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The oxidative DNA base damage in testes of rats after intraperitoneal cadmium injection

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Abstract

Cadmium is known to be a carcinogenic metal that especially its compounds have sufficient evidence in both humans and experimental animals beneath its environmental effects. Testis tissue is highly sensitive to the effects of cadmium. It is proposed that cadmium also increases oxygen derived free radicals and lipid peroxidation. As indicators of oxidative DNA damage, 6 oxidative DNA bases were determined by using Gas Chromatography/Mass Spectrometry-Selected Ion Monitoring technique. 45 Sprague-Dawley rats (225-300 g) were used as experimental animals and were divided into 3 groups of 15 rats. A single 2 mg NaCl/kg body wt, 0,5 and 1,25 mg $CdCl_2$/kg body wt were injected intraperitoneally to control, low and high dose groups, respectively. 5-OH Cytosine, 8-OH Adenine and Fapy Guanine lesions were elevated significantly in high dose group in the first day. A clear dose-response relationship was seen between dose groups and 8-OH Adenine levels related with time in all periods. There was a significant dose-response relationship in 2-OH Adenine, Fapy Guanine and 8-OH Guanine, especially in the second week suggesting the inhibition of XPA protein by cadmium after first week. In contrast, the observation of a significant decrease of 5-OH Cytosine levels after first week showed that cadmium could not affect the enzymes repairing the cytosine base lesions.

Introduction

Cadmium is an inorganic toxic heavy metal of continuing environmental and occupational concern with a wide variety of adverse effects. A considerable number of studies (Kipling & Waterhouse 1967, Thun *et al.* 1985, Stayner *et al.* 1992, Waalkes 2000) and reviews (Waalkes *et al.* 1992, Satoh *et al.* 2002) on cadmium toxicity have been carried out. International Agency of Research on Cancer (IARC) evaluated and classified cadmium and cadmium compounds under Group 1 as human carcinogens (IARC 1993). Epidemiological and experimental studies points out that cadmium and its compounds are related with carcinogenesis. It is proposed that occupational exposure to cadmium produces lung and prostate cancers in workers (Kipling & Waterhouse 1967, Thun *et al.* 1985, Kazantzis *et al.* 1988, Stayner *et al.* 1992, Waalkes & Rehm 1994, Waalkes 2000). Due to the widespread occurrence of this metal compounds at workplaces and in the environment, much effort has been made to elucidate the molecular basis for its carcinogenic potential. However underlying mechanisms for initiation of the carcinogenic process are incompletely understood. Studies and epidemiological investigations in experimental animals proves that exposure of cadmium is a causative factor for lung, prostate and testis cancer. It is proposed that cadmium causes oxidative DNA damage by various possible mechanisms such as formation of reactive oxygen species (ROS), increasing lipid peroxidation (Kasprzak 1991), induction of oxidative stress via glutation consumption (Bagchi *et al.* 1996) and inhibition of the enzymes responsible from the DNA repair. Cadmium also causes oxidative mutations in mammalian cells (Yang *et al.* 1996) and these mutations contribute to the initiation and promotion phases of carcinogenesis.

There are various studies related with the cadmium-induced inhibition of DNA repair enzymes (Kasprzak *et al.* 1992, Dally & Hartwig 1997, Kasprzak *et al.* 1997, Hartmann & Hartwig 1998, Asmuβ *et al.* 2000). In bacterial systems; formamidopyrimidine-DNA glycosylase (Fpg protein) and UvrA protein, in mammalian systems; Xeroderma Pigmentosum Group A (XPA protein), DNA ligase III enzyme and poly (ADP-ribose) polymerase (PARP enzyme), in human; 8-oxo-dGTPase MTH1 and Uracil-DNA N-glycosylase enzymes are responsible from the DNA repair mechanism. All of these enzymes and proteins have a structure called zinc-finger structure. Zinc element takes place in the structure of these enzymes. The studies proved that *in vitro* cadmium replaces with zinc and inhibits the repairing mechanism and it is expected that this replacement might have a role in the *in vivo* carcinogenecity of cadmium (Hartwig *et al.* 1996, Dally & Hartwig 1997, Hartmann & Hartwig 1998, Asmuβ *et al.* 2000).

Metallothionein protein is the key element of the cellular immune system and it has the ability to bind with cadmium as with the other metals and make them ineffective (Frieberg *et al.* 1986). Since the initial reports in the 1970's, a considerable number of publications have discussed the involvement of metallothionein as a protective agent against tissue damage from cadmium in reproductive tissues. The metallothionein content of testis tissue is not as high as liver and kidney so testis tissue becomes the most sensitive organ to the effects of cadmium.

