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Original Article

Assessment of DNA damage in the heroin addicted population of Khyber Pakhtunkhwa, Pakistan using alkaline comet assay

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Abstract

The study was conducted to gain knowledge about the assessment of DNA damage in the population of heroin addicts and also to learn about the related health complications in drug abusers. A total of 103 samples of blood were collected from 65 heroin addicted subjects and 38 control subjects. For evaluation of DNA damage in the heroin addicted group, a comet assay technique was carried out. The results demonstrated that significant DNA damage 167.35±16.49 ($P<0.05$) was found in subjects who were addicted to heroin as compared to the control group having cells without any DNA damage 53.02±25.03. The population which were positive for human immunodeficiency virus infection had the highest total comet score values followed by asthma and hepatitis C virus. The total comet score increased significantly with age group. Significantly, the highest genotoxicity was concluded in the heroin addicted population.

Keywords: heroin addicted population, DNA damage, comet assay, genotoxicity

1. Introduction

Drug addiction is also called drug syndrome and has many complications. The general risk factor, which is characterized by signs and symptoms, is absence of certainty, peer pressure, miserable family conditions, and easy approach toward drugs due to irresponsible guardians (Lone & Mircha, 2013). Heroin is among the most commonly used and hazardous addictive opiates. It can damage the DNA inside the brain cells and lymphocytes (Li *et al*., 2006; Supic, Petrovic, Milicevic, Trajkovic, & Bukumiric, 2013). A study reported that the prominent negative effects on heroin addicted people are a decline in DNA damage repair pathways and the interchange of sister chromatids (Shafer, Falek, Donahoe, & Madden, 1990). Different investigations helped understand that medication which was illicit in nature enhances DNA damage, fracture, methylation and histone change in heroin dependent individuals (Vassiliades *et al*., 2008). It has been demonstrated that illegal drugs such as the use of heroin as well as morphine increase cell apoptosis along with causing termination of normal functionality of the immune system. Delayed investigations have shown that addiction to heroin is linked to genetic polymorphism. The recycled opium and heroin items impact the degree of UA, FBS, Ca +2, K+2) and the level of cholesterol that is present in the serum of human blood (Siilivan *et al*., 2013). The sedative victimizer has indicated a decline in the levels of testosterone and GnRH, along with sperm quality being badly affected. If the situation becomes grave, it could lead to serious problems such as hypogonadism. Medication metabolites have a poisonous impact that causes or is more likely to cause hepatotoxicity. Different studies conducted which helped us to understand that medications which are taken or consumed in the form of heroin or consumed in the

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form of morphine have always been perilous as compared to drugs that are consumed in a stimulant fashion, such as cocaine, etc. (Kumar, Chakraborty, & Das, 2012). Numerous procedures are viable through which one can determine different working agents that could possibly be the reason for damage done to DNA. The Comet assay is one of the exact techniques to determine DNA damage. It was created by Singe, McCoy, Tice, & Schneider, (1988) and Andreoli, Rossi, Marcon & Crebelli (1997). In Comet's method of determining the level of DNA damage, it can be estimated as the length of the DNA tail. DNA damage is viewed as higher when the length of the DNA tail is longer.

Many international studies have shed light on the fact that addiction and continually taking heroin can cause DNA damage, but very little literature is available regarding it in Pakistan. Consequently, the current investigation was intended to approach the evaluation of DNA damage in the heroin addicted population of Khyber Pakhtunkhwa, Pakistan, using an Alkaline comet essay.

2. Material and Methods

2.1 Study area

The samples were collected from three different districts: Peshawar, Charsadda and Nowshehra. Samples were collected according to the maximum heroin addict population of the districts.

2.2 Study population

A total of 103 collected subjects were included in the study, with the lowest age of 14 years and the highest age of 55 years. Study population was divided into two groups; the addict group included subjects who were addicted to heroin from one year. No specific amount of heroin usage by addicted subject was noted, and subjects were included according to the duration of heroin usage. The control group included subject who were not addicted to heroin and were considered as normal person. In the addict group, 65 subjects were included with a mean age value of 34.2 + 11.1 years, while in the control group, 38 subjects were included with a mean age of 36.18-+13.20 years. Among 38 individuals in the control group, 16 were students, 12 were employed, and the remaining 10 were unemployed. Patients with hepatitis C virus, human immunodeficiency virus and asthma were included in the study population.

