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Effects of Cadmium on Hepatocellular DNA Damage, Proto-Oncogene Expression and Apoptosis in Rats[1]

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Objective To study the effects of cadmium on hepatocellular DNA damage, expression of proto-oncogenes c-myc, c-fos, and c-jun as well as apoptosis in rats. **Methods** Cadmium chloride at the doses of 5, 10, and 20 μmol/kg was given to rats by i.p. and there were 5 male SD rats in each group. Hepatocellular DNA damage was measured by single cell gel electrophoresis (or comet assay), while expression of proto-oncogenes c-myc, c-fos, and c-jun in rat hepatocytes were measured by Northern dot hybridization. C-Myc, c-Fos, and c-Jun were detected with immuno-histochemical method. Hepatocellular apoptosis was determined by TUNEL (TdT-mediated dUTP Nick End Labelling) and flow cytometry. **Results** At the doses of 5, 10, and 20 μmol/kg, cadmium chloride induced DNA damage in rat hepatocytes and the rates of comet cells were 50.20%, 88.40%, and 93.80%, respectively. Results also showed an obvious dose-response relationship between the rates of comet cells and the dose of cadmium chloride (r =0.9172, P<0.01). Cadmium chloride at the doses of 5, 10, and 20 μmol/kg induced expression of proto-oncogenes c-myc, c-fos, and c-jun. The positive brown-yellow signal for c-myc, c-fos, and c-jun was mainly located in the cytoplasm of hepatocytes with immunohistochemical method. TUNEL-positive cells were detected in cadmium-treated rat livers. Apoptotic rates (%) of cadmium-treated liver cells at the doses of 5, 10, and 20 μmol/kg were (17.24 ±2.98), (20.58±1.35), and (24.06±1.77) respectively, being significantly higher than those in the control. The results also displayed an obvious dose-response relationship between apoptotic rates and the dose of cadmium chloride (r=0.8619, P<0.05). **Conclusion** Cadmium at 5-20 μmol/kg can induce hepatocellular DNA damage, expression of proto-oncogenes c-myc, c-fos, and c-jun as well as apoptosis in rats.

Key words: Cadmium; DNA damage; Proto-oncogene; Apoptosis

INTRODUCTION

Cadmium, a ubiquitous toxic heavy metal with a long biological half life, to which most individuals are exposed, poses health and environmental problems. Increased human exposure to cadmium occurs in both occupational and non-occupational environments. Workers may be exposed to cadmium and cadmium compounds in a variety of occupational settings such as smelting, refining of zinc, electroplating, manufacturing of cadmium alloys, nickel-cadmium batteries and welding[1-2]. Under living conditions, cadmium is present in food, water and environment. Water and soil can be contaminated with industrial waste or the deposition of sewage sludge on agricultural lands. Aquatic organism and plants accumulate cadmium. For this reason, food consumption is a primary source of exposure for the general population[3]. In addition, all forms of tobacco contain cadmium and tobacco smoke is another source of exposure in humans[4].

Experimental and epidemiological studies have provided evidence that cadmium is carcinogenic by all routes of exposure to it so far tested, and exposure has been associated with tumors of the lung, prostate, testes and hematopoetic system[5]. In men, chronic exposure to low levels of cadmium results in damage to liver and kidney and is associated with neoplastic disease and aging. Cadmium has been classified by the International Agency for Research on Cancer as a category I (human) carcinogen. But the role of cadmium in carcinogenesis in humans and animals

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has not been definitely established[1,5].

Although the exact mechanism of cadmium toxicity is unclear, cadmium may induce oxidative damage in different tissues by enhancing lipid peroxidation[6]. Cadmium exposure can produce various direct and indirect genetoxic effects, such as DNA stread breaks, DNA-protein cross-linking, oxidative DNA damage, apoptosis, and inhibition of DNA repair[7-9]. In this study we investigated the effects of cadmium on DNA damage, apoptosis and expression of proto-oncogenes c-myc, c-fos, and c-jun in rat liver.

