Long-Term Methadone Intake and Genotoxicity in Addicted Patients

Mohsen Rezaei1,2,*, Forouzan Khodaei1, Mehdi Sayah Bargard1, Mahsa Abasinia1

1. Background

Control of pain in a successful treatment is an important point and requires careful consideration of appropriate dosage, type of medication and cognitive disease (1). Using opioids to control pain in acute conditions is different with chronic conditions. In these cases, other factors must be considered, particularly the rate of tolerance and physical dependence (2).

Methadone hydrochloride (synthetic derivative of diphenyl heptane) is an opioid analgesic used for moderate to long-term use of methadone and higher risk of side effects may produce metabolites able to cause mutation. With chronic exposure to some physical, chemical or biological conditions, mutation probability can be increased by several times. These include medicines that can damage the DNA of cells (6). Using some compounds in a long-term (7) or simultaneous usage of some medications may produce metabolites able to cause mutation. Results regarding methadone genotoxicity are controversial and need a comprehensive evaluation to obtain unequivocal conclusions. Brambilla and colleagues in 2009 studied the effects of genetic toxicity and carcinogenicity of 120 analgesics, anti-inflammatory and anti-pyretic. Due to long-term use of methadone and higher risk of side effects, it was selected for evaluation and finally positive results were demonstrated in three assays as below:

1. Gene mutation, mouse lymphoma L5178Y cell, Tk locus
2. Chromosomal aberrations, mammalian spermatogonia in vivo
3. Dominant lethal test, mice (7).

Results:
The mutagenicity index in addicted group was robustly higher than healthy volunteers. Fortunately, this significant difference was lower than positive control.

Conclusions:
Genome instability in addicted patients was demonstrated in this study. Controversially, considering incoherent results of previous studies and our data, more studies in longer duration of methadone use are needed to elucidate the consequence.

Keywords: Methadone; Comet Assay; Mutagens
Gorazd et al. studied genetic toxicity of methadone in 23 patients recovering methadone maintenance therapy. Lymphocytes of two control groups were exposed to known mutagenic agents. Higher proportion of cells with elevated numbers of chromatid exchanges demonstrated methadone genotoxicity (8). In another study, Skowronska et al. evaluated genotoxic effect of methadone on peripheral blood lymphocytes of 12 addict patients; sampling was performed during and at beginning of maintenance therapy (9). Surprisingly, DNA breaks and damages were reported to be reduced in methadone treated groups. Same results were reported by Zimmering et al. and Badr et al., using drosophila melanogaster and dominant lethal mutation consecutively (10, 11).

Apoptotic effects of methadone may directly or indirectly contribute to its proposed DNA toxic effects. In this regard, Friesen and colleagues found that methadone is a potent inducer of programmed cell death in leukemic cells and Non-leukemic lymphocytes. Methadone treatment resides and resistance to chemotherapy decreased (12).

2. Objectives

Given controversial results regarding methadone genotoxicity, we investigated the effect of methadone genotoxicity. Altogether, research on mutagenesis of methadone may uncover its potential adverse effects contributing to overall health of patients.

3. Patients and Methods

HEPES, Na$_2$HPO$_4$ and Disodium EDTA were obtained from Sigma (USA), C$_{15}$H$_{28}$NO$_3$Na, Tris Hydrochloride and Triton x-100 from Merck (Germany), LMPA and Agarose normal from Fermentas (EU) and Methadone from Exir (Tehran, Iran).

3.1. Patients and Healthy Subjects

This study was performed to evaluate mutagenic effect of methadone in addicted patients referred to Imam Khomeini Hospital in Ahvaz. Total samples of 90 subjects were divided into dichromate treated group, no treated healthy volunteers group and test group. Each group included 30 subjects as follows:

- No treated healthy volunteers group: normal subjects without any history of opioid consumption in the last six months. All individuals filled a detailed questionnaire according to the published protocol by the International Commission for Protection against Environmental Mutagens and Carcinogens.
- Dichromate treated group: 500 μM of dichromate was added to samples as a positive agent for induction of DNA breakage (13).
- Test group: all 30 subjects in this group met the inclusion criteria.