2.3 Sample collection

The study sample were collected from three different districts; Peshawar, Charasadda and Nowshehra. Sample were collected at day time. Approximately 3 ml of blood sample was collected in a syringe per individual. To prevent coagulation, samples were transferred to EDTA tubes. Within 3-4 hours, samples were carried to molecular laboratory for assessment of DNA damage.

2.4 Alkaline comet assay

For the assessment of total DNA damage (single and double strand) and modification of alkali labile DNA, a technique called the Alkaline Comet Assay described by Sing *et al.* (1988) was performed. Duplicate comet assay slides in molten standard melting agarose (NMA) (0.7) were prepared for each sample and microscopic slides of conventional glass were dipped. placed in a tray to air dry and the tray were rubbed from the bottom in order to remove the extra agarose. Preparation and labelling of slides was done a day before use and kept at room temperature. About 70 µl low melting point agarose (0.7%) was taken and mixed with 15 µl of cell suspension and kept for 5 minutes at 0°C with coverslips after spreading on the top of pre coated slides. The cover slip was stripped and a second coating of 85 µl low melting point agarose was applied to completely fill any remaining gap. Agarose was kept at 0 ° C with a cover slip for 5 minutes.

2.5 Cell lysis

After solidification, cell lysis was performed. The freshly developed chilled lysing solution containing 2.5M NaCl, 10mM Tris, 100mM Na2EDTA, 1% Triton X-100, 10% DMSO and pH 10 was packed. After removing cover slips, the slides were carefully put into a chilled lysing solution. All the steps were carried out in the dark and at a 4 °C temperature to prevent accidental DNA harm.

2.6 Electrophoresis and neutralization

After cell lysis, a buffer was prepared containing 300 mM NaOH, 1mM EDTA and 13 p. slide was immersed for electrophoresis and were placed for 20 minutes to allow DNA denaturation. The electrophoresis was performed for 20 min at 300mA and 25V. In order to avoid any form of unintended DNA impact, slides were protected from direct exposure to light. Each slide was washed three times (400 mM Tris, pH 7.5) for 5 min for neutralization.

2.7 Comet tail visual scoring

Photographs which could be viewed like slides under florescent microscopy (Nikon Eclipse 80 I equipped with a 450 490 nm excitation filter) after staining with 70 µl Acridine orange dye (20 µg ml) and placed for 5 mints. Visual assessment of DNA damage was done on the basis of comet tail appearance. Comet DNA was categorized into five classes. Class 0; represents the intact position of the nucleus, showing no damage to DNA. Class 1; represents tiny or little damage to DNA because of small tail appearance. Class 2; shows medium-sized DNA damage because of a medium-sized tail appearance. Class 3; shows maximum DNA damage because of the greater visual appearance of the tail. Class 4; shows all of the DNA being damaged because complete DNA was visualized in the tail position (Figure 1). The total comet score (TCS) was then determined by the formula, TCS = 0 (n) +l (n) +2 (n) +3 (n) +4 (n), where "n" shows the number of cells that were present in each set of class. In this way, the score made by every slide was recorded during the interval of 0 (intact) and the maximum range of 400 (greatest harmed), as alluded to by Collins. After recording the Comet DNA visual scoring, the DNA damage of the addict group subject was compared with the DNA of normal subject in the control group.

