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Short communication

CHROMOSOMAL INSTABILITY IN PERIPHERAL BLOOD LYMPHOCYTES OF PATIENTS WITH REPRODUCTIVE FAILURE ASSESSED BY MICRONUCLEUS ASSAY

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We investigated chromosomal instability in peripheral blood lymphocytes (PBL) of patients with reproductive failure in respect to age, smoking habits, gender, miscarriages, and semen parameters. The study involved 36 individual cases of reproductive failure (18 men and 18 women) attended at the Clinical Centre of Kragujevac, Serbia, and 30 healthy subjects (15 men and 15 women). Micronuclei (MN) frequency was estimated in PBL using the cytokinesis-block micronucleus (CBMN) assay. The baseline MN frequencies were significantly higher ($p=0.031$; $p<0.001$) in male [(9.22 ± 4.70) MN per 1000 BN cells] and female patients [(13.50 ± 2.5) MN per 1000 BN cells] than in male and female healthy controls [(6.27 ± 2.66) MN per 1000 BN cells; (6.80 ± 2.98) MN per 1000 BN cells]. The mean baseline MN frequency did not significantly differ between miscarriage groups and between patients with and without normal values of semen parameters. The correlations between poor sperm concentration ($<20x10^6$ mL^{-1}), rapid progressive motility (<25 %), normal morphology (<30 %), and MN frequencies were negative, but not statistically significant. We found that only gender significantly influenced the MN rates in analysed patients. There were no significant differences between age groups and between smokers and non-smokers in patients and control samples. We conclude that the increase in baseline MN frequency in PBL of patients with reproductive failure corresponds to the increase in chromosomal damage, which occurs as a result of complex events that cause reproductive disorders.

KEY WORDS: *age, gender, infertility, micronuclei, semen quality, smoking*

Infertility is defined as the inability to conceive after twelve months of regular unprotected sexual intercourse. It may be related to a variety of genetic (1, 2), as well as nongenetic factors (3-5).

Previous studies confirmed higher frequency of chromosomal abnormalities in the sperm of men with abnormal semen parameters (6, 7). Chromosomal abnormalities were also found in peripheral blood lymphocytes of infertile men (8, 9). Two comet assay studies (10, 11) reported a significantly higher level of DNA strand breaks in the sperm of infertile men compared to the controls. Duzcan et al. (12) suggested a greater incidence of sex aneuploidy in somatic cells of oligozoospermic men.

Female infertility is also associated with genomic instability. High incidence of genomic instability in lymphocytes of women with polycystic

ovary syndrome was revealed in the studies of Yesilada et al. (13) and Moran et al. (14). Moreover, couples with a history of spontaneous abortions and idiopathic infertility tend to have an increased micronuclei frequency in lymphocytes (15).

Micronuclei (MN) are defined as small, round nuclei clearly separated from the main cell nucleus which forms from acentric chromosome fragments or whole chromosome(s) during cell division. The frequency of MN as an index of chromosomal damage and genome instability is widely used for evaluating the genotoxic impact of demographic, habitual (16), occupational, and environmental factors (17, 18). Moreover, Bonassi et al. (19) consider that MN frequency may serve for predicting individual cancer risk.

Assuming that MN persistence in cells reflects chromosomal instability, the objective of this study was to evaluate spontaneous chromosomal damages in peripheral blood lymphocytes (PBL) of patients with reproductive failure and healthy controls by using cytokinesis-block micronucleus assay (CBMN) in respect of the factors that may affect MN frequency (i.e. age, smoking habits, gender, miscarriages, and semen parameters).

MATERIALS AND METHODS

Study populations

This study has been approved by the Ethics Committee of the Clinic of Kragujevac (No 01-4886). It included 36 individual cases of reproductive failure (18 men and 18 women) refereed to the Clinical Centre of Kragujevac, Serbia. All participants were informed about the aim of the study and completed a standardised questionnaire that included standard demographic, medical, lifestyle, and occupational questions.