In this study, we investigated the potentials of cadmium to induce *in vivo* oxidative DNA damage in testis tissues of rats after intraperitoneal (i.p.) cadmium injection. As sensitive indicators of oxidative DNA damage, we determined the oxidative DNA bases quantitatively by using Gas Chromatography/ Mass Spectrometry-Selected-Ion Monitoring (GC/MS-SIM) method.

Materials and methods

Animals and treatments

In the present study, 5 weeks old 45 Sprague-Dawley male rats (225–300 g) were used as experimental animals. They were purchased from Gülhane Military Medical Academy. All animals were housed at Ankara University Faculty of Pharmacy Experimental Animals Care Unit. They were feeded by pellet rat chow produced by Turkish Chow Industry Ankara Chow Factory. Furthermore, the rats were maintained at a temperature of 21 °C with lighting from 6 a.m. to 6 p.m. daily by a timer in order not to be affected by environmental conditions.

The rats were divided into three groups of 15 rats. One group of rats received a single 2 mg NaCl/kg body wt (control group). Rats from the treatment groups received a single i.p. injection of 0,5 (low dose) and 1,25 mg $CdCl_2$/kg body wt (high dose group), respectively. The rats were killed 1 day; 1 week and 2 weeks post injection. Their testes were quickly removed and stored in a deep freezer at -80 °C until analyses.

Isolation of DNA

The DNAs of the testis tissues of rats were isolated by Promega Wizard Genomic DNA Purification Kit. 600 μl of Nucleus Lyzis Solution was added to tubes containing the tissues and they were homogenized on ice with a Teflon pestle fitted homogenizer. These lysates were transferred into 1.5 ml eppendorfs. After 30 min incubation, 3 μl RNase solution was added and these mixtures were incubated at 37 °C for 30 min. Then 200 μl of Protein Precipitation Solution was added to the solutions and centrifuged at 10.000 g for 10 min for precipitation of proteins. The supernatants were carefully removed to other eppendorfs containing 600 μl of room temperature isopropanol. The solutions were gently mixed by inversion until the white thread-like strands of DNA form a visible mass. The solutions were centrifuged at 10.000 g for 2 min and the supernatants were decanted carefully. 600 μl of room temperature 70% ethanol was added for washing DNA then the solutions were centrifuged at 10.000 g for 2 min. Ethanol was aspirated carefully; eppendorfs were inverted on a clean absorbent paper for drying. 100 μl of DNA Dehydration Solution was added and the solutions were incubated overnight at room temperature. These solutions were lyophilized before the GC/MS-SIM technique was performed.

Determination of DNA Damage by GC/MS-SIM technique

The 6 oxidative DNA bases (5-OH Cytosine, Fapy Adenine, 8-OH Adenine, 2-OH Adenine, Fapy Guanine and 8-OH Guanine) were measured by GC/MS-SIM technique according to Dizdaroğlu's method in his laboratory (Dizdaroglu 1994).

Lyophilized DNA samples were solved in 10 mM phosphate buffer (pH = 7.4) and the isotope-labeled

Table 1. The amounts of the 6 modified bases per 10^6 DNA bases.

Oxidative DNA Bases	Oxidative DNA bases/10^6 DNA base ± S.D.[c]								
	1 day			1 week			2 weeks		
	Control n=5	0,5 mg/kg dose n=5	1,25 mg/kg dose n=5	Control n=5	0,5 mg/kg dose n=5	1,25 mg/kg dose n=5	Control n=5	0,5 mg/kg dose n=5	1,25 mg/kg dose n=5
5-OH Cyt	12,8 ± 3,8	12,8 ± 2,5	18,8[a,b] ± 5,8	22,3 ± 4,4	30,2 ± 6,2	24,8 ± 4,3	19,5 ± 4,4	25,3 ± 4,1	20,0[b] ± 5,2
FapyAde	11,9 ± 2,2	11,8 ± 3,2	13,6 ± 1,7	17,6 ± 3,4	18,8 ± 1,8	14,2[a,b] ± 1,0	17,5 ± 5,2	27,1[a] ± 6,4	26,3[a] ± 6,0
8-OH Ade	2,5 ± 0,6	3,1 ± 0,6	10,8[a,b] ± 2,8	4,3 ± 1,6	7,2[a] ± 1,7	15,7[a,b] ± 4,0	12,2 ± 3,8	24,5[a] ± 4,8	24,3[a] ± 7,0
2-OH Ade	4,0 ± 0,9	5,0 ± 0,6	4,1 ± 1,4	6,5 ± 0,6	4,9[a] ± 1,1	4,0[a] ± 1,8	7,0 ± 0,4	10,2[a] ± 1,8	11,7[a] ± 3,3
FapyGua	29,1 ± 3,4	29,3 ± 4,1	53,6[a,b] ± 4,0	38,2 ± 5,3	50,2[a] ± 4,6	30,8[a,b] ± 4,6	24,9 ± 7,0	46,5[a] ± 4,3	47,1[a] ± 9,2
8-OH Gua	11,4 ± 1,2	13,3 ± 1,2	12,2 ± 2,8	13,2 ± 2,2	15,6[a] ± 1,4	13,2[b] ± 1,8	18,8 ± 2,4	20,3 ± 5,2	25,2[a,b] ± 1,9

