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Evaluation of the effect of methadone maintenance treatment program on the frequency of DNA lesions revealed as micronuclei and sister chromatid exchanges

Ocena wpływu kuracji metadonowej na częstość uszkodzeń DNA ujawnianych jako mikrojądra i wymiany chromatyd siostrzanych

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The aim of the present paper was to examine the effect of substitution methadone treatment on the frequency of DNA lesions using a micronuclear and a sister chromatid exchange tests. The material under study were lymphocyte cultures from peripheral blood, collected 3 times at 3-month intervals from 24 patients. The obtained results may suggest that methadone treatment combined with a change in lifestyle significantly reduces the risk of genome lesions.

Celem pracy było zbadanie wpływu metadonowej kuracji substytucyjnej na częstość występowania uszkodzeń DNA określanych przy pomocy testów mikrojądrowego i wymian chromatyd siostrzanych. Materiałem do badań były hodowle limfocytów z krwi obwodowej pobieranej 3 razy w kwartalnych odstępach od 24 pacjentów. Wyniki badań mogą prowadzić do wniosku, że kuracja metadonowa w połączeniu ze zmianą trybu życia istotnie wpływa na obniżenie ryzyka uszkodzeń genomu komórek.

Introduction

The programme of maintenance treatment with methadone is an alternative for drug addicts. It consists in replacing an illegally obtained drug with methadone, which is tantamount to keeping drug addiction under control [10]. Such a solution arouses a lot of controversy, also among specialists [20]. Opponents in the first place hold to the principle that "drug addiction should not be treated using other drugs", whereas supporters point to amelioration of the programme participants' health condition, improvement of the quality of their life [15] and, above all, separation from everyday contacts with the subculture of drug addiction [5]. Some doctors even express an opinion that regular treatment with methadone ensures the biochemical balance of the patients' organism [18].

Despite all its positive effects, methadone is a narcotic, hence a non-physiological substance which is alien to the organism. As has already been suggested by some earlier studies [9,24], it may also be a mutagen and may induce changes in cell DNA structure, detectable by standard cytogenetic tests.

The genotoxic activity of opioids [21,23], especially morphine and heroine both in their pure state and in the form of mixtures used by drug addicts, has been widely acknowledged. On the other hand, the aim of the present study was to examine changes in the frequency of genome lesions during a substitution treatment with methadone, and on this basis to assess the risk factor of mutation occurrence.

Materials and methods

The study was carried out on 57 lymphocyte cultures from peripheral blood, collected from 24 patients (16 men and 8 women, aged between 21 and 46; 32.7 years old on an average) on the occasion of routine clinical tests within a methadone programme run by the Drug Addiction Outpatient Clinic at the L. Rydygier Hospital in Kraków (table I). That group of patients were subjected to longitudinal studies at 3-month intervals: I – June 2003, II – October 2003, III – February 2004. The cultures obtained from 11 patients were developed three times, and those from another 11 patients twice (either the patients' condition did not permit the experimenter to collect blood, or the culture conditions made it impossible to perform a full cytogenetic analysis). Two persons were examined only once because of their withdrawal from the programme for breaking the rules (taking forbidden psychoactive drugs).

The cultures of lymphocytes from 1 ml of peripheral whole blood was carried out according to the modified method of Moorhead et al. [14] using the RPMI 1640 culture medium ("BIOMED" Lublin), enriched with calf serum (INC Biomedicals Inc.), and mitogen LF-7 ("BIOMED" Kraków) at a volume of 0.4 ml per 10 ml of the culture. The cultures were incubated in a thermostat at a temperature of 37°C for 72 hrs. Afterwards the cells were treated with a hypotonic solution (0.075 M KCl) and fixed in acetic alcohol.

Two kinds of cytogenetic tests were performed for each patient: a micronuclear (MN) test, and a sister chromatid exchanges (SCE) test. In the case of the former, the cytokinesis blocker – Cytochalasine B ("SIGMA") was added to the culture after a 48-hour incubation period, and the preparations were stained with Giemsa dye ("SIGMA"). For the latter test, the slides obtained as a result of the culturing carried out by a standard method were stained twice: with the fluorochrome Hoechst 33258 ("SERWA") according to *Perry* and *Wolff* [17] and with Giemsa dye.