The mean age of male patients was (35.22±6.68) years (range from 27 to 48 years). All of them underwent sperm analysis and three standard sperm parameters (sperm concentration, rapid progressive motility, and morphology) were determined. Numbers of spermatozoa per unit volume refers to the sperm concentration. Actively moving spermatozoa, linearly or in large circles, were scored for determining the progressive motility, while spermatozoa without malformations (head, neck, and tail defects) were scored for estimating the proportion of spermatozoa with normal morphology. Sperm concentration ≥20 $x10^6$ mL^{-1}, spermatozoa with rapid progressive motility ≥25 %, and normal sperm morphology ≥30 % were referred to as normal values. The samples of five patients revealed azoospermia, four were diagnosed with abnormalities only in one parameter, two in two parameters, and three in all three major parameters. Four men from couples with idiopathic infertility were assigned to the infertile group despite normal sperm parameter values.

All male patients kept standard dietary habits. Nine of them were smokers.

Mean age of female patients was (31.67±3.50) years (range from 24 to 37 years). Eleven of them were smokers and only one patient kept diet. Seven women had a history of miscarriages (ranged from 1 to 2).

The control sample included 15 age-matched healthy fertile male donors aging from 27 to 50 years [mean age (38.27±7.76) years], and 15 age-matched healthy fertile female donors, aging from 24 to 36 years [mean age (32.4±2.99) years] without any recent exposure to known mutagenic agents. Fifteen of them (7 men and 8 women) were smokers.

Cytokinesis – block micronucleus test (CBMN)

We applied the CBMN test, as proposed by Fenech (20), for the analysis of MN frequency in peripheral blood lymphocytes. Heparinised whole blood was cultivated in the complete medium (PBMax Karyotyping, Invitrogen, USA) for a total of 72 h at 37 ºC. Cytochalasin B (Sigma-Aldrich, USA), at a final concentration of 4 μg mL^{-1}, was added to cultures after 44 h of incubation. Using the standard procedure, cells were harvested to prepare microscopic slides that were then stained in the 2 % Giemsa solution (Alfapanon, Novi Sad, Serbia). The MN frequencies were determined by analysing 1000 BN cells per person following the standard criteria for scoring MN in BN cells as described by Fenech (21).

Statistics

Data were presented as mean ± standard deviation (S.D.). The differences between baseline MN frequencies in lymphocytes in male patients and healthy men, healthy women and healthy men, among male samples, and among healthy female samples

were compared using the Student′s t-test. The differences between MN frequencies of female patients and female controls, male and female patients, and among female patients were determined using Mann-Whitney U test. The relationship between age, smoking status, and gender and MN frequency in both patients and control samples was determined by multiple linear regression analysis. The relationship between poor semen parameters and MN frequency was determined by Pearson′s correlation. Level of significance was $p<0.05$.

RESULTS

Results of the CBMN assay in PBL of patients with reproductive failure and PBL of healthy controls are shown in Tables 1-5.

We found significantly higher baseline MN frequencies ($p<0.001$) in female patients [(13.50±2.50) MN per 1000 BN cells] in comparison with healthy female controls [(6.80±2.98) MN per 1000 BN cells]. The mean MN frequency in male patients was significantly higher ($p = 0.031$) than in control healthy men [(9.22±4.70) MN per 1000 BN cells vs. (6.27±2.66) MN per 1000 BN cells]. In all samples gender affected MN rates, but only in patients was the sample statistically significant ($p=0.011$).

In all analysed samples, BN cells with 1 MN were most frequently present. Cells with 2 MN were less common, while BN cells with 3 MN were found only in the patient sample (Table 3).

The mean MN frequency did not differ between smokers and non-smokers both in male patients and healthy men. Age had also no effect on the MN frequency in both analysed samples of men - patients and healthy controls.

