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Research paper

Cocaine effects on generation of reactive oxygen species and DNA damage: formation of 8-hydroxydeoxyguanosine in active abusers

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Abstract

Cocaine abuse continues to be a major public health problem in the world. An upper numbers of individuals are initiating cocaine use with a stable rate of growth each year with an increasing number of people with cocaine related problems. Following cocaine oxidative pathways a ROS formation are generated. Oxidative stress has been demonstrated to play an important role in cocaine addiction and toxicity due to its oxidized metabolites produced by cytochrome P450 during cocaine biotransformation. The ROS induced genotoxicities include DNA damage, gene mutation, chromosome aberrations and micronuclei formation. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) an oxidative modified DNA product, is the most representative product that may reflect oxidative damage induced by ROS. The present study was designed to investigate whether a systemic cocaine administration and its metabolism increase 8-OHdG production. Our findings clearly showed that cocaine promoted the ROS formation with significant increased of urinary 8-OHdG and MDA with a decreased of total scavenging capacity (TSC).

Keywords: *Cocaine Abusers; ROS; DNA Damage; 8-OHdG; MDA.*

1. Introduction

It is known that oxidative DNA damage occurs continuously in living cells and as a results of oxygen metabolic process and reactive oxygen species (ROS) play a pathogenic role in carcinogenesis by inducing oxidative DNA damage, modulating gene expression, altering different signaling pathways and leading to a deregulation of cell proliferation and apoptosis ((Marx (2004), Bartsch & Nair (2006),' Liou & Storz (2010)). ROS have an extremely short half-life and the direct determination of them in tissue or body fluids is impracticable, while the measurement of biomarkers of oxidative modified cellular constituents in biological samples is a promising strategy.

Many potential targets of oxidative damage are microRNAs (miRNAs), a family involved in post-transcriptional gene regulation, differentiation, cell proliferation, cell death, and carcinogenesis. MiRNAs, as oxidative DNA damage, are also involved in the regulation of telomerase activity, which is up-regulated in mutated cells. Telomeric DNA indeed is particularly rich in guanine residues and under ROS attack, is highly prone to 8-Hydroxy-2'-deoxyguanosine (8-OHdG) formation. 8-OHdG an oxidative modified DNA product is the most representative product that reflects oxidative damage induced by ROS. An increased production of 8-OHdG has been found after exposure to various carcinogens in target organs of mice, rats and human cells (Przybyszewski et al.(1998), Arima et al.(2006)). Higher mean values of 8-OHdG have been found in DNA from cancer than non-cancer tissue. In fact an higher concentration of 8-OHdG than healthy controls was observed in various malignant neoplasm such as gastric cancer (Hirahashi et al. (2014)), urinary bladder carcinoma (Soini at al. (2011)), lung cancer (Caliskan-Can et al.(2008)), and diabetes mellitus (Ece et al. (2012)) and chronic hepatitis (Nishida et al. (2013)) suggesting that a variety of diseases are characterized by an increased levels of oxidative damage of DNA. In the occupational exposure studies the increase of 8-OHdG was related to volatile organic compounds exposure (Kim et al (2011)), in foundry workers (Lin et al. (2011)), in male electrical and electronic equipment dismantling workers exposed to high concentrations of polychlorinated dibenzo-p-dioxins and dibenzofurans, polybrominated diphenyl ethers, and polychlorinated biphenyls (Wen et al (2008)). A positive association between ROS and levels of 8-OHdG has been found in lifestyle changing such as alcohol, tobacco, and morphine exposure (Zhang et al. (2004)). Drugs of abuse are known to lead to the formation of ROS and alter neuronal functions. However, few studies engaged in assessment of this oxidative biomarker for a population who has a voluntary addiction of cocaine. Moreover, no data are available regarding the correlation between higher concentration of 8-OHdG and addiction of cocaine. Cocaine is a powerfully additive drug of abuse and the epidemic abuse is a social and health problem worldwide. In an old research Karreman at al. (1959) invoked in this bioactive substance an electrochemical phenomenon with electron transfer. Electron transfer agents with oxygen can give rise to oxidative stress through generation of ROS. In fact the metabolites of cocaine play a role in toxicity and addiction involving ROS. Cocaine is metabolized by two different pathways in human. Hydrolysis of the ester groups is the major metabolic pathways but is not important in the toxicology of cocaine. Instead an oxidative metabolism of cocaine involving the amine moiety has attracted most attention relative to same toxic response. In fact hepatic injury from cocaine generally is regarded as attributable to one or more