MATERIALS AND METHODS

Animal Handling

Male Sprague-Dawley rats weighing approximately 200±50 g were used in all the experiments. Rats were housed in polycarbonated cages with compressed fiber bedding. Commercial pelleted diet and water were provided. Four groups were used and each group had five animals. The first (control) group received no cadmium treatment, the second one received $CdCl_2$ at a dose of 5 μmol/kg, the third group received $CdCl_2$ at a dose of 10 μmol/kg and the last one 20-μmol/kg $CdCl_2$ prepared in 0.9% NaCl. The animals in the control group received an equal volume of 0.9% NaCl. The animals were sacrificed two days after the initial injections and liver was removed immediately for use.

SCGE Assay

DNA damage in liver induced by cadmium was detected using single cell gel electrophoresis or comet assay. Comet assay was carried out according to the procedure developed by Singh *et al*. with slight modifications[10]. Briefly, fully freezed slides were covered with 80 μL of 0.65% normal melting point agarose (NMA) in Ca^{2+} and Mg^{2+} free PBS (pH7.4) as the first layer. After solidification, 80 μL of mixture of freshly prepared cell suspension (10 μL, 1×10^6/mL-5×10^6/mL) with 75 μL of 0.65% low melting point agarose (LMA) in Ca^{2+} and Mg^{2+} free PBS (pH7.4) was rapidly pipetted onto the first layer as the second layer, and then covered with coverslips and kept at 4℃ for 5 minutes. After removal of the coverslip, 80 μL of 0.65% LMA (without cells) was added onto cell layer as the third layer. After solidification at 4℃ and removal of coverslip, the slides were lysed in freshly prepared ice-cold lysing solution for 1 hour, then the slides were removed and placed in a horizontal electrophoresis tank side by side avoiding space with the agarose end facing the anode. The tanks was filled with freshly prepared electrophoresis solution, and the slides were left in the solution for 20 minutes to allow the unwinding of DNA and expression of alkali labile damage before electrophoresis. Electrophoresis was conducted at 4℃ for 20 minutes using 25 v and the current was adjusted to 0.3 A by rising or lowering the buffer level. After electrophoresis, the slides were neutralized 3 times in 0.4 mol/L Tris-HCl buffer (pH 7.5), then 45 μL of 20 μg/mL ethidium bromide was added to each slide and covered with a coverslip. The slides were placed in a humidified air-tight container at 4℃ and examined within 2 days at 400× magnification using a fluorescence microscope (Nikon, Japan). Images of 100 randomly selected cells were analyzed. The degree of DNA damage was graded by naked eye into 5 categories according to the amounts of DNA in the tail: Grade 0: no damage, <5%; Grade 1: low level damage, 5%-20%; Grade 2: medium level damage, 20%-40%; Grade 3: high level damage, 40%-95%; Grade 4: total damage, >95% (Fig. 1).

Flow Cytometry

Apoptosis induced by cadmium was also evaluated by the determination of sub-G1 cells. At the end of various designated treatments, cells were washed, fixed and permeated with 70% ice-cold ethanol at 4℃ for 2 hours. Cells were then incubated with freshly prepared propidium iodide (PI) staining buffer (0.1% Triton X-100, 200 μg/mL RNase A, and 20 μg/mL PI in PBS) for 15 min at 37℃, followed by flow cytometry analysis of 20 000 cells in each group. The histogram was abstracted and percentage of cells in the sub-G1 phase was then calculated to reflect the percentage of apoptotic cells.

TUNEL

Apoptosis was demonstrated by TUNEL (TdT-mediated dUTP-digoxigenin Nick End Labeling) assay. The 3'-hydroxy end of DNA fragments was labeled with deoxy-UTP-digoxigenin and the digoxigenin was detected using specific antibodies and the peroxidase-diaminobenzidine system. TUNEL assay was performed using a test kit from Boehringer Mannheim according to the manufacturer's instructions. Briefly, sections were deparaffinized in xylene and hydrated in graded alcohol series. Endogenous peroxidase was blocked with 2% H_2O_2 in PBS. Terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-labelled dUTP were applied to the sections for 1 hour at 37℃. Sections were washed in buffer and treated with peroxidase-conjugated anti-digoxigenin antibody for 1 h at room temperature. Sections were stained lightly in diaminobenzidine (DAB), dehydrated in alcohol series,

cleared in xylene and mounted in Permount.

