85V, 1h.

A. Multiplex A PCR reaction, lane 1: patient with microdeletion of AZFbc region (del sY127 and del sY254); lane 2: sample wild-type; lane 3: wild type- woman control sample; lane 4: wild type-man control sample; lane 5: blanc; lane 6: GeneRuler 1kb DNA ladder

B. Multiplex B PCR reaction, lane 7: patient with microdeletion of AZFbc region (del sY134 and del sY255); lane 8: sample wild-type; lane 9: wild type- woman control sample; lane 10: wild type-man control sample; lane 11: blanc.

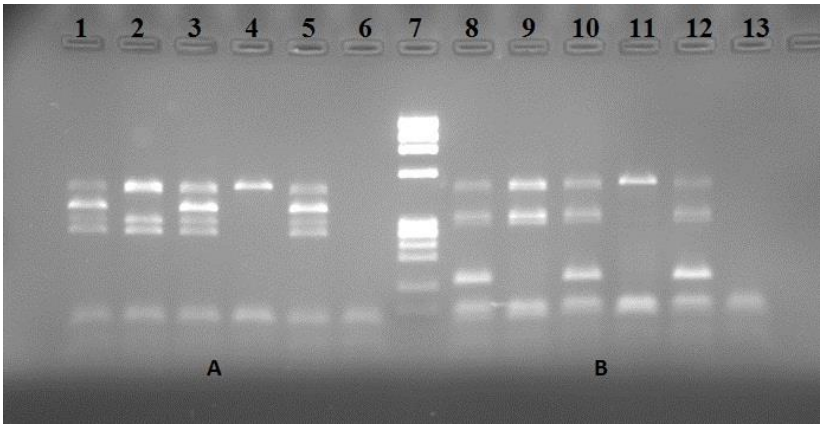

Figure 2. PCR-multiplex products for detection microdeletions in AZF region, 2% agarose gel, 85V, 1h.
A. Multiplex A PCR reaction, lanes 1 and 3: sample wild-type; lane 2: patient with microdeletion of AZFc region (del sY254); lane 4: wild type- woman control sample; lane 5: wild type- man control sample; lane 6: blanc; lane 7: ΦX174 DNA-Hae III Digest DNA ladder
B. Multiplex B PCR reaction, lanes 8 and 10: sample wild-type; lane 9: patient with microdeletion of AZFc region (del sY255); lane 11: wild type- woman control sample; lane 12: wild type- man control sample; lane 13: blanc.

In addition, the high molecular diversity observed using SSR and ISSR has also been described in the analysis of mulberry cultivars from South Korea and India (KALPANA *et al.*, 2012; KRISHNAN *et al.*, 2014a; BANERJEE *et al.*, 2016) and wild mulberry populations from India and China using SRAP markers (BAJPAI *et al.*, 2014; HU *et al.*, 2015). The propagation system through seed (GARCÍA *et al.*, 2006) should be the responsible of this great diversity.

DISCUSSION

Chromosomal aberrations, single-gene mutations and Y-chromosome microdeletions are important genetic factors found to be causes of male infertility. Individuals with karyotype abnormalities, either numerical or structural, have a predisposition to chromosomally abnormal conceptions, often associated with reduced fertility due to a failure of achieving a successful pregnancy. According to the literature, incidence of chromosomal abnormalities in healthy fertile men is approximately 0.6% (HARTON and TEMPEST, 2012). In normozoospermic infertile men, frequencies of chromosomal aberrations are significantly higher, being found in 2-8% (on average 3%: sex chromosomes 1.4% and balanced structural aberrations 1.6%) (FORESTA *et al.*, 2002). Further, frequencies found worldwide for infertile men with reduced sperm number are higher, ranging from 2.2-27% (PENNA VIDEAU *et al*., 2001; FORESTA *et al*., 2002; 2005; VICDAN *et al*., 2004; YATSENKO *et al*., 2010).

Results of this study showed that chromosomal aberrations were presented in infertile men multiple times more than in fertile men in worldwide population (HARTON and TEMPEST, 2012). We analysed 110 patients from Serbia with reduced sperm count, 13 of which (11.82%) had chromosomal anomalies with similar frequencies of both numerical and structural

aberrations (Table 1). This high frequency is in the range of higher rates of previously described rates in other populations 2.2-14.3% (VICDAN *et al*., 2004; ARCHANA, 2009; YATSENKO *et al*., 2010). The highest prevalence, as expected, had patients with Klinefelter syndrome (5.45%), two of them not being clinically recognised until karyotype analysis was done. Sex chromosomes abnormalities were predominating, with frequency of 9.09%. This was similar to the results of the studies done in other populations where numerical and structural autosomal chromosome aberrations were also less common (FORESTA *et al*., 2002; YATSENKO *et al*., 2010; OCAK *et al*., 2014).