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of its oxidative metabolites (Shi et al. (2012)) such as nor-cocaine, norcocaine nitroxide, N-hydroxynorcocaine, norcocaine nitrosonium, cocaine iminium, and formaldehyde. Although it has been shown that the cocaine depresses mitochondrial respiration, decreased mitochondrial membrane potential and enhances mitochondrial production of ROS (Cunha-Oliveira et al. (2013)). In the body the toxicity of cocaine, due to its oxidative metabolism, is reported in liver with generation of ROS and lipid peroxidase (Oztezcan et al. (2000), Vitcheva (2012)), in central nervous system (Sharan et al. (2003)), in cardiovascular system with the release of superoxide (Moritz et al. (2003), Vergeade et al. (2012)), in various form of renal disease where nitroxide and N-hydroxy derivative of norcocaine are believed to play a key role (Kovacic et al. (2002), Valente et al. (2012)). Several studies reporting the deleterious effects of cocaine and its oxidized metabolites un numerous cutaneous manifestations (Portugal-Cohen et al. (2010), Molgo et al. (2014), Menick & Salibian (2014)) and also skin ulcers (Shawwa et al. (2013)). These different skin insults resulted in common cellular biochemical pathways involving inducible nitric oxide synthase (iNOS) and xanthine oxidase (XO) and these enzyme are known to produce the radicals nitric oxide (NO●) and superoxide (O2●), respectively that combine together to form a peroxynitrite (ONOO-) which is considered as a harmful oxidant, that can attack a wide range of biological molecules (Szabo (2003)). Several studies involving the iNOS and XO in cocaine induced oxidative damage. The administration of cocaine enhanced iNOS expression in different organs and increased XO activity mainly in heart (Isabelle et al. (2007)) and liver (Aoki et al. (1997)).

Many studies have focused that another the cause of ROS formation is hypoxia. Is known that hypoxia induce severe oxidative stress and this is one of the major mechanism of tissue damage. Some investigators have found that hypoxia increased mitochondrial activation of Nuclear Factor–kappa B (NF-kB), one of oxidative stress-sensitive transcription factors. In fact the activation of NF-kB induce over-expression of apoptosis, inflammatory genes and also have a protective role by enhanced antioxidant enzyme expression such as SOD (Ergaz et al. (2005)). In order to protect the organism from oxidant damage about 90 genes have been identified that can be up-regulated in response to hypoxia (Harris (2002)).

The present study is aimed at investigating the human urinary excretion of 8-OHdG in response to various lifestyle habits as cocaine addiction and smoking in a sample of healthy people who had no known occupational exposure to carcinogens. In order to find the usefulness of this biomarker as early predictor of disease risk at population we examined the ability of cocaine to induce oxidative DNA damage by examine the 8-OHdG, the antioxidant power of the non-enzymatic defense of plasma that may be useful to providing an index of ability to resist oxidative damage. The malondialdehyde (MDA), a marker for lipid peroxidation, and cotinine determination were also measured in urine.

2. Materials and methods

2.1. Subject/sample collection

A total of 76 volunteers are included in the study. Subjects enrolled in this study have previously signed an informed consent, and a personal interview was conducted (in anonymous form for privacy rights) and information about socio-demographics, smoking history, cocaine consumption, occupational exposure, diet and family history of cancer were collected. The individuals recruited ranged in age from 20 to 65 years. There were no restrictions for study eligibility but subjects with concurrent disease or those taking medications capable of interfering with free radical production, such as non-steroidal anti-inflammatory drugs (NSAIDs) or antioxidants (vitamin C), were excluded from the study. The volunteers were divided in: cocaine group, smokers group, and control group, with 27, 23, and 26 for each group, respectively. The cocaine group was formed by cocaine abusers of the Addition Service of local Hospital, claimed that cocaine was their illicit drug of choice, used cocaine by any route for at least 2 years with self-administration of cocaine at least four times per month. Cocaine abusers were excluded if their urine toxicology screen is positive for illicit substances other than cocaine and its metabolites. As cigarettes smoking are more common in the cocaine group, the smokers group was create to know the real provision of cocaine addiction. The control subjects (not exposed to cocaine or tobacco products) were recruited to provide urine as reference specimens to define normal concentration of 8-OHdG, MDA, cotinine, and ferric reducing antioxidant power (FRAP). All recruited control participant claimed that they did not use cocaine or tobacco products, had no others living with them who used cocaine or tobacco, and as far as possible did not frequent areas where tobacco smoked was prevalent for at least 1 week before specimen collection. All participants in the study claimed to drink no more than one glass of wine (170 mL, 11% in alcohol) or beer (300mL, 5% in alcohol) at meals.