Male patients were grouped according to their values for each analysed parameter. Statistical analyses for each analysed parameter (sperm concentration, rapid progressive motility, morphology) did not show any significant difference between the groups of

Table 1 *General characteristics, micronuclei frequency and MN distribution in male patients with reproductive failure*

No	Age / year	Smoking status (+/-)	Occupation	Semen parameters			MN per1000 BN cells	Distribution of MN		
				Sperm concentration / $x10^6$ mL^{-1}	Rapid progressive motility / %	Normal morphology / %		1 MN	2 MN	3 MN
1.	32	-	worker	5.50	37	22	3	3	0	0
2.	44	+	lawyer	0	0	0	13	11	1	0
3.	30	+	policeman	0	0	0	9	9	0	0
4.	35	-	engineer	51.37	38	26	9	7	1	0
5.	48	+	car mechanic	1.10	9	19	6	6	0	0
6.	48	-	instructor	58.9	34	35	17	14	0	1
7.	41	-	seller	0	0	0	4	2	1	0
8.	29	+	farmer	89.50	39	45	9	9	0	0
9.	29	+	doctor	5.90	7	28	6	6	0	0
10.	28	-	seller	0	0	0	16	13	0	1
11.	40	+	worker	27.6	30	48	12	10	1	0
12.	31	+	driver/ technician	8.58	28	32	1	1	0	0
13.	38	-	economist	50.25	21	45	7	7	0	0
14.	36	+	locksmith	6.10	35	22	14	12	1	0
15.	36	-	doctor	0	0	0	14	8	3	0
16.	32	-	engineer	141.25	25	41	9	9	0	0
17	27	+	driver	43.00	5	53	13	9	2	0
18.	30	-	seller	13.875	18	24	4	4	0	0
Mean ± S.D.	35.22 ± 6.68			27.94 ± 38.75	18.11 ± 15.41	24.44 ± 18.31	9.22 ± 4.70	7.78 ± 3.67	0.56 ± 0.86	0.11 ± 0.32

MN - micronuclei, BN - binucleated

Table 2 *General characteristics, micronuclei frequency and MN distribution in female patients with reproductive failure*

No	Age / year	Smoking status (+/-)	Dietary status (+/-)	Occupation	Miscarriages	MN per 1000 BN cells	Distribution of MN		
							1 MN	2 MN	3 MN
1.	31	-	-	engineer	0	21	19	1	0
2.	35	+	-	technician	0	17	12	1	1
3.	36	+	-	technician	1	12	12	0	0
4.	32	+	-	cook	0	13	13	0	0
5.	34	-	-	nurse	0	15	13	1	0
6.	33	-	-	doctor	2	16	16	0	0
7.	31	+	-	technician	2	12	12	0	0
8.	37	-	-	nurse	0	14	12	1	0
9.	29	+	-	locksmith	0	11	9	1	0
10.	25	+	-	worker	0	12	10	1	0
11.	30	+	-	worker	0	13	13	0	0
12.	28	-	-	seller	2	14	12	1	0
13.	33	+	-	cleaner	0	13	11	1	0
14.	33	-	+	seller	1	12	12	0	0
15.	32	+	-	technician	1	11	11	0	0
16.	24	+	-	worker	0	13	11	1	0
17.	35	+	-	housewife	1	13	13	0	0
18.	32	-	-	seller	0	11	11	0	0
Mean ±S.D.	31.67± 3.50					13.50±2.50	12.33± 2.22	0.5± 0.51	0.05± 0.23

MN - micronuclei, BN - binucleated

Table 3 *Micronuclei frequency and MN distribution in analysed patients and healthy controls*