[a]Significantly different from control ($P < 0,05$).
[b]Significantly different from low dose (0,5 mg/kg $CdCl_2$ body wt) ($P < 0,05$).
[c]Standard deviation.

base analogs were added as internal standards. DNA pellets were dried under vacuum at −80 °C and were hydrolyzed by passing N_2 on them in a hydrolysis tube with 0.5 ml 60% formic acid. Hydrolysis procedure was undertaken at 140 °C for 30 min. Hydrolysates were transferred into small bottles, frosted in liquid nitrogen and lyophilized at −85 °C for 18 h. For derivatization, trimethyl silylation method was used. Lyophilized DNA hydrolysates were heated with 0.1 ml 1% trimethylchlorosilane included bis(trimethylsilyl) trifloroacetamide and acetonitrile compound (4:1, v/v) at 120 °C for 30 min. After heating, the samples were allowed to cool down to room temperature before proceeding. Equal amounts were taken from samples and injected to gas chromatography and the measurements were performed. 5% phenyl methyl silicone gum coated fused-silica capillary colon was used. After measurements the DNA lesions amount per 10^6 DNA bases were calculated.

The measurement of cadmium amounts in testes

The quantitative measurement of cadmium amounts in testes was performed by flameless Atomic Absorption Spectrometer (AAS) technique. Before performing this technique, the samples were digested. For digestion, 1 ml 65% HNO_3 and 250 μl 30% H_2O_2 were added to each 500 mg testis tissue samples and digestion was performed by using Milestone mls 1200 mega, High Performance Microwave Digestion Unit (HPMDU). Tissue cadmium levels were determined by using Varian, Model 30/40 Atomic Absorption Spectrometer. For this technique, a Varian GTA-96 graphite furnace atomizer was attached to the spectrometer and the equipment was set to background correction (deuterium lamp) mode. The determination of cadmium was performed at a wavelength of 228 nm. Stock solutions of cadmium (1.000 μg/ml) were used as calibration solution by diluting in appropriate deionized water to provide an adequate quantification range.

Results

After isolation of the DNA samples from the testis tissues of rats, the oxidative DNA damage was determined by measuring of 6 oxidative DNA bases by GC/MS-SIM technique. Oxidative DNA bases were 5-OH Cytosine (5-OH Cyt), 4,6-Diamino-5-formamidopyrimidine (Fapy Adenine) (FapyAde), 8-OH Adenine (8-OH Ade), 2-OH Adenine (2-OH Ade), 2,6-Diamino-4-hydroxy-5-formamidopyrimidine (Fapy Guanine) (FapyGua) and 8-OH Guanine (8-OH Gua). The stable isotope ana-

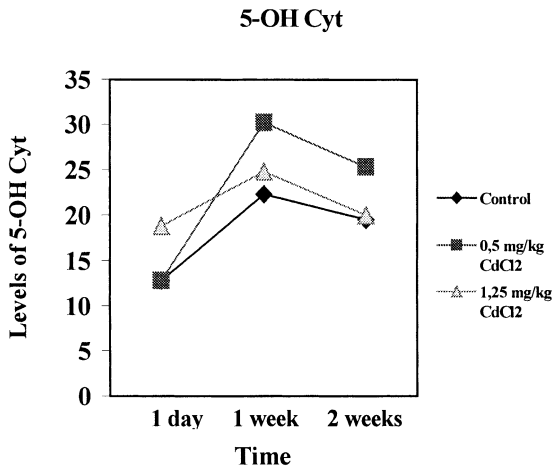

Figure 1. The levels of 5-OH Cytosine versus time.