For monitoring the oxidative stress, the urine is considered an ideal biological sample because it can be obtained none invasively. Every 15 days, for a period of two months, the urine of the subjects with addition were analysed for evaluate the effective abuse of cocaine, while for the urine of controls were analysed for cotinine. After this pre-study period, a spot urine samples were collected from all volunteers in sterile 120 mL urine collection cups and were aliquoted into 10 mL polypropylene tubes. Specimens were store at –20°C until analysis.

The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Declaration of Helsinki. The demographical characteristics of subjects are reported in table 1.

Table 1: Characteristic of Subjects Included in the Research

	Controls		Smokers		Cocaine abusers	
Total	26		23		27	
	Male	Female	Male	Female	Male	Female
Age (x±sd)	46,5±6,8	38,4±7,7	50,4±3,5	44,2±5,7	47,8±6,6	46,3±5,9
Gender (%)	15(57,7)	11(42,3)	10(43,5)	13(56,5)	17 (63)	10 (37)
BMI kg/m^2 (x±sd)	20,8±5,5	21,4±3,1	23,6±3,9	22,1±3,4	22,9±5,7	24,1±6,4
Alcohol drinking	3/15	2/11	7/10	8/13	15/17	8/10

2.2. Urine analysis for 8-OHdG

The urinary levels of 8-OHdG was performed by high-performance liquid chromatography with electrochemical detection (HPLC-ED) and the separation conditions used were based on previous work (Inaba et al. (2011)) with modifications. Each sample was thawed and centrifuged at 1500xg for 10 min at 4°C to remove any precipitates before the clean-up procedure by solid-phase extraction (SPE). Briefly, an aliquot of 3.0 mL of urine was mixed with 0.2 mL of 10mM KH2PO4 (pH 4.0). The acidified urine samples were incubated for 10 min at 4°C and then centrifuged at 3000 g for 10 min at 4°C and supernatant was collected. Waters Oasis®HLB Vac cartridges (with 60 mg of packing material) were used for clean-up. OASIS®cartridges were preconditioned with 3 mL of methanol and then with 3 mL of 10mM KH2PO4. To avoid contamination and maximize the recovery, the cartridge was totally dried under vacuum after each step of clean-up. The sample solution (2 mL) was loaded onto the preconditioned SPE cartridge and passed through the cartridge under light vacuum. The column was washed with 3 mL of deionised water and 8-OHdG was eluted with 2mL of 30% acetonitrile in 50mM KH2PO4. After that, the eluate was evaporated to dryness under vacuum and the residue was dissolved in 100 μL of mobile phase. An aliquot of 20 μL was injected onto the HPLC. The HPLC chromatograms for 8-OHdG were recorded and integrated, as specified above, and were quantified by comparing the peak areas

with those obtained from external standards analysed on a daily basis. The accuracy of the measurement estimated from the recovery of an added 8-OHdG standard was 92-97%.

2.2.1 Apparatus and chromatographic conditions

HPLC analysis was carried out using a chromatographic system composed of the following: a model 515 pump (Waters, Milford, MA, USA) and a model 7725i sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20 µL loop. The detector system consisted of a Coulochem II model 5100A electrochemical detector with a model 5021 conditioning cell and a model 5011 analytical cell (ESA, Bedford, MA, USA). The conditioning cell (set to -350 mV) was placed between the column and the analytical cell; it was used to minimize the background noise of mobile phase due to the electro active components. Oxidizing potentials of the analytical cell were set at -50 mV for the first electrode (E1), and at +450 mV for the second electrode (E2). Chromatographic data management was automated using software Millennium32 (Waters, Milford, MA, USA). The analysis was performed on an analytical 150 x 4.6 mm i.d. reversed-phase Kromasil KR100 – 5C18 (5 µm particle size) column (Eka Chemicals AB – Bohus – Sweden), protected by a 20 x 4.6 mm i.d. (40 µM particle size) disposable Pelliguard pre-column (Supelco, Bellefonte, PA, USA). Analyses were performed at room temperature. The mobile phase consisted of a mixture of 10mM of Na2HPO4 containing (0.1 mM of EDTA, 100 mM of citric acid, and 2 mM of heptanesulfonic acid) and methanol (90:10, v/v); the pH was adjusted at 3.0 with 100 mM NaOH. The mobile phase prior to use was filtered through an 0.22 µm filter (Whatmann, Ltd, Maidstone, UK), while methanol through a FA 0.5 µm filter (Millipore, Bedford, MA, USA). The traces of organic materials were removed from the water by passing the water through a Sep-Pak Vac cartridge. This treatment reduces the electrode background currents. The mobile phase was degassed using an in-line degasser (Waters, Milford, MA, USA) and delivered at a flow rate of 0.8 mL/min.