The incidence of chromosomal abnormalities in the group of 72 infertile patients with azoospermia was 13.89%, which is in agreement with the other studies findings, where these aberrations were presented up to 15% (FORESTA *et al*., 2002; ARCHANA, 2009). Numerical aberrations had higher frequency, due to the fact that the commonest anomaly was Klinefelter syndrome, with a prevalence of 6.95%, which is also in agreement with literature data (YATSENKO *et al*., 2010). One patient in azoospermic group had 47, XYY karyotype (1.39%), with controversial clinical significance. Infertility in such cases may be associated with Y chromosome gene dose effect (gain or loss), altered meiotic segregation and sperm apoptosis and necrosis (YATSENKO *et al*., 2010). Results from other studies showed lower incidence of 47, XYY karyotype in infertile men (0.26%) (MAFRA *et al*., 2011). Structural karyotype abnormalities that have been identified in this azoospermic group included three patients with aberrations of Y chromosome (4.17%) and one patient with reciprocal autosomal translocation (1.39%) (Table 2). Similar frequencies were detected in other studies done worldwide (FORESTA *et al*., 2002; YATSENKO *et al*., 2010; AKBARI *et al*., 2012).

In the group of 38 patients from Serbia with severe oligospermia abnormal karyotype was detected in three patients (7.89%), one with numerical (47, XXY, 2.63%) and two with structural aberrations (reciprocal autosomal translocations, 5.26%) (Table 1, Table 2). Oligospermic men were investigated as isolated group and there is a limited data described in the literature. Data from the literature suggested lower frequency of Klinefelter syndrome in patients with oligospermia then in azoospermic men, which is also found in our population. Our results showed higher proportion of structural rearrangements than aneuploidies in patients with oligospermia, which is in agreement with other studies (ranging from 0.8-6.6%) (ARCHANA, 2009; YATSENKO *et al.*, 2010; AKBARI *et al*., 2012; MASSART *et al*., 2012). Differences in frequencies found can be explained by a small number of analysed patients, but there was a trend of chromosomal anomalies more present in patients with severe oligospermia then in normozoospermic infertile men.

Number of data published in the literature showed that Y-chromosome microdeletions of AZF regions were frequently associated with a failure of spermatogenesis, found to be the second most common cause of disruption of spermatogenesis, after Klinefelter's syndrome. The analysis of the individual genes in the AZF regions in infertile men is essential for the accurate determination of the genotype-phenotype correlation (FORESTA *et al*., 2002; KRAUSZ *et al*., 2014). For example, the diagnosis of complete deletions of AZFb or AZFbc is related to virtually zero chance for testicular sperm retrieval. On the other side, in men with azoospermia and AZFc deletion there is approximately 50% chance of retrieving sperm from testicular sperm extraction (SIMONI *et al*., 2004; KRAUSZ *et al*., 2014).

In this study all patients with normal karyotype were further analysed for the presence of microdeletions. The overall frequency of AZF microdeletions was 7.22%, while in control

fertile group of men from Serbia microdeletions was not found (RISTANOVIC *et al*., 2007). The AZFc region was the most frequently deleted (6.19%), followed by the deletions of AZFbc region (1.03%) (Table 3). These data are in agreement with the results studies done in other populations. In general, frequency of AZF microdeletions is 10%, going up to 15% in patients with oligospermia and up to 20% in azoospermic men (FORESTA et al., 2002). According to the literature, the ranges of Y-chromosome microdeletions in infertile men vary very widely from 1-55% (OCAK et al., 2014). The differences in reported frequencies may reflect variations in the sample group, ethnic and geographic differences and selection of STS markers (NG *et al*., 2009; KRAUSZ *et al*., 2014). For example, overall frequency found in this group of primary infertile patients with reduced sperm count was lower compared to previously done analysis of an isolated group of patients from Serbia who unsuccessfully underwent ICSI (15.6%) (RISTANOVIC *et al*., 2007).