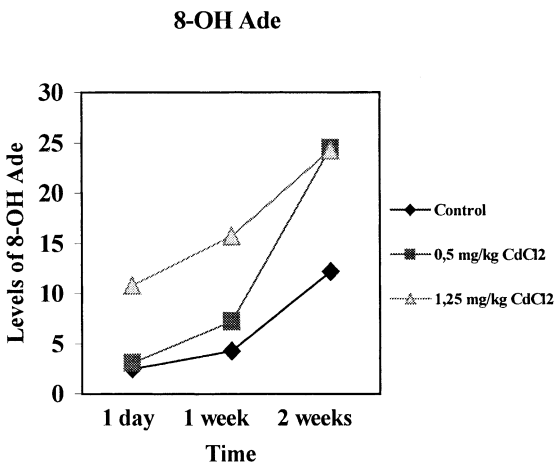

Figure 3. The levels of 8-OH Adenine versus time.

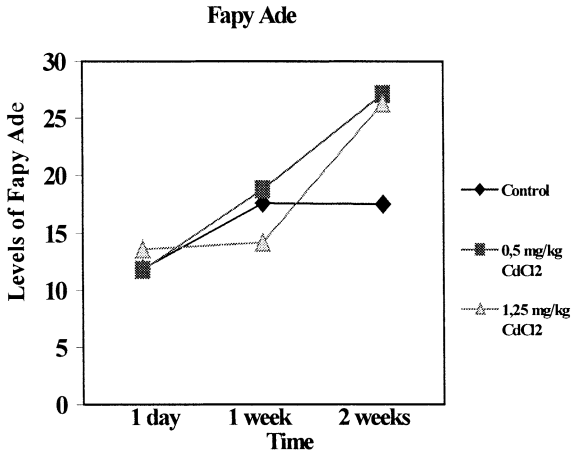

Figure 2. The levels of Fapy Adenine versus time.

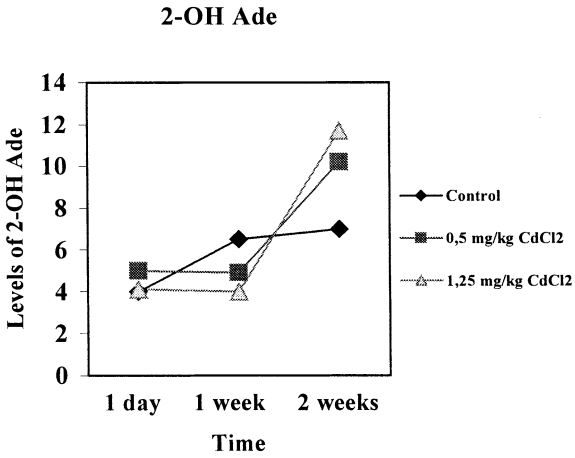

Figure 4. The levels of 2-OH Adenine versus time.

logs of these oxidative bases were used as internal standards and DNA damage was calculated as the amounts of the 6 modified bases per 10^6 DNA bases. The amounts of lesions of the 6 modified bases per 10^6 DNA bases were given in Table 1.

A significant elevation was observed in 5-OH Cytosine base, only in the first day in the high dose group compared with control and low dose groups. After first week, a significant decrease in 5-OH Cytosine levels was seen. Figure 1 shows the levels of 5-OH Cytosine versus time. Fapy Adenine levels were significantly increased with time but a significant dose-response relationship was not observed between the dose groups. Only in the second week, there was a significant elevation in the dose groups compared with the control group. Figure 2 shows the levels of Fapy Adenine versus time. A clear dose-response relationship was seen between dose groups and 8-OH Adenine levels related with time in all periods. This base seems to be mostly affected from the effects of cadmium and it reflected the effects of this metal very well in this study. Figure 3 shows the levels of 8-OH Adenine versus time. A dose-response relationship was not observed for 2-OH Adenine base in the first day and first week but especially in the second week, a significant enhancement was obtained between the dose groups and the control group. In Figure 4, the levels of 2-OH Adenine versus time are shown. A significant elevation was observed in Fapy Guanine levels in the first day in the high dose group compared with the control and low dose groups. After first week, a dose-response relationship was seen between the dose groups and control group. In Figure 5, the levels of lesions of Fapy Guanine versus time are shown. 8-OH Guanine levels were significantly increased with

