Chromosomal Abnormalities and Programmed Cell Death in Patients with Psychoactive Substance Dependence

Sabry N[1], Korraa S[2], Mobasher M[1], El Rakhawy M[1]

[1]Department of Psychiatry, Faculty of Medicine, Cairo University, Cairo, Egypt
[2]Department of Radiation, National center for research and technology, Atomic center Authority, Cairo, Egypt.

ABSTRACT

Background: Changes in gene expression in brain reward regions are thought to contribute to the pathogenesis and persistence of drug addiction. Cytological examination has routinely been used for several decades as a tool to monitor occupational and environmental exposure to agents known to damage DNA, thereby causing mutations, which can result in cancer (genotoxins). **Objective:** This study considers the evidence suggesting that chronic exposure to drug addiction might be genotoxic. **Subjects and methods:** Peripheral blood lymphocytes are studied for the presence of biomarkers that may be associated with persistence and chronicity of drug use and the addictive state. Sixty two subjects were included in the study: 30 male psychoactive dependant inpatients diagnosed according to SCID criteria after subsidence of withdrawal symptoms, and 32 mentally healthy male volunteer relatives matched for age and sex. Urine samples were taken from all subjects for urine analysis for substances of dependence was performed using the semi-quantitative ELIZA technique. Blood samples were drawn for cytogenetic analysis of lymphocytes after being incubated in the presence of PHA for 48 hours, and then stained by special techniques to asses apoptosis and chromosomal aberrations. **Results:** There is an evident statistically significant difference between patients with psychoactive substance dependence and controls on three recorded cytogenetic lymphocyte findings in blood samples. Patients with drug dependence have higher degrees of programmed cell death, apoptosis, and (mean+ SD 5.48±1.7) compared to (1.2±0.93). Patients with addiction have a significantly higher number of micronuclei (9.1±8-9) compared to normal controls (0.69±0.5). The mean number of chromosomal aberrations in the addict group was 3.94±0.92 while in the control group was 2.39±0.33. **Conclusion:** This study demonstrates obvious chromosomal abnormalities in the peripheral blood of drug addicts mostly suggested to be a result of chronic drug intake. It is hypothesized that these changes could be a reflection of similar central changes in the brain that contribute to the process of addiction and its consequences.

Key words: Psychoactive substance dependence, Chromosomal abnormalities, Programmed cell death.

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INTRODUCTION

Regulation of gene expression is one mechanism by which drugs of abuse can induce relatively long-lasting changes in the brain to cause a state of addiction[1-2]. These

changes in gene expression are believed to affect brain reward regions. Recent studies have begun to focus on the molecular mechanisms by which drugs of abuse and related environmental stimuli, such as drug-associated cues or stress, alter specific gene expression included in the genome. Increasing evidence suggests that these changes in gene expression are stable and include alteration of chromatin structure in neurons on specific gene promoters. This novel mechanistic insight might open new avenues for improved treatments of drug addiction[2-3].

The frequency of chromosomal aberrations is measured in human peripheral blood lymphocytes. This is done via conventional cytogenetic assays in the metaphase (second stage of cell division of mitosis or meiosis). During this stage, the chromosomes, each consisting of two chromatids, are arranged in the equatorial plane of the spindle prior to separation. Cytological examination has routinely been used for several decades as a tool to monitor occupational and environmental exposure to agents known to damage DNA, thereby causing mutations, which can result in cancer (genotoxins). Most mutagens and genotoxic carcinogens are efficient inducers of chromosomal alterations in exposed cells. Two important classes of aberrations namely structural and numerical, are recognized and both are associated with congenital abnormalities and neuoplasia in humans[4].

Numerical aberration can be estimated in metaphase by counting the number of fluorescent in situ hyperidization technique (FISH)-painted chromosomes. Micronuclei are formed by lagging chromosome fragments or whole chromosomes during anaphase cell division. The nature of micronuclei whether they possess a centeromere can be determined[4]. Micronucleus assay in human exfoliated cells has been widely used to detect genotoxic effects of environmental mutagens, infectious agents and hereditary diseases[5-6]. There is ample evidence of the value of this biomarker for the identification of occupational and environmental hazards[7-11].