RNA Extraction

Total RNA was isolated using TRLzol reagent. Briefly, 10-100 mg of fresh liver tissue was homogenized in a homogenizer. Following homogenization, the homogenate was stored for 5 min at 4℃ to permit the complete dissociation of nucleoprotein complexes. Then 0.2 mL of chloroform was added, samples were covered tightly, shaken vigorously for 15 s and placed on ice at 4℃ for 5 min. The mixture was centrifuged at 12 000 g (4℃) for 15 min. After centrifugation, volume of the aqueous phase was 40%-50% of the total volume of the homogenate plus chloroform. The aqueous phase was carefully transferred to a fresh tube, an equal volume of isopropanol was added and stored for 10 min at 4℃. The samples were centrifuged at 12 000 g for 10 min. The RNA precipitation formed a white pellet at the bottom of the tube. After removal of the supernatant, the RNA pellet was washed twice with 1 mL of 75% ethanol by swirling and subsequent centrifugation for 5 min at 7500 g (4℃). At the end of the procedure, the pellet was dried under a vacuum for 5-10 min. At last, the RNA pellet was dissolved in 50-100 μL DEPC-treated water by swirling for 1 min. Purity of the RNA was determined by the ratio of the absorbance at 280 and 260 nm. The RNA yield was calculated based on the absorption at 260 nm.

Northern Dot Hybridization

Aliquots of total RNA (60 μg) were denatured for 15 min at 68℃ in 50% formamide, 17% formaldehyde, and 1×saline sodium citrate (SSC; 0.015 mol/L sodium citrate, 0.15 mol/L NaCl, pH7). Then, denaturated RNA was blotted on nylon membranes with successive decreasing dilutions using dot-blot apparatus. RNA was fixed by irradiating the membranes with uv light. The cDNA probe of c-myc, c-fos, and c-jun was labelled with digoxigenin by random priming labeling kit. RNA blots were prehybridized for 2h at 42℃ in 20% formamide with 3×saline sodium phosphate and ethylene diaminetetraacetic acid solution, 1% SDS, 5×denhardt's in the presence of 0.1 mg/mL salmon sperm DNA. RNA blots were then hybridized for 18h at 42℃ in the same solution containing 9% of dextran sulfate and the digoxigenin-labeled probe. Membranes were then washed four times at 20℃ for 5 min in 2×SSC, 0.1% of SDS, and then, two times at 55℃ for 20 min in 1×SSC with 0.1% of SDS. For color reaction, diaminobenzidine (DAB) was used. Levels of mRNA were quantified by densitometry.

Immunochemistry

The sections were deparaffinized in xylene and hydrated in grated ethanol continuously. Then the sections were covered with 3% hydrogen peroxide in PBS to block the endogenous peroxidase activity for 10 min and pretreated in citrate buffer (0.01 mol/L, pH 6.0) under microwave heating for 20 min to retrieve the antigen. Normal goat serum was added to the slides for 30 min at room temperature. Then the sections were incubated overnight at 4℃ with monoclonal primary antibody, anti-c-Myc, anti-c-Fos, and anti-c-Jun (Santa Cruez). SP kit was purchased from Beijing Zhongshan Biotechnology Ltd. (China). After the slides were washed three times in PBS for 5 min each, the biotinylated goat secondary antibody was added for 30 min followed by avidin-biotinylated peroxidase complex for another 30 min at room temperature. After being washed with PBS, the slides were stained with DAB and then counterstained with haematoxylin.

Statistical Analysis

The data were tested with statistical programs. Student's *t*-test or chi-square test was used and $P<0.05$ was defined as statistically significant.

RESULTS

DNA Damage Induced by Cadmium in Hepatocytes

Table 1 shows the effects of cadmium chloride at the doses of 5, 10, and 20 μmol/kg on the grades of DNA damage using the comet assay. Statistically significant differences were seen between three dose levels and the control using chi-square test, $P<0.01$. There was an obvious dose-response relationship and statistical analysis yielded a relative coefficient value of 0.9172 for the dose range examined. Figure 1 displays grades of DNA damage induced by cadmium in hepatocytes of rats.