Table 2. The ng/g amounts of cadmium in testes related with time

	Control	Low dose (0.5 mg/kg body wt)	High dose (1.25 mg/kg body wt)
1 day	7.118 ± 2.99	141.354 ± 16.38^{a}	$245.740 \pm 14.48^{a,b}$
1 week	6.426 ± 1.37	174.206 ± 6.85^{a}	$320.776 \pm 57.27^{a,c}$
2 weeks	5.836 ± 2.77	190.680 ± 15.34^{a}	$361.318 \pm 41.96^{a,b}$

[a]Significantly different from control ($P < 0,0001$).
[b]Significantly different from low dose group (0,5 mg/kg $CdCl_2$ body wt) ($P < 0,0001$).
[c]Significantly different from low dose group (0,5 mg/kg $CdCl_2$ body wt) ($P = 0,0005$).

time compared with the control group. Especially in the second week, there was a significant enhancement in the high dose group compared with the control and the low dose groups and there was an about two-fold increase compared with the first day. In Figure 6, the levels of 8-OH Guanine versus time are shown.

A significant relationship was obtained between the cadmium concentrations in testis tissue with time and also there was a clear dose-response relationship between the dose groups. In the first day, the cadmium concentration in the low dose group is about 20 fold and in the high dose group it is about 35 fold compared with control. In the first week, this ratio elevates to 27 and 50 respectively and in the second week, the ratios are 33 and 62 respectively. These results are a clear evident that testis tissue becomes the target organ to the effects of cadmium. In Table 2, the ng/g concentrations of cadmium in testes are shown.

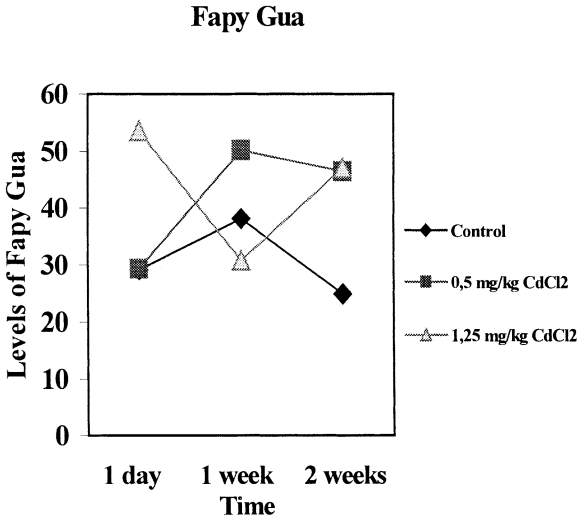

Figure 5. The levels of Fapy Guanine versus time.

Discussion

Cadmium has got carcinogenic effects beneath its environmental effects. IARC evaluated cadmium and its compounds as human carcinogen and classified in Group 1 (IARC 1993). There are several studies suggesting sufficient evidence in humans (Kipling & Waterhouse 1967, Elinder *et al.* 1985, Thun *et al.* 1985, Kazantzis *et al.* 1988, Stayner *et al.* 1992, Waalkes & Rehm 1994, Waalkes 2000) and sufficient evidence in animals (Poirier *et al.* 1983, Takenaka *et al.* 1983, Waalkes *et al.* 1988, Glaser *et al.* 1990, Waalkes & Rehm 1994).

To our best knowledge, the results of the present *in vivo* study reveal for the first time the single i.p injection of cadmium-induced oxidative DNA base damage in animal organs. A clear dose-response relationship was observed between the doses and cadmium concentrations in testes (Table 2). These significant enhancements might be the result of accumulation of cadmium

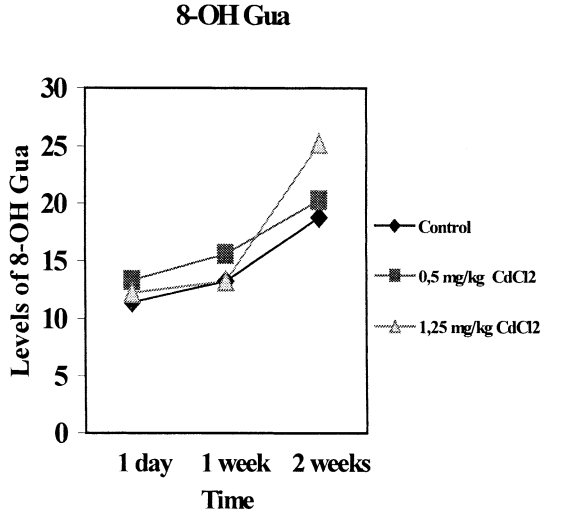

Figure 6. The levels of 8-OH Guanine versus time.