Apoptosis is a process of cell death, which is characterized by DNA cleavage, nuclear condensation and fragmentation, plasma membrane blebbing up to phagocytosis of the cell, without induction of an inflammatory response. Apoptossis is an ultimate example of structural aberration of chromosomes. This type of cell death is important for lymphocyte development. Programmed cell death is a pathway of cell death by apoptosis, which occurs in lymphocytes deprived of necessary survival stimuli, such as growth factors or co-stimulators. Programmed cell death also called "death by neglect " is characterized by release of mitochondrial cytochrome C into the cytoplasm, activation of caspas 9 and initiation of the apoptotic pathway[12].

This study considers the evidence suggesting that chronic exposure to drug addiction might be genotoxic. Chromosomal changes measured in peripheral blood lymphocytes are studied for the presence of biomarkers that contribute to the persistence and chronicity of drug use and the addictive state.

SUBJECTS AND METHOD

This study was carried out on thirty male inpatients with substance dependence during an inpatient period of treatment for substance misuse. Patients were

interviewed and investigated after withdrawal symptoms have subsided. Patients were diagnosed as psychoactive substance dependent patients according to SCID criteria. Cases were selected consecutively from 2 private mental hospitals in Cairo during the year 2005.

Patients with co-morbid conditions on axis I, those with possible learning disability, or an organic mental disorder were excluded.

The control group consists of 32 mentally healthy male volunteer relatives of the same age range. They were collected by snowballing as known to be non drug users and were subjected to urine analysis for psychoactive substances and results were free from psychoactive substances. Their ages ranged from 17 to 55 years with a mean age of 27.70 years. Subjects of both groups gave written consents for involvement in the study.

They had neither recent infections nor active inflammatory diseases. None had received any drug with a potential effect on the immune system during the preceding 3 months. Twenty nine of the patients' groups were sero-negative for HIV. Only one patient was sero-postive. Four cases were positive for hepatitis (+ve HBVC) (table 1).

Members of both groups were subjected to the following assessment procedure:

1- ***Psychiatric assessment*** using a specially designed semi-structured interview with emphasis on substance use history. Controls were assessed to rule out a history of psychiatric illness, substance misuse, and serious medical conditions

Patients were assessed while being in hospital after withdrawal symptoms subsided.

2. ***Diagnosis according to SCID-II criteria***[13]

3-***Laboratory investigations:***

a) Urine analysis for substances of dependence using semi-quantitative ELIZA technique according to Immunanalysis Corporation considering National Institute of Drug Abuse, NIDA, cut off values.

b) Cytogenetic study: A venous blood sample was drawn from each subject and examined according to the procedure described below. The person who performed the cytogenetic analysis (SK) was blind to the status of the blood sample (Addict or control).

i- Conventional cytogenetic analysis for the detection of programmed cell death (apoptosis) and the numbers of the micronuclei.

ii- Peripheral blood is the most frequently used tissue for postnatal chromosome studies or diagnosis primarily because it is easy to obtain a sample and simple to culture. Normal circulating lymphocytes do not divide under routine culture condition but they can be stimulated to proliferate by several lectins such as phytohaemagglutinin (PHA), which stimulates the T-cell fraction of lymphocytes, has become a popular mutagen to study human chromosomes for diagnostic purposes. Initial incubation of lymphocytes in the presence of PHA for 10 to 15 hours is adequate to stimulate the potential cells to begin macromolecular synthesis and cell division. Removal of PHA from culture or washing the cells after this period does not decrease or alter the mitotic activity. The mitotic index increases up to 2 to 3% and reach a plateau within 44 hours. The dividing cells appear over a 6-day period.

However, it has been suggested that the quality of the mitotic chromosomes with regard to their condensation and morphology, deteriorates rapidly after 120 hours of incubation. Therefore, the optimal time for harvesting the lymphocyte cultures for cytogenetic studies is between 45 and 96 hours. At least 20 banded metaphase spreads should be counted and analyzed under the microscope and two metaphases should be karyotyped for routine chromosomal study[14].

Apoptosis in Circulating Lymphocytes

Separated mononuclear cells were subjected to a double staining technique. They were washed and briefly incubated with fluorescence diacetate for 15 minutes, rewashed and fixed with 70% Ethyl alcohol incubated with RNAase for 15 minutes and then stained with and propdium iodide.

Viable cells will take up fluorescence diacetate and fluoresce in green, while dead cells will allow propidium iodide to traverse the cell membrane stain with nuclear DNA giving a red fluorescence. RNA present in apoptotic cells will be removed by RNA-ase to eliminate confusion with nuclear DNA[15].