	No.	Age / years Mean±S.D. (range)	No. of analysed cells	MN per 1000 BN cells Mean±S.D. (range)	No. of BN cell with MN / %	Distribution of MN (%)		
						1 MN No. (%)	2 MN No. (%)	3 MN No. (%)
Controls	30							
Male	15	38.27±7.76 (27 to 50)	15 000	6.27±2.66 (2 to 10)	92 (0.61)	90 (0.60)	2 (0.01)	0 (0.0)
Female	15	32.4±2.99 (24 to 36)	15 000	6.80±2.98 (1 to 11)[a]	102 (0.68)	102 (0.68)	0 (0.0)	0 (0.0)
Patients	36							
Male	18	35.22±6.68 (27 to 48)	18 000	9.22±4.70 (1 to 17)[b]	152 (0.84)	140 (0.78)	10 (0.05)	2 (0.01)
Female	18	31.67±3.50 (24 to 37)	18 000	13.50±2.5 (11 to 21)[c,d]	232 (1.29)	222 (1.23)	9 (0.05)	1 (0.01)

[a] *no statistically significant difference in MN frequencies between healthy women and healthy men, p=0.609 (Student's t- test)*
[b] *statistically significant difference in MN frequencies between male patients and male controls, p=0.031 (Student's t- test)*
[c] *statistically significant difference in MN frequencies between female patients and female controls, p<0.001 (Mann-Whitney U test)*
[d] *statistically significant difference in MN frequencies between female patients and male patients, p=0.011 (Mann-Whitney U test)*
MN - micronuclei, BN – binucleated

Table 4 *Micronuclei frequencies in male patients and healthy male controls with regard to demographic, lifestyle and medical characteristics*

Groups (number)	MN per 1000 BN cells Mean±SD (range)	p value
Patients [a]		
Smoking status		
Smoker (9)	9.22±4.29 (1 to 14)	1.00
Non-smoker (9)	9.22±5.33 (3 to 17)	
Age / years		
27 to 35 (10)	7.90±4.56 (1 to 16)	0.190
36 to 48 (8)	10.87±4.61 (4 to 17)	
Semen parameters		
Sperm concentration / $x10^6$ mL^{-1}		
<20 (11)	8.18±5.25 (1 to 16)	0.250
≥20 (7)	10. 86±3.39 (7 to 17)	
Rapid progressive motile spermatozoa (grade a)		
<25 % (10)	9.20±4.44 (4 - 16)	0.983
≥25 % (8)	9.25±5.31 (1 - 17)	
Normal morphology		
<30 % (11)	8.91±4.68 (3 to 16)	0.734
≥30 % (7)	9.71±5.06 (1 to 17)	
Healthy controls [a]		
Smoking status		
Smoker (7)	6.00±2.31 (3 to 9)	0.731
Non-smoker (8)	6.50±3.07 (2 to 10)	
Age / years		
27 to 39 (9)	6.55±3.13 (2 to 10)	0.624
40 to 50 (6)	5.83±1.94 (3 to 8)	

[a] *MN frequencies between groups were compared by Student's t-test*
MN - micronuclei, BN - binucleated

patients with normal values of semen parameters and those who had lower values (Table 4).

The mean MN frequency did not differ between non-smokers and smokers in both female patients and female healthy controls. In both analysed samples of women, patients and controls, age had no effect on the MN values. The MN frequency between female patients with and without miscarriage history was not significantly different (Table 5).

Multiple linear regression analyses showed that among the analysed confounding factors (i.e., age, smoking habits, and gender), only gender had a significant effect on the MN values in the analysed sample of patients (p=0.001). Neither of the analysed factors affected MN frequency in the analysed healthy control sample.

The result of Pearson′s correlation analyses showed a negative correlation between poor values of sperm concentration (<$20x10^6$ mL^{-1}), rapid progressive motility (<25 %), normal morphology (<30 %) and MN frequencies but without statistical significance (r=-0.524, p=0.098; r=-0.545, p=0.103; r=-0.463, p=0.152).

DISCUSSION

Chromosomal instability can be defined as an increased rate of numerical and structural chromosomal changes during cell division. Different human health problems are associated with chromosomal instability in lymphocytes (22–24). Today, cytogenetics provides numerous different biomarkers for evaluating chromosomal instability and scoring of MN in lymphocytes is one that draws a lot of interest.