in testes with time because of the decreased levels of metallothionein in this tissue. Thus, the testis becomes the target tissue to the effects of cadmium. The reason of this is not clearly clarified yet, recent studies have questioned the existence of metallothionein in the testes and this has been the subject of considerable controversy. Some authors like Waalkes *et al.* consider that the lack of expression of metallothionein in this tissue would explain the sensitivity to cadmium exposure (Waalkes *et al.* 1992). On the other hand, some researchers like Nordberg *et al.* found out that the expression of metallothionein showed different profile characteristically in control and cadmium treated animals. (Nordberg *et al.* 2000). In this *in vivo* study, it was attempted to determine the DNA damage occurred in testes after single intraperitoneal injection of cadmium by measuring the oxidative DNA bases. There were recent similar studies done by Kasprzak and his coworkers in 1992 and 1997 with nickel and in 1994 with cobalt (Kasprzak *et al.* 1992, Kasprzak *et al.* 1994, Kasprzak *et al.* 1997). Various studies reported that the production of reactive oxygen species (ROS) and oxidative tissue are associated with the toxicity of cadmium (Ochi *et al.* 1987, Koizumi & Li 1992, Bagchi *et al.* 1995, Hassoun & Stohs 1996, Novelli *et al.* 1998). It is also known that cadmium causes indirect damages by inducing lipid peroxidation and oxidative stress on DNA molecule (Muller 1991). DNA is very sensitive to the effects of oxidative stress. Especially, oxygen derivated free radicals like $OH^{\cdot}$ causes formation of a number of modified bases and sugars in DNA (Jaruga *et al.* 1994). In the present study, of the 6 oxidative testicular DNA bases quantified, they were significantly increased by cadmium treatment and related with time.

Cadmium inhibits nucleotide excision repair involved in the removal of a broad spectrum of DNA lesions induced by environmental mutagens as well as the repair of oxidative DNA damage. There are several studies related with this inhibition induced by cadmium (Hartwig *et al.* 1996, Dally & Hartwig 1997, Hartmann & Hartwig 1998, Asmuβ *et al.* 2000). Cadmium performs the inhibition of repair effect by replacing with zinc, which takes part in the structure of the repairing enzymes. It is expected that this replacement might have a role in the *in vivo* carcinogenecity of cadmium (Dally & Hartwig 1997, Asmuβ *et al.* 2000). In the present study, the observation of a significant decrease of 5-OH Cyt levels after first week showed that cadmium could not affect the enzymes repairing the cytosine base lesions. There was a significant elevation in this base only in the first day and in the high dose group. Although, the levels of FapyAde were significantly elevated related with time, a significant dose-response relationship was not observed between the dose groups and FapyAde levels. A clear dose-response relationship was seen between dose groups and 8-OH Ade levels related with time in all periods. There was a significant dose-response relationship in 2-OH Ade, FapyGua and 8-OH Gua, especially in the second week suggesting the inhibition of XPA protein by cadmium after first week. 8-OH Gua levels were significantly increased with time compared with the control group and in the second week, there was a significant enhancement in the high dose group compared with the control and low dose groups and there was an about two-fold increase compared with the first day. Dally and Hartwig proposed that, the Fpg and XPA protein specifically removes the 8-OH Gua lesions (Dally & Hartwig 1997) so it was expected that after inhibition of repair the best dose-response relationship would be seen in 8-OH Guanine base in every period. However, we found the clearest dose-response relationship in 8-OH Ade for all periods. On the other hand, in 8-OH Gua, a clear relationship was only obtained after first week. In the further similar studies, 8-OH Ade base should be considered to measure because it was founded that this base was mostly affected from cadmium and it reflected the effects of this metal very well in this study.

In conclusion, the elevation of 5-OH Cyt, 8-OH Ade and FapyGua in the high dose in the first day might be the result of the increase in oxidative stress induced by cadmium. On the other hand, except 5-OH Cyt, a dose-response relationship was observed in modified bases especially after first week. This might be the result of the inhibition of XPA protein that repairs DNA by cadmium. This inhibition caused an increase in amount of DNA base products and oxidative DNA damage. Unfortunately, there is no enough study and data about the *in vivo* oxidative DNA damage of cadmium so further studies and investigations are needed to confirm the findings those were found in the recent studies.

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