Hepatocellular Apoptosis Induced by Cadmium in Rats

Table 2 shows the effects of cadmium chloride at the doses of 5, 10, and 20 μmol/kg on hepatocellular apoptosis using flow cytometry. There was a statistically significant difference between the cadmium-treated groups and the controls in apoptotic rates. Statistical analysis yielded a relative coefficient value of 0.8619, $P<0.05$.

TUNEL method was utilized to detect individual cells in the three cadmium-treatment groups. Representative results are depicted in Fig. 2. The black,

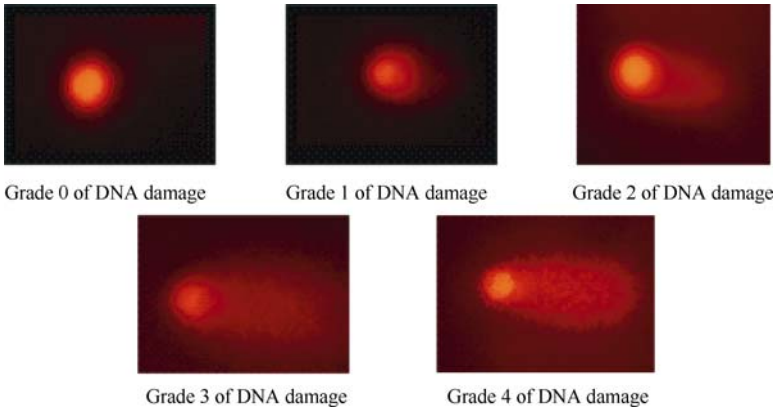

FIG. 1. Grades of DNA damage induced by cadmium chloride in rat hepatocytes. The degree of DNA damage was graded into 5 categories according to the amounts of DNA in the tail: Grade 0, no damage, <5%; Grade 1, low level damage, 5%-20%; Grade 2, medium level damage, 20%-40%; Grade 3, high level damage, 40%-95%; Grade 4, total damage, >95%.

TABLE 1

Effects of Cadmium on DNA Damage in Rat Hepatocytes

Groups	Cell Number	Grade of DNA Damage					Rates of Comet Cells
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	
$CdCl_2$ (5 μmol/kg)	500	249	106	58	56	31	50.20**
$CdCl_2$ (10 μmol/kg)	500	58	67	99	107	169	88.40**
$CdCl_2$ (20 μmol/kg)	500	31	49	57	149	214	93.80**
Control (0 μmol/kg)	500	450	39	6	5	0	10.00

Note. $^{**}P<0.01$, *vs* Control.

TABLE 2

Apoptosis Induced by Cadmium in Rat Hepatocytes

Groups	Animal Number	Apoptotic Rates
$CdCl_2$ (5 μmol/kg)	5	17.24±2.98**
$CdCl_2$ (10 μmol/kg)	5	20.58±1.35**
$CdCl_2$ (20 μmol/kg)	5	24.06±1.77**
Control (0 μmol/kg)	5	2.22±0.43

Note. $^{**}P<0.01$, *vs* Control.

brown and dark-brown immunoreaction's products, indicative of TUNEL positive outcomes, were observed in the groups treated with cadmium chloride at the doses of 5, 10, and 20 μmol/kg.

Expression of Proto-oncogenes c-myc, c-fos, and c-jun Induced by Cadmium in Rat Hepatocytes

Table 3 shows the effects of cadmium chloride at the doses of 5, 10, and 20 μmol/kg on expression of proto-oncogenes c-myc, c-fos, and c-jun in rat hepatocytes. Value of mRNA was expressed as integrated optical density (IOD) using densitometry. IODs of c-myc and c-fos mRNA induced by cadmium chloride at the doses of 10 and 20 μmol/kg were higher than those in the control, $P<0.01$. IODs of c-jun mRNA induced by cadmium chloride at the doses of 5 and 20 μmol/kg were significantly different from those in the control, and the value of cadmium chloride at the dose of 10 μmol/kg was higher than that in the control, but had no statistical significance, $P>0.05$, suggesting that cadmium could induce expression of proto-oncogenes c-Myc, c-Fos, and c-Jun in rat hepatocytes, but the mode was different in these three kinds of proto-oncogenes. The positive brown-yellow signal for c-Myc, c-Fos and c-Jun was mainly located in the cytoplasm of hepatocytes with immunohistochemistry method. Representative results are depicted in Fig. 3.