Inclusion criteria were methadone use 60 to 120 mg daily, age of 18 to 65 years, minimum period of three years for methadone consumption and IQ above 75 (10, 11). Consents were obtained from all subjects participated in this study according to university Ethics Committee.

Exclusion criteria were use of any medicines such as benzodiazepines, amphetamine derivatives, antipsychotics, mood stabilizers, use of substances such as marijuana, crack, opiates, tramadol, mental disorders (personality disorders, mood disorders, anxiety disorders, psychoses), organic disease and positive results for HIV Ab, HCV Ab or HBS Ag (7, 10, 11).

3.2. Isolation of Peripheral Blood Lymphocytes

Two milliliters of peripheral blood were collected from each patient in heparinized tubes. Whole blood with Hank’s solution was diluted to a ratio of 1 to 2.5 mL of sterile Ficoll solution (in the dark and temperature of 2-8°C), added to a centrifuge tube and diluted blood poured slowly to form a layer on top of the tube. The tubes were centrifuged for 20 minutes 1000 rpm (at this centrifugal brake or stopping using hand was avoided). After centrifugation, mononuclear cells (including lymphocytes) settle as a white layer between Ficoll and plasma. Lymphocytes were collected using pipette and transferred to another tube. The tube was filled with Hank’s solution and centrifuged at 440 rpm for 10 minutes. The supernatant discarded, deposited again on Hank’s solution added and mixed gently and centrifuged again for 15 minutes at 230 rpm. Besides, washing repeated with 110 rpm again. At this point, fully isolated lymphocytes achieved (14).

3.3. Comet Assay

Afterwards, 100 µL of cell suspension premixed with low melting point agarose (LMA 1%) poured on a slide coated with normal melting point agarose (NMA 1%) and covered with cover slips. Slides were kept for 15 to 20 minutes horizontally in the ice tray to solidify. Next, the cover slips were removed from the slides and placed in a lysis solution (14.61 g NaCl, 3.72 g EDTA, 0.125 g Tris ,0.9 g NaOH, 1 g sodium lauryl sarcosinate %1, DMSO 10%, Triton x-100 % and pH = 10) for one hour and then washed by deionized water, kept for 20 minutes in electrophoresis buffer (NaOH 12 g, EDTA 372.0 g, dH$_2$O to 1000 mL and pH = 13) and electrophoresed at 25 V and 300 mA for 20 minutes and then washed with neutralized buffer (Tris 1.12 g, dH$_2$O to 250 mL and pH = 7.5) for five minutes three times. Slides were immersed in ethidium bromide solution for five minutes and according to the method described by Speit and Hartmann (15), which is based on the original work of Singh et al. (16), slides were analyzed by fluorescence microscope. The extent and distribution of DNA damage indicated by comet assay was evaluated by examining cells. The cells were visually scored into comet classes according to tail size class (17-19): 0 = no tail, 1 = tail shorter than the diameter of the head (nucleus), 2 = tail length 1 to 2x the diameter of the head and score 3 = tail longer than 2x the diameter of the head. Comet with-
out head and those with nearly all the DNA in the tail or with a very wide tail were excluded from the evaluation, because they probably represented dead cells (20, 21). Tail length and the mutagenic index were calculated according the following formula MI = (0 NMC+ 1 SMC+ 2 MMC+ 3 LMC)/200, or we could express it as NMC = No migration cells (score 0), SMC = Short migration cells (score 1) MMC = Medium migration cells (score 2), LMC = Long migration cells (score 3) (22).

3.4. Statistical Analysis

Using SPSS software, Chi-square was performed to compare the groups.

4. Results

Peripheral blood was collected from 30 addicted patients screened to be exclusively on methadone and lymphocytes were separated to assess the genotoxicity by the comet assay. Percent of mutagenicity index (MI) was significantly lower in the test group compared to positive control and conversely, it was higher compared to the negative control group. Addicted patients showed more than two fold MI% compared to negative control or normal samples. Damaged cells in the test group were significantly higher compared to negative control. Altogether, instability of genome was demonstrated for addicted patients according to scores obtained for damaged cells.