Our results show that mean MN frequencies in both male and female patients increased in comparison with mean MN frequencies of healthy male and female controls. The approximately 1.5-

Table 5 *Micronuclei frequencies in female patients and healthy female controls with regard to demographic, lifestyle and medical characteristics*

Groups (number)	MN per 1000 BN cells Mean±SD (range)	p value
Patients [a]		
Smoking status		
Smoker (11)	12.73±1.62 (11 to 17)	0.117
Non-smoker (7)	14.71±3.25 (11 to 21)	
Age / years		
24 to 31 (7)	13.71±3.35 (11 to 21)	0.854
32 to 37 (11)	13.36±1.96 (11 to 17)	
History of miscarriages		
Yes (7)	12.86±1.68 (11 to 16)	0.433
No (11)	13.91±2.91 (11 to 21)	
Healthy controls [b]		
Smoking status		
Smoker (8)	6.25±3.24 (1 to 11)	0.465
Non-smoker (7)	7.43±2.76 (4 to 11)	
Age / years		
24 to 32 (6)	6.83±2.99 (3 to 11)	0.973
33 to 36 (9)	6.78±3.15 (1 to 11)	

MN – micronuclei, BN - binucleated
[a] MN frequencies between groups were compared by Mann-Whitney U test
[b] MN frequencies between groups were compared by Student's t- test

fold MN increase in male patients and more than a two-fold MN increase in female patients indicated greater chromosomal damages in PBL of patients with reproductive failure. Recently, the association between MN frequency in PBL and impaired reproductive history has been reviewed by Fenech (25).

Similar, De Palma et al. (26) demonstrated an increase in sex chromosome aneuploidy rate in peripheral leukocytes of patients with impaired spermatogenesis and suggested that they had generalised defective cell division mechanism. Higher MN frequency in analysed patients with reproductive failure may be explained by abnormality in the mechanism that controls division.

Increased MN frequencies in patients may also be the consequences of oxidative stress (OS), which has been considered a contributing factor to infertility. The events that lead to MN formation in the cells may be induced by OS (19).

In our study, we obtained a great inter-individual variation in MN frequencies. Age, smoking, gender, dietary habits, and individual susceptibility to environmental or occupational mutagens are factors that account for the MN frequency variation. According to Fenech (27), age is an important demographic contributing factor. The same age effect on MN was reported in the studies of Ishikawa et al. (16) and Mahrous (28). In our study, age had no effect on MN frequency in both healthy subjects′ and patients′ samples. Trkova et al. (15) explained the lack of significant correlation between age and MN frequency by age homogeneity of tested samples. Besides this explanation, the lack of effect of age on MN frequency in analysed samples might be due to a small range of years. However, these results have to be confirmed in larger samples primarily designed to evaluate the effect of age.

Gender, as one of the contributing factors, was analysed in many studies. In most of them, MN frequencies have been reported to be higher in women than in men (27, 29); generally by 1.2 to 1.6 times (27). This appearance could be explained by over-prevalence of X chromosome in female MN (30). Although in our study, women had higher MN frequency in both healthy control and patient samples, a significant difference in MN frequencies was evident only in the patient sample. Women had approximately 1.5-fold higher MN frequency than analysed male patients.

In addition to age and gender, smoking habits may modulate MN frequency. Bonassi et al. (31) reported a significant effect of smoking on MN frequency only in the heavy-smoker group. In the present study, we did not find a statistically significant difference in MN frequency between smokers and non-smokers. No association between smoking habit and MN frequency was also observed in the study of Hessel et al. (32) and Costa et al. (33). It is possible that exposure to genotoxic substances from cigarette smoke induces serious impairments of cell division or cell death. However, negative results of CBMN assay in smokers might be explained by the "escape" of highly damaged cells from scoring. Contrary to these, micronucleated cells with one MN and less genome damages have a better chance of surviving (34). Thus, Bonassi et al. (31) explain that if damaged cells do not divide, they will not form binucleated cells, so these will not be scored for MN. Furthermore, adaptive response is often mentioned as a possible reason for lower MN frequency in the PBL of light - medium smokers (31, 35).