Preparation of micronuclei was done according to the method of FISH[16]. Briefly, lymphocytes, separated from l0ml punctured heparinized blood, were stimulated to divide with 5 μg/ml phytohaemagglutinin (Gibco) in RPMI media (Gibco) containing 15% (v/v) inactivated foetal calf serum. Incubation was at 37^0C in a humidified atmosphere containing 10% carbon dioxide.

Cytochalasin B was added to the culture (to give a final concentration of 4.5μg/ml) 44 hours after the commence of culture. Harvesting was carried out 28 hours after the addition of cytochalasin B. Cells were fixed with Carnoy's fixative and stained with Giemsa. 200 binucleated cells were scored for each individual (fig. 2).

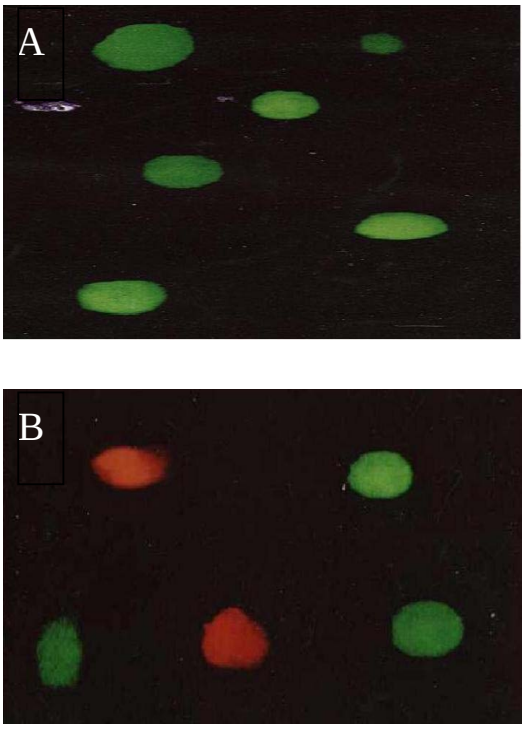

Figure (1) a) Viable cells will take up fluorescence diacetate and fluoresce in green, b) Dead cells will allow propidium iodide to traverse the cell membrane stain with nuclear DNA giving and red fluorescence

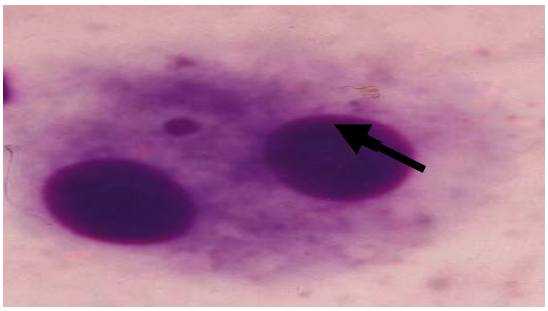

Figure (2): A micronuclei in a cytokine blocked binucleated lymphocyte from blood of an addict

The analysis of structural chromosomal aberrations was carried out according to the well known conventional method of

biological dosimetry. Forty eight hours cultures of phytohaemagglutinin-stimulated (to proliferate) lymphocytes were incubated in the presence of colchicine for the last three hours. Subsequent harvesting, fixation with Carnoy's fixative and staining with Giemsa was carried out. 100 metaphases were analyzed for each individual. Chromosomal damage was classified as acentric fragments, dicentrics, rings, chromatid and chromosome breaks (fig. 3).

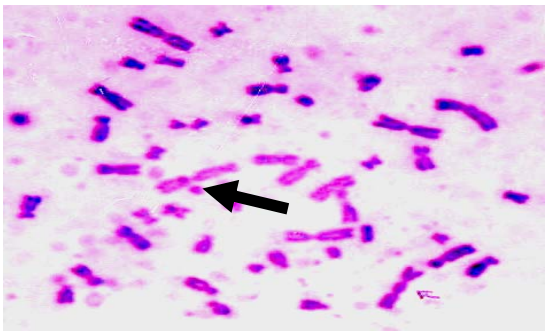

Figure (3): A metaphase spread of a lymphocyte from blood of an addict

Statistical analysis:

The data were coded, and entered on an IBM compatible computer using the statistical package for the Social Sciences SPSS version 16. Results are presented as mean ±SD and as percentages for nominal data.