5. Discussion

Brambilla et al. studied the carcinogenic effects of 120 different drugs including analgesics, anti-inflammatory and antipyretics. Methadone, because of its predominant and long-term use for treating severe pain, and its possible side effects was investigated. Results were deemed positive in the following three areas: gene mutation, chromosomal aberrations and dominant lethal test in mice (7).

Gorazd and Kamenczak studied genetic toxicity of methadone in lymphocytes cultures of 34 patients treated with methadone. Micronuclei occurrences along with number of chromatids’ changes taking place in the metaphase indicate genetic mutation. Two control groups with elements of genetic mutation were used. Increased ratio of number of cells to immense chromatid changes are indicators of genetic toxicity of methadone, although these changes are not so apparent in other groups facing mutagen (8). As indicated by these studies, genetic mutation in patients treated with methadone is controversial and therefore in this study we investigated its possible genotoxic effect. Lowest concentration of sodium dichromate, which causes a break in DNA strand is 50 µM, correlating to the dosage can increase to its maximum amount of 500 µM. Higher concentrations of dichromate would cause the curve of DNA strand breaks vs. dichromate concentrations to turn into plateau. The reason for this phenomenon could be cross binding of DNA-DNA and protein-DNA, which would prevent DNA migration or could be satura-
by controlling proteins of apoptosis such as BCL-XL and p53 in this process. This study showed that a reduction in cell energy level would result in cell necrosis (24).

Garcia-Fuster et al. studied external and internal apoptosis pathways in prefrontal cortex of persons using methadone and heroin. Their findings showed that in such individuals these pathways were not activated in an abnormal manner. Instead, slowing or continuous manipulation of some parts of these pathways (downregulation cytochrome C and upregulation BCL-2) induces anti-apoptosis activity (25).

Friesen et al. indicated methadone as a strong inducer of death in leukemia cells. Methadone controls the proliferation of leukemia cells and induces apoptosis in them. Contrary to leukemia cells, non-leukemic lymphomas after treatment with methadone remain healthy and alive. The study showed that methadone kills leukemia cells and eliminates resistance to chemotherapy and apoptosis (12).

There was a significant difference between mutation indicators of the experimental and negative control groups (P < 0.0001). Individuals who used methadone during this study had a less stable lymphoma genome compared to healthy individuals. Although on this subject and the reasons for instability of genome, study showed conflicting results, but continuous manipulation of immune system via methadone can induce or modify cellular death. Proving genotoxic effects of methadone requires further studies for a longer time of usage. To get more conclusive findings from this and previous studies, it is necessary to:
- Perform an extensive review of methadone usage periods and its effects on genome and cellular deaths.
- Conduct studies reviewing methadone usage and its relation to autoimmune diseases and allergies.
- More detailed review of reasons for instability of genome by methadone as means of new clinical targets.

Authors’ Contributions
Study concept and design: Mohsen Rezaei. Analysis and interpretation of data: Mohsen Rezaei. Sample collection: Mehdi Sayah Bargard. Practical work: Mahsa Abasinia. Drafting of the manuscript: Forouzan Khodaei and Mohsen Rezaei. Critical revision of the manuscript for important intellectual content: Mohsen Rezaei. Statistical analysis: Mohsen Rezaei.

Funding/Support
This work was supported by a grant (NO. 768) from the Deputy of Research, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