DISCUSSION

DNA lesions induced by metals consist of DNA single- and /or double-strand breaks, DNA-DNA cross-links, DNA-protein cross-links and base modifications. Under standard conditions, the comet assay detects the amount of cells with DNA single-strand

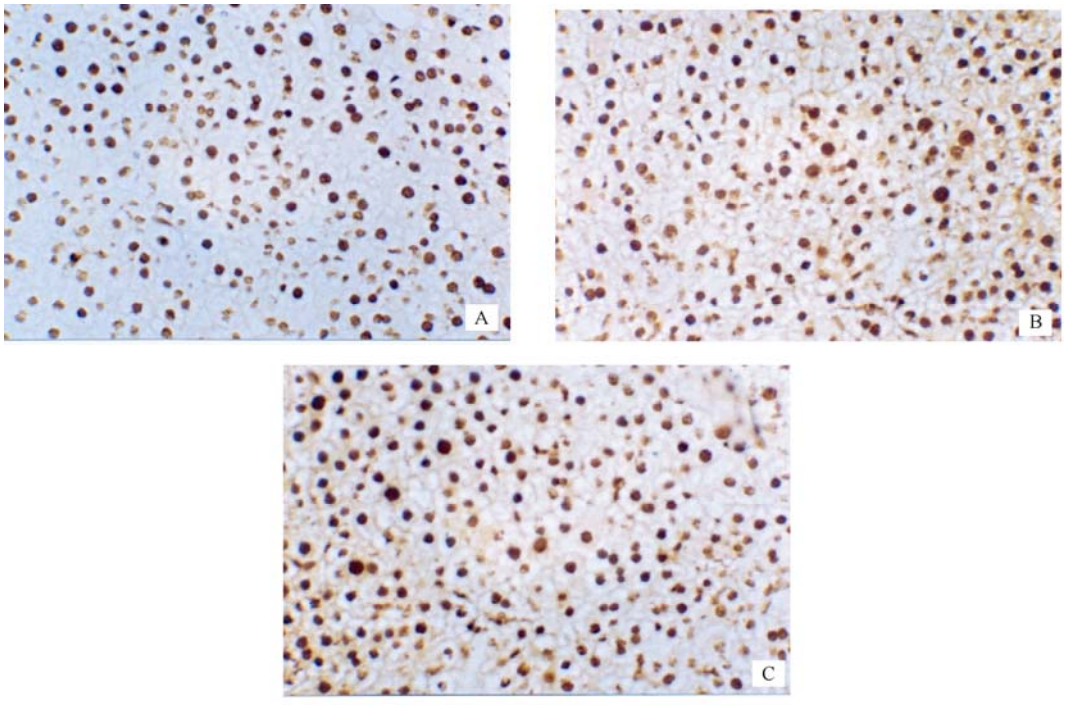

FIG. 2. Apoptosis induced by cadmium chloride at the doses of 5 (A), 10 (B), and 20 μmol/kg (C). Typical apoptotic changes such as cell membrane blebbing, chromatin condensation and formation of apoptotic bodies were observed in cadmium-treated groups.

TABLE 3

Effects of Cadmium on Proto-oncogene Expression in Rat Hepatocytes ($\bar{x} \pm s$)

Groups	Animal Number	Value of Integrated Optical Density (IOD)		
		c-myc mRNA	c-fos mRNA	c-jun mRNA
$CdCl_2$ (5 μmol/kg)	5	$18.16 \pm 9.10^{\triangle}$	$13.85 \pm 5.19^{\triangle}$	$22.42 \pm 3.45^{**}$
$CdCl_2$ (10 μmol/kg)	5	$26.62 \pm 5.14^{**}$	$18.79 \pm 3.13^{**}$	$14.51 \pm 3.59^{\triangle}$
$CdCl_2$ (20 μmol/kg)	5	$24.74 \pm 3.49^{**}$	$19.64 \pm 4.52^{**}$	$20.03 \pm 3.91^{**}$
Control (0 μmol/kg)	5	8.43 ± 2.76	6.99 ± 0.40	7.42 ± 1.05