2.3. Serum ferric reducing antioxidant power (FRAP)

The antioxidant power of urine was determined using FRAP assay (Benzie & Strain 1996). Briefly, 50 µL of the urine was added to 1.5 mL of freshly prepared and pre-warmed (37°C) FRAP reagent (300 mM acetate buffer, pH = 3.6, 10 mM TPTZ (tripyridyl-s-triazine) in 40 mM HCl and 20 mM FeCl3*6H2O in the ratio of 10:1:1) and incubated at 37°C for 10 min. The absorbance of the sample was read against reagent blank (1.5 mL FRAP reagent + 50 µL distilled water) at 560 nm with a spectrophotometer (Lambda 2. Perkin-Elmer, Milano Italy). Aqueous solutions of known Fe (II) concentration (FeSO4*7H2O) were used for calibration of the FRAP assay and antioxidant.

2.4. Malondialdehyde (MDA) assay

Malondialdehyde is the most prevalent product during oxidative stress originate from highly reactive lipid hydroperoxides (Spickett et al. (2010)) and MDA levels were determined for the extent of lipid oxidation using a commercial kit with thiobarbituric acid reactive substances (TBARS) assay (OxiSelect™ TBARS assay kit, Cell Biolabs, Inc., DBA Italia, Milano, Italy).
Briefly, after a spin at 5000g. For 10 min., to remove insoluble particles, 100µL of urine of volunteers were assayed directly. After the incubation of TBA reagent in a boiling water bath for 50 minutes, the resulting supernatant was measured at 532 nm with a spectrophotometer (Lambda 2. Perkin-Elmer, Milano, Italy). MDA standard curve was created to evaluate the results. The average value of three analyses was taken.

2.5. Urine analysis for cotinine

Cotinine is a metabolite of nicotine and due to its longer half-life has been used as reliable marker for smoking status. For the analysis of cotinine an aliquot of 10 µL of urine is used in a solid phase competitive ELISA kit (Sigma-Aldrich, Milano, Italy). The absorbance was read at 450 nm with a spectrophotometer (Lambda 2. Perkin-Elmer, Milano Italy) within 15 minutes after adding the stopping solution. A cut-off of 15 ng/mL of cotinine was used to differentiate smokers from controls.

2.6. Urine analysis for creatinine

Creatinine values may differ between individuals due to age and sex differences in the ratio between skeletal muscle and total body lean mass, and they are also affected by exercise and diet. For this the excretion values of 8-OHdG, cotinine, MDA and FRAP were normalized to the metabolic rate to account for the effects of antioxidants and individual differences. The creatinine level in the urine sample was measured between samples from different individuals and between different collection times. Creatinine in urine samples (1mL) was measured using an according Jaffe's picric acid assay kit.

2.7. Statistical analysis

Presented data are the mean ± standard deviation (SD). For comparison group data are used one-way ANOVA to determined concentrations difference of biomarkers of oxidative stress. Followed post hoc Tukey's HSD (Honestly Significant Difference) test was applied to statistically compare differences between the treatment groups. Results were considered as significantly different using a value of p<0, 05 for refusing the null hypothesis. To examine the oxidative stress biomarkers compared to the subjects characteristics, the Pearson's correlation analysis was used.

3. Results

A total of 76 participants, 23 smokers, 27 cocaine abused, and 26 healthy volunteers were included in the study. The mean urinary concentrations of 8-OHdG, MDA and FRAP of all the three groups are illustrated in figure 1, 2, 3, respectively.
The urinary concentrations of 8-OHdG in cocaine abusers group was significantly higher than that of controls (p<0,001) and smokers group (p<0, 01). Also the concentrations of 8-OHdG of smokers group was significant versus controls group (p<0, 01) (figure 1).