References
1. Buffum MD, Hutt E, Chang VT, Craine MH, Snow AL. Cognitive impairment and pain management: review of issues and challenges. J Rehabil Res Dev 2007;44(2):215-30.
2. Lewis NL, Williams JE. Acute pain management in patients receiving opioids for chronic and cancer pain. Cont Educ Anaesth Crit care pain. 2005;5(4):127-9.
3. Lacy C, Armstrong L, Goldman MP, Lance L. Drug Information Handbook. 18 ed: Lexi-comp; 2009.
4. Katzung B, Masters S, Trevor A. *Basic and clinical pharmacology*: Twelfth ed. McGraw-Hill; 2012.
5. Peles E, Schreiber S, Naumovsky Y, Adelson M. Depression in methadone maintenance treatment patients: rate and risk factors. *J Affect Disord.* 2007;99(1-3):213–20.
6. Jun S. DNA Chemical Damage and Its Detected. *Int J Chemistey.* 2010;2(2):263–4.
7. Brambilla G, Martelli A. Genotoxicity and carcinogenicity studies of analgesics, anti-inflammatory drugs and antipyretics. *Pharmacol Res.* 2009;60(1):13–17.
8. Gorazd M, Kamenczak A, Pach J, Schmager J, Wronka I. [Preliminary research on opioid mutagenicity]. *Przegl Lek.* 2003;60(4):245–8.
9. Skowronska A, Groszek B, Schmager J. [Micronuclei as markers of DNA damage in patients addicted to opioid drugs]. *Przegl Lek.* 2004;61(4):241–3.
10. Zimmering S. Evidence for the absence of a mutagenic effect of methadone in germ cells of Drosophila melanogaster. *Mutat Res.* 1979;66(2):133–4.
11. Badr FM, Rabouh SA, Badr RS. On the mutagenicity of methadone hydrochloride. Induced dominant lethal mutation and spermatocyte chromosomal aberrations in treated males. *Mutat Res.* 1979;68(3):235–49.
12. Friesen C, Roscher M, Alt A, Mittner E. Methadone, commonly used as maintenance medication for outpatient treatment of opioid dependence, kills leukemia cells and overcomes chemoresistance. *Cancer Res.* 2008;68(15):6059–64.
13. Trzciak A, Kowalik J, Malecka-Panas E, Drzewoski J, Wojwodzka M, Iwanenko T, et al. Genotoxicity of chromium in human gastric mucosa cells and peripheral blood lymphocytes evaluated by the single cell gel electrophoresis assay. *Mutat Res.* 2000;46(2):235–49.
14. Andreani G, Gagnon D, Lodge R, Tremblay MJ, Richard D. An in vitro co-infection model to study Plasmodium falciparum-HIV-1 interactions in human primary monocyte-derived immune cells. *J Vis Exp.* 2012(66).
15. Speit G, Hartmann A. The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol Biol.* 1999;133:203–12.
16. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175(1):184–91.
17. Jalili M, Hatami A, Kalantari H, Kalantar E. Mutagenicity assessment of two herbal medicines, Urtan and Carmint in human leukocytes by single cell gel electrophoresis. *Saudi Pharm.* 2006;14:129.
18. Junqueira APF, Perazzo FF, Souza GHB, Maistro EL. Clastogenicity of Piper cubeba (Piperaceae) seed extract in an in vivo mammalian cell system. *Genet Mol Biol.* 2007;30(3):66–61.
19. Kalantari H, Jalali M, Moein E. In Vitro Evaluation of Mutagenic Effect of Vitagun in Human Leukocytes by Single Cell Gel Electrophoresis. *Jundishapur J Nat Pharm Prod.* 2007;2:26–31.
20. Hartmann A, Speit G. The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). *Toxicol Lett.* 1997;90(2-3):183–8.
21. Heibatullah K, Marzieh P, Arefeh I, Ebrahim M. Genotoxicity determinations of coriander drop and extract of Coriander Sativum cultured fibroblast of rat embryo by comet assay. *Saudi Pharm.* 2008;16:85–8.
22. Kobayashi H. A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay. *MM Commun.* 1995;3:145–151.
23. Hodges NJ, Adam B, Lee AJ, Cross HJ, Chipman JK. Induction of DNA-strand breaks in human peripheral blood lymphocytes and A549 lung cells by sodium dichromate: association with 8-oxo-2-deoxyguanosine formation and inter-individual variability. *Mutagenesis.* 2007;21(5):467–74.
24. Perez-Alvarez S, Cuenca-Lopez MD, de Mera RM, Puerta E, Karachitos A, Bednarczyk P, et al. Methadone induces necrotic-like cell death in SH-SY5Y cells by an impairment of mitochondrial ATP synthesis. *Biochim Biophys Acta.* 2010;1802(1):1036–47.
25. Garcia-Fuster MJ, Ramos-Miguel A, Rivero G, La Harpe R, Meana JJ, Garcia-Sevilla JA. Regulation of the extrinsic and intrinsic apoptotic pathways in the prefrontal cortex of short- and long-term human opiate abusers. *Neuroscience.* 2008;157(1):105–19.