The significant differences in means for lymphocyte subsets between patients and controls were analyzed by unpaired student t-test. Statistical correlation between the duration of drug dependence and percentages of lymphocyte subsets were determined by Pearsons[17]. Linear Regression analysis has been done for the effect of age on the lymphocyte analysis

RESULTS

The two studied groups are matched as regards age and socioeconomic status. Being relatives, they shared the same family history of drug abuse and psychiatric illness as recorded in (table 1). The fundamental difference between the two groups is the history of psychoactive substance dependence, which has been rolled out in the control group, both by the absence of the history of substance dependence and negative urine screening for substance abuse.

Table 1: Sample Characteristics

Characteristic	Count	Percent
Mean Age	Substance misuse Controls	27.7 ± 8* 27.3 ± 7.7*
Positive Family History of Mental Disorder	20/30	66.7%
Positive Family History of Substance Misuse	3/30	10%
HIV positive	1/30	3.3%
Hepatitis positive	4/30	13%

Concerning the psychoactive substance dependence group; the duration of substance abuse ranged from 1 to 25 years with a mean 9.3years (SD=5.99). All patients were hospitalized between one and 20 times (means 5.5±8.14). The average duration of hospitalization was 1.41 months. 14 patients (47%) reported a history of previous overdose.

Table (2) shows that all patients were multiple substances abusers. All combined cannabinoid abuse with other substances. 26 patients (87%) were on different pharmaceuticals. Twenty two cases (73%) of the addicts were mainly heroin dependent, but none of them started their

addiction by heroin. The major type of complications encountered in these cases was family problems as it was reported by 93% of patients. Other social problems come second in 73% of addicts, and occupational problems were found in 63% of the addict group, followed by legal problems in 24% of the patients. Health problems were present in only 13% of the patients were sero- positive for hepatitis and 3% was HIV positive.

Table 2: Patients' group Psychoactive Dependence Characteristic

Types of substance:		
Cannabis	30/30	100%
Pharmaceuticals	26/30	87%
Heroin	22/30	73%
Alcohol	13/30	43%
Complications Of Substance Misuse:		
Legal	7/30	24%
Family	28/30	93%
Social	22/30	73%
Work	19/30	63%

Table (3) shows the differences in the lymphocytic changes between the two studied groups. The studied changes include the Apoptosis (irreparable DNA damage which leads to programmed cell death), the presence of multiple (micro) nuclei and chromosomal aberrations. There is an evident statistically significant difference between cases and controls in all three aspects of cytogenetic study.

Concerning the degree of apoptosis the mean number in patients with drug addiction was 5.48±1.7 compared to 1.2±0.93 in controls which is statistically significant (P<0.001).

The mean number of micro-nuclei in patients with drug addiction was 9.1±8-9 compared to 0.69±0.5 in the normal control group which is also statistically significant and p<0.001. It is noteworthy that the variation in the addict group in the number of micro nuclei judged by the wide standard deviation is quite high which suggests that this abnormality may be restricted to a subgroup of patients with drug addiction.

The mean number of the chromosomal aberrations in patients with addiction is 3.94±0.92 while in the control group is 2.39±0.33. The difference is also statistically significant P<0.001.

Chromosomal damage was classified as acentric fragments, di-centrics, rings, chromatid and chromosome breaks. A-centeric and di-centrics chromosomes indicate the occurrence of breakage and reunion that occurred in specific bands on sister chromatides. Ring chromosomes breakage occurred at band in the short arm and in the long arm of chromosome. With deletion of the segments distal to these bands, the broken ends have joined to form a ring chromosome[14].

Linear Regression analysis was performed in order to confirm if age is related to the degree of apoptosis. This analysis revealed an insignificant effect which means that the difference between patients and controls is related to substance misuse (B=.033+.042, Beta=.102, t=.774. P= .442)

* t = .21, P<0.83, 95% CI -3.57-4.4 n.s.

Table 3: Chromosomal abnormalities and Apoptosis in Patients with Psychoactive Dependence and Normal Controls

Finding	Group	Mean ± SD	t	P<	95% Confidence Interval
Apoptosis	Addicts Controls	5.48 ± 1.7 1.2 ±.93	12.06	0.001	3.56-4.99
Micro-nuclei	Addicts Controls	9.1 ±. 8.9 0.69 ±. 0.51	5.18	0.001	5.2-11.7
Chromosomal aberrations	Addicts Controls	3.94 ±. 0.92 2.39 ±. 0.33	19.6	0.001	3.1-3.9

DISCUSSION

This study confirms the possibility of DNA damage and alteration in patients with substance dependence. The occurrence of this damage and alteration may be one of the mechanisms through which the drug dependent state is maintained. Our findings indicate that patients with substance abuse have a significantly higher incidence of programmed cell death in peripheral lymphocytes that cannot be accounted for by age differences from healthy controls. Patients with substance dependence also have a larger number of micronuclei inside the lymphocytes, and significantly more aberrations in chromosomal structure as observed during cell division.