Note. $^{\triangle}P>0.05$, $^{**}P<0.01$, *vs* Control.

breaks[11]. Our results showed that cadmium chloride at the doses of 5, 10, and 20 μmol/kg could cause DNA single strand breaks in hepatocytes of rats and there is an obvious dose-response relationship. Several possible mechanisms may be involved in the production of DNA damages. Studies have provided evidence that reactive oxygen species (ROS) are involved in DNA damage induced by carcinogenic metal ion[12]. Cadmium has been shown to enhance lipid peroxidation in cultured cells and animals[13-14]. Almazan *et al*.[15] showed that treatment with cadmium causes a more pronounced reduction in intracellular glutathione levels and a significantly higher free radical accumulation in progenitors. Cadmium can decrease intracellular glutathione content and activities of cellular antioxidant enzymes, superoxide dismutase, peroxidase and catalase, leading to the accumulation of ROS and an increase in intracellular oxidative stress in cadmium exposed CRL-1439 normal rat liver kidney cells[16-17]. The result is also in agreement with the recent report of Szuster-Ciesielska *et al*.[18], who found that cadmium induces formation of superoxide ion and hydrogen peroxide in HeLa human tumor cells and bovine aorta endothelial cells. Although Cd belongs to the group of transition elements, it almost always adopts only one oxidation state, 2+. Thus, in most chemical reactions it behaves similarly to main group metals. In particular, it does not induce production of ROS through a fenton-like reaction[19]. Mitochondria are the major source of ROS production in cells. The ROS produced include superoxide radical, hydrogen peroxide and hydroxyl radical[20]. About 1%-4% of total mitochondrial oxygen consumed is incompletely reduced and leads to the production of ROS[21]. Free radical scavengers and antioxidants have been

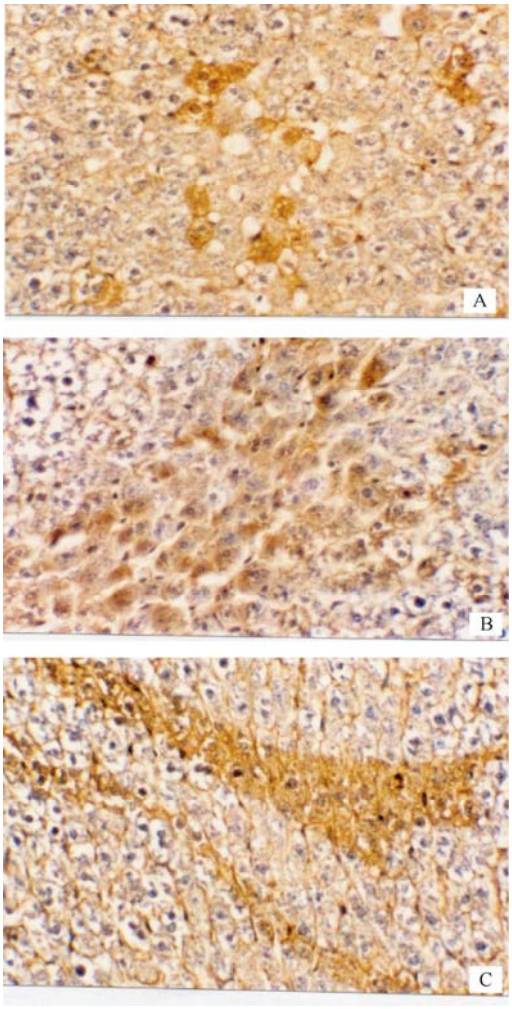

FIG. 3. Positive signal for c-Myc (A), c-Fos (B) and c-Jun (C) induced by cadmium chloride with immunohistochemistry method. The positive brown-yellow signal was mainly located in the cytoplasm of hepatocytes. ×400.