In the group of 62 Serbian patients with azoospermia, incidence of microdeletions was 6.45%. The AZFc region was the most frequently deleted (4.84%) and deletion of AZFbc region was found in one patient (Table 3). These results agree with the frequencies obtained in different countries, for example Brazil, Slovakia, Turkey, Slovenia, Macedonia (PETERLIN *et al*., 2002; PLASESKI *et al*., 2003; BEHULOVA *et al*., 2011; MASSART *et al*., 2012). High incidences of microdeletions (over 40%) in azoospermic men were found mostly in studies conducted on a small number of patients, for example in Iran, Brazil and Netherlands (BEHULOVA *et al*., 2011)

In our group of patients with severe oligospermia, microdeletions was detected in three patients (8.57%), all having deletion of AZFc region (Table 3). Higher frequency observed in comparison with group of azoopermic men can be explained by a smaller number of patients. But it is known that deletion of AZFc region is compatible with residual spermatogenesis and therefore can be found often in oligospermic men. Results of the analysis of patients with oligospermia in neighbouring and other worldwide populations showed similar data (ranging from 1.5-15%) (FORESTA *et al*., 2002; PETERLIN *et al*., 2002; MAFRA *et al*., 2011)

At the end, the significance of using these genetic tests is not only to explore the cause of the infertility but also to assess the risk of a given couple to transmit its genetic variants. In cases when chromosomal abnormality is present, there is a higher risk for having a child with chromosomal disorder. When Y chromosome microdeletions are present, they can be transmitted to the sons. It was also observed that these patients are at the higher risk for having 45,X female children (FORESTA *et al*., 2002; KRAUSZ *et al*., 2014)

CONCLUSION

In summary, our results demonstrate the higher frequency of chromosome aberrations and Y-microdeletions in infertile men from Serbia with azoospermia or oligospermia compared to fertile men. Findings are in concordance with proposed protocols by which these patients should be first karyotyped. In cases of normal karyotype, analysis of AZF microdeletions is recommended.

Karyotype and Y-microdeletions analyses have both diagnostic and prognostic value in men with azoospermia or oligospermia, therefore it is necessary to offer genetic counselling to infertile couples before planning the family.

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UČESTALOST GENETIČKIH UZROKA IDIOPATSKOG MUŠKOG STERILITETA U SRBIJI- DESETOGODIŠNJE ISKUSTVO JEDNE USTANOVE

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Izvod

Jedan od uzroka steriliteta, značajnog medicinskog problema današnjice, vodi poreklo od poremećaja reproduktivne sposobnosti muškarca. Zbog sve veće učestalosti steriliteta nepoznate etiologije („idiopatski sterilitet"), citogenetičke i molekularno genetičke analize su danas esencijalne u dijagnostičkoj proceduri ispitivanja uzroka muškog steriliteta. Osnovni cilj ovog rada je detekcija tipa i učestalosti hromozomskih aberacija i mikrodelecija Y hromozoma kod muškaraca iz Srbije sa dijagnostikovanim idiopatskim sterilitetom. Tokom desetogodišnjeg perioda (2006-2014), od 823 bračna para, uzrok steriliteta kod 110 parova je bio prisustvo azospermije/teške oligospermije kod muškog partnera. Svim muškim partnerima je urađena analiza kariotipa standardnim tehnikama citogenetike. Određivanje prisustva/odsustva mikrodelecija AZF regiona Y hromozoma pomoću metode multipleks PCRa, urađeno je kod svih pacijenata sa normalnim citogenetičkim nalazom (97) i kod tri pacijenta sa citogenetički vidljivom aberacijom na Y hromozomu. Učestalost hromozomskih aberacija kod svih ispitivanih pacijenata (110) iznosila je 11,82% (Klinefelterov sindrom kao najčešće detektovana aberacija sa učestalošću od 5,45%). Aberantan kariotip je u grupi ispitanika sa kliničkom dijagnozom azospermije uočen u 13,89% slučajeva, dok je 7,89% detektovano kod pacijenata sa teškom oligospermijom. Ukupna učestalost mikrodelecija AZF regiona iznosila je 7,22%. Detektovana su dva tipa mikrodelecija: AZFc i AZFbc (učestalost 6,19% i 1,03%, respektivno). Rezultati ove studije su pokazali veću učestalost hromozomskih aberacija i mikrodelecija na Y hromozomu kod muškaraca iz Srbije sa azospermijom i teškom oligospermijom. Takođe, ovi rezultati potvrđuju značaj primene ovakvih genetičkih testova u okviru genetičkog savetovanja bračnih parova sa problemom steriliteta u našoj zemlji.

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