Ready-for-use slides from each culture were ana-

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Table I
Characteristics of the patients.
Charakterystyka pacjentów.

			Probe I (VI 2003)		Probe II (X 2003)		Probe III (II 2004)	
No.	Gender	Age	Day of therapy	Dose of methadone [mg]	Day of therapy	Dose of methadone [mg]	Day of therapy	Dose of methadone [mg]
1	m	33	30	35	156	35	253	35
2	m	33	129	65	253	75	358	80
3	m	28	134	80	267	70	375	70
4	f	30	207	45	excluded from programme			
5	f	25	226	60	354	80	460	80
6	m	30	297	50	excluded from programme			
7	m	35	456	60	582	60	690	60
8	m	29	475	40	594	35	703	35
9	f	21	-	-	621	10	727	10
10	f	34	-	-	791	100	897	95
11	m	33	-	-	877	50	975	60
12	f	39	-	-	890	95	998	100
13	m	45	-	-	900	130	1021	130
14	m	30	886	60	1012	50	1117	60
15	f	40	-	-	1146	60	1246	60
16	m	29	1088	70	1222	70	1328	70
17	m	30	1132	20	-	-	1359	20
18	m	33	-	-	1336	35	1435	40
19	f	27	1237	80	1356	70	1464	80
20	m	37	1249	100	1369	80	1472	80
21	f	33	1252	70	1371	60	1473	60
22	m	34	-	-	1371	60	1466	60
23	m	30	-	-	1415	55	1517	55
24	m	46	-	-	1417	140	1518	140

lysed under a light microscope, and – depending on the test used – the frequency of micronuclei in 1000 binuclear cells, or the number of sister chromatid exchanges in 30 complete, well-stained metaphasic plates was determined.

Results and discussion

Micronuclear test

Micronuclei (MN) are visible in the cytoplasm of binuclear cells as cylindrical structures whose shape and stainability resemble those of main nuclei, but which are considerably smaller in size (1/3 to 1/16 of the cell nucleus diameter). They are formed as a result of activity of a mutagenic/genotoxic factor which damages the structure of chromosomes in such a way that during karyokinesis, besides daughter nuclei, there remain in the cytoplasm acentric chromosome fragments or complete chromosomes which form micronuclei. The presence of micronuclei testifies to the exposure of cells to a genotoxic factor whose effect has not been removed by repair mechanisms.

The results of studies conducted by a micronuclear test are shown in table II.

Three patients were excluded because of the possibility of conducting the test with one repetition only, and so was a woman-patient no. 12 in whom an almost threefold increase in the frequency of micronuclei was detected in the second probe.

The means calculated in successive trials for the whole group show that in the course of treatment there takes place a decrease in the frequency of micronuclei. The differences between probes I and III, as well as between II and III are statistically significant, which suggests that a regular treatment with methadone contributes to the lowering of the level of lesions cumulated in the genetic material due to an earlier opioid abuse.

The individual frequencies of micronuclei showed a downward tendency in 14 cases; a similar tendency was displayed by the means for the group. However, in the case of 7 patients those results were not in line with a general trend. The information obtained from the Drug Dependence Outpatient Clinic helped to find a possible cause of that phenomenon. In patients no. 1, 5 and 9, random controls showed the presence of amphetamine in urine, which may explain the increased number of micronuclei in the samples due to additionally taking the very substance. Patient no. 16 was staying in the Detoxification Ward after drug overdose, shortly before blood sampling; patient no. 23 revealed symptoms of alcohol dependence, and so did woman-patient no. 12 (excluded from the statistical analysis) who additionally took considerable amounts of tranquilizers and ten or so days before the second blood test showed the presence of benzodiazepines in urine which, apparently, had a significant influence on the observed, surprising increase in the frequency of micronuclei in that test. Only in woman-patient no. 21 the attempt to identify a possible cause of the increase in the number of micronuclei in trial III failed. This could, however, be connected with the lifestyle, the quality of consumed foods, or with other mutagens coming from environment.