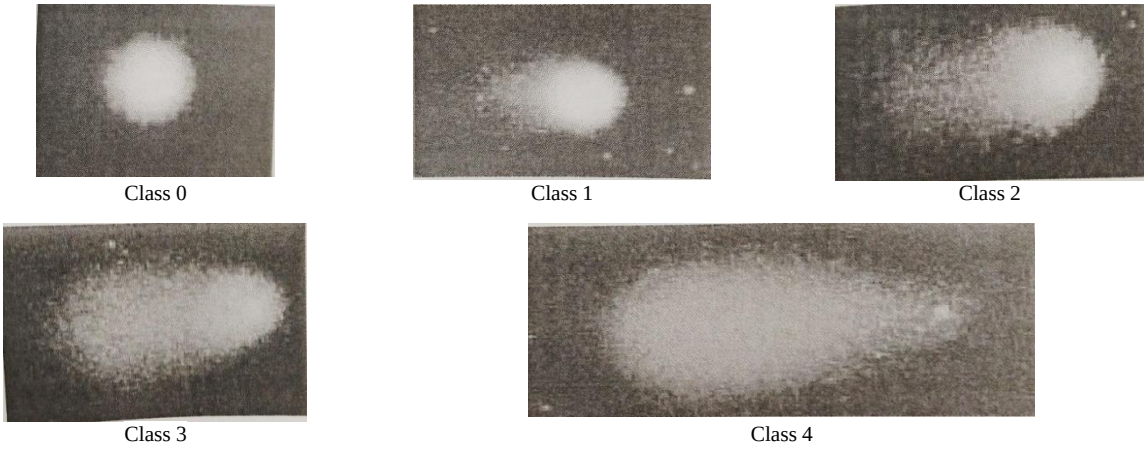

Figure 1. Cell showing different class and intensity of DNA damage (Class 0; represents the intact position of the nucleus, showing no damage to DNA. Class 1; represents tiny or little damage to DNA because of small tail appearance. Class 2; shows medium-sized DNA damage because of a medium-sized tail appearance. Class 3; shows maximum DNA damage because of the greater visual appearance of the tail. Class 4; shows all of the DNA being damaged because complete DNA was visualized in the tail position).

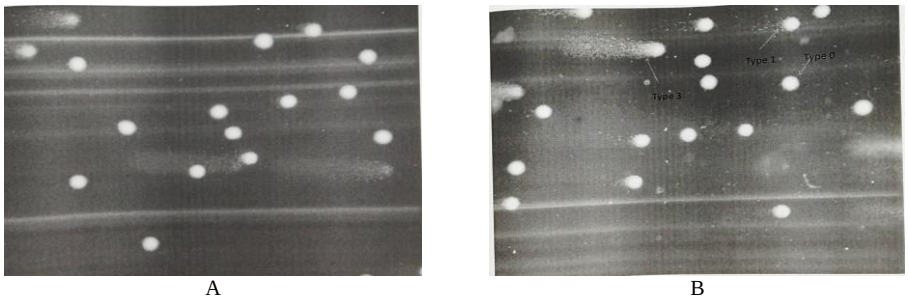

Figure 2. (A) Acridine orange staining cells showing Comet. (B) Lymphocyte cell DNA visualization from control subject

3. Results

The main characteristics of the study population showing age, occupation, routes of heroin administration and health status of heroin addicts are summarized in Table 1.

The degree of Comet score showing DNA damage was checked in the current study in both addict and control groups. Highest TCS value were found in the addict group as compared to the control group. The mean value of the total comet score between the addict and control group were 167.35±16.49 versus 53.02±25.03 (P= 0.005) (Table 2).

Comet DNA was classified into five classes; The highest DNA damage was found in Class 4, in the heroin addict subjects. It was 14.24+5.50 which was statistically higher than in the control subject of 3.68+3.24 (P <0.005). Difference of TCS value distribution according to health status and routes of heroin administration were also noted in the current study. The population which were positive for HIV infection had the highest TCS values, 189.00±4.63, followed by asthma and HCV. The current study also indicated that the most common routes of heroin administration were smoking and the highest TCS values were found in those addicts who use heroin intravenously (Table 3).

Comet score value distribution was also studied in different age groups. Highest TCS value of 180.68 ± 18.3 was found in the age group 46-55 as compared to the control group, followed by the age groups 36- 45 (171.87 ± 13.04), 26-35 (164.75 ± 9.00) and 14-25 (152.88 ± 10.44) (Table 4).

4. Discussion

In the current study, the degree of comet assay was studied in the subject and control group. Significant DNA damage frequency were found in the group which was addicted in comparison to the other group which had not developed the addiction, or in other words, the control group.

Mean values of total comet score between addicts and control group were 167.35±16.49 versus 53.02±25.03 (P= 0.001). In a similar study performed by Shafer *et al*. (1990) in a heroin addict population, a visible and pronounced lack of DNA capacity to reconstruct itself along with a fragile immune system was found. Drugs which are illegal to be used in ordinary cases, such as heroin, damaged the structure of DNA and were found to be responsible for the damage relating to disorders such as Genotoxicity (Shafer *et al*., 1990). In 1991, Falek *et al* in thier studies revealed that the use of drugs such as opiates could lead to abnormality in the chromosome repairing capability, which could consequently give rise to a deficiency in the repairing capacity of DNA (Falek, Donahoe, Madden, & Shafer, 1991).