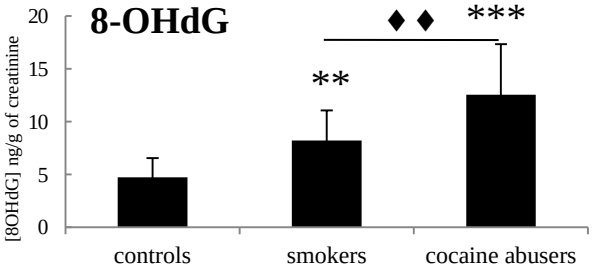


Fig. 1: Urinary Concentrations of 8-Ohdg. Values are Means ± S.D.Statistically Significant (** P<0, 01; *** P<0, 001 Versus Controls; ♦♦ P<0, 01 Versus Smokers).

Smoking (p<0, 01) and cocaine abusers (p<0,001) groups were significantly positively associated with urinary MDA respect to control group. Moreover cocaine abuser group showed a higher levels of MDA compared to smokers group (p<0, 05) that enhanced the lipid oxidation (figure 2).

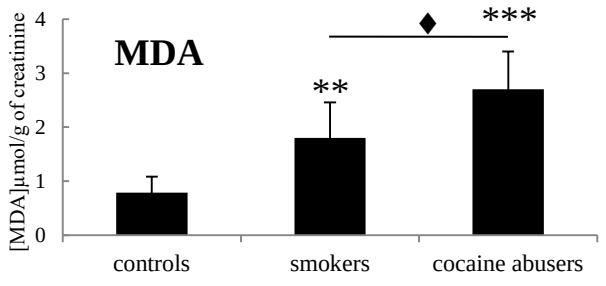


Fig. 2: Urinary Concentrations of MDA. Values are Means ± S.D.Statistically Significant (** P<0, 01; *** P<0,001 Versus Controls; ♦ P<0, 05 Versus Smokers).

The urinary concentrations of antioxidant power (FRAP) were significantly different in the groups of cocaine abusers and smokers versus controls group with a significant difference of p<0,001 and p<0, 05 respectively. Also, significant difference was present between cocaine abusers and smokers groups (p<0, 05) (figure 3).

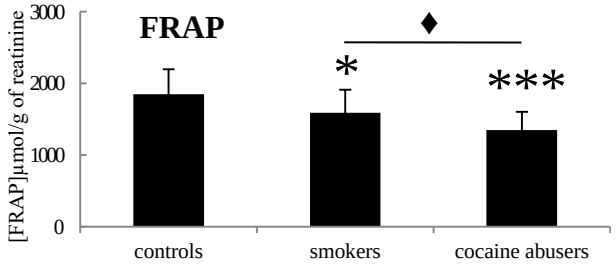


Fig. 3: Urinary Concentrations of FRAP. Values are Means ± S.D.Statistically Significant (** P<0, 01; *** P<0, 001 Versus Controls; ♦ P<0, 05 Versus Smokers).

As expected the high cotinine concentrations were revealed only in the smokers and cocaine abusers groups with no significative differences between them. All the concentrations of cotinine in urine of control group were <15, 0 ng/g of creatinine.

The Pearson's correlation analysis no revealed any significant correlation between age, gender, BMI, and moderate alcohol drinking with oxidative biomarkers.

4. Discussion

Most human cells usually produce low level of ROS and maintain a tightly controlled redox homeostasis with normal cell function, but in moderate oxidative stress, ROS production promotes cellular dysfunctions, while, during periods of profound extreme oxidative stress the protective antioxidant mechanism are overwhelmed. Mechanism of involvement of ROS in the pathology of disease is known and the monitoring of oxidative stress is quite important in the cocaine abuse study. Cocaine, primarily used illegally as recreational drugs, can cause oxidative injury both with metabolism that hypoxia. In our study we detect an increase of DNA oxidative damage and a significant increase in the MDA levels as well as decreases of antioxidants. DNA is very susceptible to radicals induced damage and ROS can modify amino acid side chain leading to formation of 8-OHdG the most commonly measured product of DNA oxidation.