Sister chromatid exchanges (SCE) test

SCE is a process of symmetric exchanges of the homologous chromatid fragments of a replicating chromosome in the period of time preceding mitotic division. These exchanges do not cause alterations in the general morphology of chromosomes, nor do they change the loci arrangement [12, 16,19]. It is presently believed that SCE take place at the points of initiation of DNA replication, or very close to them [22].

Evaluation of the frequency of SCE occurrence has been widely applied in practice, above all as a sensitive cytogenetic test permitting estimation of the mutagenic properties of chemical compounds and physi-

Table II
Frequency of micronuclei in three successive probes (s.d. – standard deviation).
Częstość mikrojąder w trzech kolejnych badaniach (s.d. – odchylenie standardowe).

No.	‰ MN in probe I VI 2003	‰ MN in probe II X 2003	‰ MN in probe III II 2004
1	17	34	13
2	22	-	9
3	14	-	8
4	14	excluded from programme	
5	-	12	19
6	13	excluded from programme	
7	25	19	8
8	11	-	10
9	-	2	5
10	-	28	14
11	-	13	4
12*	-	20	56
13	-	-	11
14	28	12	7
15	-	24	8
16	14	16	12
17	10	-	6
18	-	15	12
19	26	-	13
20	18	16	15
21	25	13	21
22	-	20	14
23	-	9	13
24	-	17	8
Mean	18.23	16.67	10.95
s.d.	6.22	7.78	4.35

* Patient no 12. has been excluded from the statistical analysis

cal factors. It is used as a quantitative indicator of mutagenesis for monitoring persons exposed to the activity of already known or putative mutagenic agents; at the same time, it testifies to the action of repair mechanisms in the cell [4,7,11,16,19,22,25].

In order to determine the mean frequency of sister chromatid exchanges per one metaphase plate, a method increasing the sensitivity of the SCE test, proposed by Cerrano and Moore in 1982 [after: 2], was used. It consists in differentiating high-frequency cells (HFC) from baseline cells (BLC). It is assumed that HFC are a subpopulation of cells much more sensitive to the action of mutagenic factors [2], or revealing the effect of chronic exposure to a potential genotoxic factor [3]. To establish a boundary between the BLC and the HFC lines individually for each patient, the following formula was used:

max BLC = x + 1,31 s.d.

(where x = a mean number of exchanges per a metaphasic plate; s.d. = standard deviation, 1,31 = critical value t0 for the one-way Student test, cutting off the extreme 10% area of normal distribution at 29 degrees of freedom – 30 metaphases were analysed in each individual).

The results obtained using the SCE test are shown in table III. The mean BLC and HFC values calculated for each patient, as well as for the whole group under study are presented.

An analysis of the dependence of those two parameters on the time of treatment and methadone dosage, conducted by a regression method on the basis of individual data, showed – like in the case of micronuclei - a weak negative dependence regarding the first variable (r = -0.24) and a weak positive dependence regarding the second one (r = 0.19). This implies that the number of exchanges to one metaphasic plate slightly diminishes during treatment, whereas patients treated with higher doses of methadone reveal both a higher basic SCE/BLC level and greater participation of cells with an increased number of exchanges (HFC).

Group means, calculated for individual trials, show an interesting distribution. The BLC values, which are the highest in trial I, are diminished in trial II and increased again in the last test (trial III). An analysis of variance showed statistically significant differences between probes I and III, as well as between II and III. A possible explanation the this phenomenon are changes in the season-related nutrition mode (probe II - June) and a shorter exposure to chemical mutagens in the form of preservatives or other supplements, related to it.

The percentage values of HFC in the three successive tests take on a similar shape; on the other hand, there are no significant differences between them from the viewpoint of statistics. Thus the magnitude of cell fractions with a great number of exchanges is maintained at a relatively stable level. It may therefore be concluded that the cells are affected by a permanent factor, possibly methadone, given daily to patients and having an impact on changes within the genome. Studies conducted by Bozkurt et al. [3] confirm these conclusions.

The conducted studies helped to assess the effect of the time of duration of a substitution treatment on the reduction of the risk of patients' exposure to the genotoxic action of opioids taken before the start of the methadone programme.