Our study shows the frequent route for the intake of heroin were found to be smoking, which accounted for 43.07%, second was through intravenous, which amounted to 30.8%, and lastly, intranasal, which contributed 26.15% to the total pool respectively. The greatest of all the values relating to TCS is found among those addicts who were in regular use of heroin by intravenous means 172.00±24.36 and those who

Table 1. Main character of the study population

Main character	Heroin addicts	Control group (n)	TCS value (n)
Subject number	65	38	-
Mean age	34.2±11.1	36.2±13.2	-
Occupation			
Students		16	
Employed		12	
Unemployed		10	
Routes of heroin administration			
Intravenous	20	-	172.00±24.36
Smoking	28	-	171.28±9.02
Intranasal	17	-	156.00±6.58
Health status			
HIV	13	-	189.00±4.63
Asthma	16	-	168.06±19.61
HCV	16	-	165.37±7.20
Normal	20	-	154.30±7.39

Table 2. Degree of comet score in subject and control group

Comet class	0	1	2	3	4	TCS
Addicts group (n=65)	19.8 ±7.00	35.93±10.36	15.4±6.40	14.49±5.50	14.24±4.49	167.35±16.49
Control group (n=38)	72.39±11.36	13.6±5.97	6.28±4.33	4.02±3.91	3.68±3.24	53.02±25.03

P = 0.005, n = number of subject, TCS = total comet score

Table 3. Comet Score distribution according to the routes and health status of heroin administration

Subject	Addicts group (n)	TCS value (n)
Routes		
Intravenous	20	172.00±24.36
Smoking	28	171.28±9.02
Intranasal	17	156.00±6.58
Health status		
HIV	13	189.00±4.63
Asthma	16	168.06±19.61
HCV	16	165.37±7.20
Normal	32	154.30±7.39

n = number of subject, TCS = total comet score

Table 4. Comet score distribution according to age group (mean ± SD)

Subject	Addicts group	Control group
Age group	TCS	TCS
12-25 years	152.88±10.44	44.4±17.5
26-35 years	164.75±9.00	49.0±24.8
36-45 years	171.87±13.04	53.3±9.7
46-55 years	180.68±18.37	72.0±33.5

Significance different is P ≥ 0.001 as compared to control group., TCS = total comet score

smoked, following the lead with a numerical value of 171.28±9.02 and 156.00±58, respectively. The preferred methods for intake of heroin are by means of smoking along with intravenous intake as compared to intramuscular and intranasal (Hosztafi, 2001). Among the Indochinese addict population, the prefered administration route for heroin is smoking rather than by other routes for heroin administration (Swift, Maher & Sunjic, 1999).

The current study also shows increases in the severity of infection. Similarly, the highest TCS values were found in those addicts who were positive for HIV, followed by asthma and HCV. The results of the current study show similarity with other studies. Opiate users have serious health issues like human immune deficiency virus, hepatitis C virus and mortality because of their individual drug addiction and are at high risk of developing such infections (Fischer *et al*., 2002). Hepatitis C virus remains a high risk due to the drug equipment sharing (Clausen, Waal, Thoresen, & Gossop, 2009; Friedman, Newton, & Klein, 2003).

A significant effect of DNA damage was shown in the current study. DNA damage was significantly increased with age. The highest DNA damage of 180.68+ 18.37 were found in the age group 46-55 years as compared to other age groups. Other data widely shows that DNA damage repair capacity is slow down among different age groups. A recent study shows that in mice there is a significant DNA damage in micro nucleotide erythrocytes and leukocytes is noted in different age groups (Heuser, Andrade, Pere, Braga, & Chies, 2008).

5. Conclusions

The study shows that addicted people who are using illicit drugs like heroin have greater damage to DNA as compared to the general population. The study also concluded that the health of the addicted population is negatively affected. They have complications like acquired immune deficiency syndrome, hepatitis C virus, hepatitis B virus and asthma. Furthermore, this study also concluded that the highest TCS value is found among those addicts who used heroin intravenously. It is essential to regulate the trafficking and smuggling of these illicit drugs and also to educate our society about the genotoxicity caused by these illicit drugs in order to promote an infection free environment and a healthy lifestyle.

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