A direct evidence for ROS, being involved in the process of dependence, is that cocaine can directly stimulate the formation of superoxide. Kloss et al. (1984) have shown that superoxide is produced when cocaine is metabolized by cytochrome CYP3A4. Superoxide is believed to reduce ferric iron to ferrous iron, which then reacts with hydrogen peroxide to form DNA-reactive hydroxyl radical. Since hydrogen peroxide is readily generated when superoxide accepts one electron in the presence of a water molecule, the metabolites of cocaine could induce the hydroxyl radicals (Inoue & Kawanishi 1987). Cytochrome CYP3A4 and mitochondria may be an important source of enhanced ROS production after cocaine treatment. In fact, in mitochondrial isolated from rats liver treated with a single dose of cocaine, have been observed to have significantly increased rates of ROS generation and this effect was significantly inhibited by pre-treatment of the rats with the cytochrome inhibitor SKF525A (Devi & Chan 1996).

In a recent paper Badisa et al (2015) the acute exposure of astroglia-like cells to cocaine causes an excessive release of ROS with a decrease in glutathione (GSH) level in dose-dependent manner. This depletion of GSH may be prevented with a pretreatment of N-acetylcysteine (NAC), a well-known antioxidant and therapeutic agent for oxidant related diseases and recently this compound has been viewed as a pharmacological drug that could provide protection against drug abuse in addicts (LaRowe et al. (2006)).

In human volunteers receiving a dose of 0.5 mg/kg of cocaine, the detected cocaine levels were between 0.1 and 1.0 µM in the serum (Javaid et al. (1982)) while the concentrations of cocaine in serum of active abusers are considerably higher reaching 0.1 mM (Siegel (1978)). Serum levels that were capable of inducing genotoxicities were comprised from 0.9 to 1.8 mM (Yu et al. (1999)). Such concentrations may seem too high even for active abusers but the lungs or nasal cavities of abusers may be locally exposed to high concentrations of cocaine through sniffing or smoking crack or cocaine powder and this could produce high levels of ROS in these cells and subsequent precancerous lesions.

As the co-abuse of cocaine and ethanol is so common, above all in the smokers and active-abusers, the consume of alcohol in experimental studies suggest that ethanol can increase DNA oxidation. The use of cocaine with ethanol leads to the formation of a hybrid metabolite of both substances known as cocaethylene. Formation and accumulation of cocaethylene seems to be able to retard the clearance of cocaine and then increase the oxidation with a greater 8-OHdG urine concentration (Wieland & Lauterburg 1995). In our study a relatively small sample size of the study groups was a limitation and does not allow forming subgroups of alcohol consumers, but the alcohol intake in the cocaine abusers group may be a further additive effect for the highest concentrations of 8-OHdG and MDA.

The increase of urinary concentration of 8-OHdG in cocaine group respect of smokers and control groups it would seem contribute to the generation of other ROS. Moreover this study focused on concurrent lipid oxidation with MDA increase and concomitant decreased of the antioxidant defence. In addition the urinary FRAP levels, that indicate the total antioxidant capacity, are significantly decreased in the active-abusers and smokers groups respect to the controls. The lower level of total non-enzymatic antioxidants may be due to the sequestration of antioxidants by products of oxidative metabolism of cocaine with a higher concentration of 8-OHdG. The elevated urinary 8-OHdG and MDA in cocaine abusers and smokers groups reflect the high levels of free radical that induce DNA and lipid damage in these subjects. This change, compared to controls, is the sum of effect of cocaine and smoke with more reducing of the total antioxidant capacity. Though the concentration of cotinine is higher in smokers than active-abusers, the concentration of 8-OHdG in the urine of cocaine addicts is significantly higher than that of smokers indicating a greater formation of ROS by metabolism and hypoxic effects of cocaine together.

The ability of defence from ROS is determined both from genetic factors and from concentration of antioxidant. Many experimental study have demonstrated the efficiency of antioxidant in reducing the adverse effects of oxidative stress and the use of antioxidant in clinical includes not only vitamin C and E but also carotenoids, flavonoids, folic acid and synthetic product as potent non toxic antioxidant. This seems to be the real approach for prevent or reduce the oxidative stress in the cocaine abusers. Furthermore, physician can be alert to these effects and potentially can recommended which detoxification treatment could be used in this addict's population to manage possible dysfunction and developed of

therapy for cocaine abuse. Future research to replicate this finding appears warranted.

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