With regard to miscarriage history, in the analysed patient sample, women with a history of miscarriages had the same level of chromosomal damages in PBL as women with no conception in reproductive history. According to the study of Milošević-Djordjević et al. (36) and an earlier study of Grujičić et al. (37), women with threaded miscarriages had an increased MN frequency in lymphocytes and this could be the explanation for MN similarity between analysed women.

In our study, male patients with poor semen parameters did not have a significantly lower MN frequency in PBL compared to patients with normal values of semen parameters. As in smokers, lower MN frequencies in these patients might be explained by greater genome damages in their lymphocytes that escaped from scoring.

Although we did not find a statistically significant association between the levels of chromosomal instability (presented in the form of MN) and poor semen quality, negative correlation between these two parameters points to a tendency of higher chromosomal damages in PBL of infertile men.

CONCLUSION

From the present study it is evident that an increase in the baseline MN frequency in PBL of untreated men and women with reproductive failure corresponds to the increase in chromosomal damage, which occurs as a result of complex events that cause reproductive disorders.

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Sažetak

PROCJENA KROMOSOMSKE NESTABILNOSTI U LIMFOCITIMA PERIFERNE KRVI BOLESNIKA S POREMEĆAJIMA REPRODUKTIVNOG SUSTAVA POMOĆU MIKRONUKLEUS-TESTA

Ispitivali smo kromosomsku nestabilnost u limfocitima periferne krvi (engl. *peripheral blood lymphocytes* - PBL) bolesnika s poremećajima reproduktivnog sustava u odnosu na parametre dobi, navike pušenja, spola, spontanih pobačaja i kvalitete sjemena. Ispitivanje je uključivalo 36 pojedinačnih ispitanika s poremećajima reproduktivnog sustava (18 muškaraca i 18 žena) u Kliničkom centru u Kragujevcu, Srbiji, te 30 zdravih ispitanika (15 muškaraca i 15 žena). Učestalost pojave mikronukleusa (MN) utvrđena je u PBL-ima primjenom mikronukleus-testa s tehnikom blokirane citokineze (engl. *cytokinesis-block micronucleus* - CBMN). Učestalosti MN bile su značajno povišene ($p=0,031$; $p<0,001$) kod muških [(9,22±4,70) MN na 1000 BN stanica] i ženskih bolesnika [(13,50±2,5) MN na 1000 BN stanica] u odnosu na osnovne vrijednosti utvrđene u muških i ženskih zdravih kontrolnih ispitanika [(6,27±2,66) MN na 1000 BN stanica; (6,80±2,98) MN na 1000 BN stanica]. Prosječna se učestalost MN nije značajno razlikovala među skupinama kod kojih je došlo do spontanih pobačaja te među skupinama koje su imale normalne vrijednosti parametara kvalitete sjemena i onih koje nisu imale takve vrijednosti. Korelacije između niske koncentracije spermija ($<20x10^6$ mL^{-1}), smanjene pokretljivosti spermija (<25 %), normalne morfologije (<30 %) i učestalosti MN bile su negativne, ali ne i statistički značajne. Utvrdili smo da je samo spol značajno utjecao na pojavnost MN u svih ispitanih bolesnika. Nije bilo značajnih razlika između dobnih skupina, kao ni između pušača i nepušača kod bolesnika i kontrolnih ispitanika. Zaključujemo da pojavnost MN u limfocitima bolesnika s poremećajima reproduktivnog sustava prati porast razine kromosomskih oštećenja koja nastaju kao posljedica složenih događaja koji uzrokuju poremećaje reproduktivnog sustava.

KLJUČNE RIJEČI*: dob, kvaliteta sjemena, spol, mikronukleusi, neplodnost, pušenje*

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