The group under examination was not homogenous in respect of sex, time of participation in the programme and the dose of methadone. Therefore, before the obtained results were interpreted, a statistical analysis of data was carried out in which those three parameters were taken into account.

The analysis of variance showed no differences between groups of women and men in all the variables examined (MN, BLC, HFC). The values of the F statistics ranged between 0.01 and 0.83 at correlation coefficient of 0.37-0.94. Therefore in a further discussion no differentiation in respect of sex was applied. Such a procedure is in line with the literature data [1,6,8,13].

To assess the two remaining parameters, an analysis of regression was used. Regarding the time of treatment, a weak, negative linear dependence was obtained for all the variables, whereas a weak positive linear relationship was found for doses of methadone. The treatment duration and the dosage were not correlated with each other; moreover, the amount of the methadone taken may vary in individual patients throughout the therapy. Therefore it was accepted that the results of the regression analysis may indicate only general directions of changes, hence more attention was focused on the comparison between the mean values calculated for each patient, as well as for the whole group under examination.

The results discussed in the present study indicate that methadone, used in the substitution therapy, is an agent with weak genotoxic activity when compared to other opioids. The number of DNA lesions caused by a long-term intake of psychoactive substances declines after their withdrawal and the start of the therapy. This is particularly evident in the micronuclear test, whereas the sister chromatid exchanges also depend on not readily identified environmental factors. Nonetheless it seems that the permanent exposure of cells to methadone

Table III

Frequencies of SCE in studied group (s.d. – standard deviation).

Częstość wymiany siostrzanych chromatyd w badanej grupie (s.d. – odchylenie standardowe).

No	Probe I (VI 2003)			Probe II (X 2003)			Probe III (II 2004)		
	x BLC	max BLC	% HFC	x BLC	max BLC	% HFC	x BLC	max BLC	% HFC
1	7.8	11.2	10.0	-	-	-	6.0	8.8	13.3
2	9.7	12.3	6.9	9.3	14.5	16.7			
3	-	-	-	-	-	-	6.4	9.9	6.7
4	6.2	10.3	17.6	excluded from programme					
5	6.1	7.6	9.1	6.4	8.8	16.7	7.5	10.5	10.0
6	excluded from programme								
7	8.6	11.7	10.0	5.4	7.3	6.7	7.9	11.8	10.0
8	-	-	-	4.4	8.6	13.3	7.0	11.2	10.0
9	-	-	-	5.4	9.0	6.7	7.3	12.0	6.7
10	-	-	-	6.4	11.0	10.0	8.3	12.6	10.0
11	-	-	-	7.0	10.4	10.0	6.7	9.3	10.0
12	excluded from the statistical analysis								
13	-	-	-	6.5	11.5	13.3	8.3	13.2	10.0
14	-	-	-	6.3	9.1	5.0	6.6	8.8	16.7
15	8.2	12.1	10.0	5.9	8.2	6.7	7.8	13.0	10.0
16	-	-	-	5.0	7.4	10.0	8.0	11.7	6.7
17	6.3	8.5	6.7	4.8	6.8	6.7	5.6	8.7	6.7
18	7.1	10.6	12.5	-	-	-	5.9	8.4	10.0
19	-	-	-	-	-	-	8.3	11.9	13.3
20	8.4	11.9	13.3	7.2	10.5	10.0	7.2	10.8	10.0
21	6.2	8.5	13.3	4.3	6.4	10.0	6.1	10.3	13.3
22	9.0	11.4	10.0	4.8	8.1	3.3	6.5	10.0	10.0
23	-	-	-	6.4	11.6	10.0	8.8	13.5	10.0
24	-	-	-	7.5	10.4	3.3	8.0	11.2	3.3
Mean	7.6	10.6	10.8	6.1	9.4	9.3	7.2	10.9	9.8
s.d.	1.28	1.64	3.15	1.28	2.02	3.90	0.92	1.55	2.88

leads up to the maintenance of the HFC fraction at a stable level. The differences in interpretation of the results of both these cytogenetic tests may also be due to the fact that micronuclei represent a different type of DNA lesions than those found in the case of sister chromatid exchanges.

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