This study does not include a differential analysis of changes in specific genes. The significant differences between patients with substance dependence and healthy controls on three parameters of chromosomal structure and cell health suggests a causal-effect relationship between substance abuse and progressive lymphocytic changes translated into chromosomal functions. However, this study cannot indicate whether such changes are a cause or an effect of drug dependence.

This study controlled for the effect of age and gender as potential confounding variables. Other confounding variables include associated physical illness, the use of multiple substances, and the possible presence of other genotoxic agents as a result of occupational exposure.

It was suggested that in humans, chemical mutagenesis may be more important than radiation in producing genetic damage. Experiments have shown that certain chemicals, such as mustard gas, benzene, some basic dyes and food additives, are mutagnic in animals. Exposure to environmental chemicals may result in the formation of DNA adducts, chromosome breaks or aneuploidy. Consequently all new pharmaceutical products are subject to a battery of mutagenicity tests which include both in vitro and in vivo studied in animals[15].

There is a considerable literature on the relationship between DNA damage and the development of dependence. Substance misuse may lead to abnormalities in protein phosphorylation which has been implicated in the process of tolerance and dependence

to opioids and cannabinoids[18]. Though previously there was a claim of the absence of any clinical consequences from using cannabinoids on the immune system, chromosomes, or cell metabolites. Contamination of marijuana by spraying with defoliants has created the clearest danger to health[19-20]. Long-term alcohol abuse results in changes in gene expression in the brain and these changes are responsible at least partly for alcohol tolerance, dependence and neurotoxicity. The identification of as-yet-unknown alcohol-responsive genes, may well confirm changes in the expression of genes which have been delineated in animal models of alcohol abuse. Initial results show selective changes in gene expression in alcoholics; of particular importance is a coordinated reduction in coding genes for myelin components[21].

The inherent diversity of human drug dependence can be used to identify genes associated with specific pathological processes, as well as to assess the effects of concomitant disease, severity of brain damage, abuse pattern, and factors such as gender and smoking history. This sample consists exclusively of male subjects, the results, therefore, cannot be generalized to female addiction. Both patients and controls are cigarette smokers; which neutralizes the confounding effect of nicotine use. Only 13.5% of the patients with drug dependence had other health problems. The effects of health problems on the findings in both groups should therefore be minimal**.**

This study showed obvious peripheral chromosomal abnormalities within the lymphocytes of patients with a drug addiction problem. These abnormalities could result from chronic drug intake, and may reflect similar central changes that contribute to the maintenance of the addict state and its consequences.

Genotoxicity may therefore be one of the possible mechanisms responsible for the chronicity of the dependency state. One possible path is through the effect of genotoxicity on the limbic system. It has been postulated that in this brain region altered and self-directed injury from microglia can lead on one hand to the enlargement of temporal brain regions in patients with substance misuse, and on the other hand explain potential self-directed neuronal damage in affected individuals[22]. It has also been confirmed in some studies[23] that methamphetamine can induce damage to the brain dopaminergic system by stimulating a process of hypersensitivity through its effect on microglial reactions. Also, methamphetamine's toxic effects may include subverting some glial cells to attack rather than preserve neurons[22]. Specifically, their results indicate that the drug incites microglia to mount an immune response against dopamine neurons.

This study is limited by the relatively small number of patients included for assessment. Most patients in this study were patients with multiple substance dependence, probably a practical reality issue that interferes with the identification of the effects of specific substances on chromosomal structure and the viability of cells. If practicable, studies directed towards identifying the effects of single substance misuse on cellular DNA will be a significant addition. Using more sophisticated genetic techniques will probably add more information on the nature of chromosomal abnormalities.

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Address of correspondence:

Sabry N. Assistant Professor of Psychiatry**,** Department of Psychiatry**,** Faculty of Medicine, Cairo University, Cairo, Egypt.

e-mail: *noha_sabry2008@yahoo.com*