reported to attenuate Cd-induced toxicity[22]. Wang *et al*.[21] investigated the effects of Cd on the individual complexes of the electron transfer chain and on the stimulation of reactive oxygen species production in mitochondria and showed that the semiubiquinones are prone to transfer one electron to molecular oxygen to form superoxide, providing a possible mechanism for Cd-induced generation of ROS in mitochondria. One notable effect of ROS is to cause DNA damages, such as DNA strand breaks and base modifications[23]. Filipic *et al*.[7] demonstrated that cadmium induces a dose dependent increase of 8-OHdG adducts, which accumulate after prolonged exposure. The findings of Xu *et al*.[24] indicate that Cd in seminal plasma could affect semen quality and oxidative DNA damage in human spermatozoa, and 8-OHdG is significantly correlated with Cd in seminal plasma.

The level of DNA damage detected at any given time point is the result of a balance between the induction of DNA damage and its repair. Cells are capable of repairing most lesions induced by physical or chemical agents. Nucleotide excision repair (NER) plays a major role in repair processes, since it recognizes and removes a wide range of DNA lesions. This complex reaction allows lesion recognition, incision of the DNA strand on both sides of the lesion, excision of the damaged oligonucleotide and DNA polymerization and ligation. Therefore, DNA strand breaks may be the result of a direct effect on the phosphate backbone itself or may be due to removal of modified or damaged bases by the repair process[25]. Several authors have provided evidence that interference with DNA repair processes may be involved in cadmium-induced genotoxicity at the biologically relevant concentration. Inhibition of repair of induced damage may influence DNA effects in the comet assay due to the persistence of DNA lesions which lead to strand breakage by themselves and/or because of an accumulation of DNA strand breaks related to ongoing DNA repair[10,26].

Protooncogenes c-myc, c-fos, and c-jun belong to immediate response genes (IEGs) that undergo early transcriptional activation when quiescent cells are exposed to mitogenic substances such as cadmium. Cadmium-induced overexpression of protooncogenes c-myc, c-fos, and c-jun has been noticed in human prostate epithelial cells[27], BALB/c-3T3 cells[28] and cultured human proximal tubule cells[29]. Significant cadmium-induced overexpression of c-myc, c-fos, and c-jun is noticeable as early as 15-30 min postexposure, reaches the highest level of induction at 2-6 h and returns to the basal level at 24-30 h[27]. In a cell transformation experiment, a prolonged overexpression of c-jun and c-myc extended up to 2 weeks, followed by a strong down-regulation of expression 8 weeks after exposure. Sustained overexpression of c-fos, c-jun, and c-myc has been observed in BALB/c-3T3 cells transformed with $CdCl_2$[28]. The influence of cadmium on the induction of IEGs has also been studied in whole animals. Researchers have noted a dose-dependent induction of c-jun gene in mice as early as 3 h following sub-cutaneous injection of $CdCl_2$[2].

Cadmium can activate the expression of proto-oncogenes c-myc, c-fos, and c-jun. However, the mechanism of cadmium is indirect. Several possible mechanisms, including effects on secondary messengers such as reactive oxygen species (ROS) and intracellular Ca^{2+}, transcription factors, cellular signal transduction cascades involving kinases, and DNA-cytosine methylation, are considered to be

responsible for the cadmium-induced expression of proto-oncogene[2]. Exposure of the nontransformed BALB/c-3T3 cells to 20 μmol/L cadmium chloride for 1 h caused elevated intracellular levels of superoxide anion, hydrogen peroxide, and calcium, with corresponding increases in the expression levels of c-myc, c-fos, and c-jun. Joseph *et al*.[28] showed that the cadmium-induced overexpression of cellular proto-oncogenes is mediated by the elevation of intracellular levels of superoxide anion, hydrogen peroxide, and calcium. Further more, the cadmium-induced overexpression of the proto-oncogene is dependent on transcriptional activation as well as on pathways involving protein kinase C and MAP kinase. The kinases activated by exposure of cells to cadmium include protein kinase C and all three classes of the mitogen-activated protein kinase (MAPK) family consisting of extracellular signal-activated protein kinase (ERK), Jun N-terminal kinase (JNK) and p38 MAP kinase[28, 30-31].

The morphological features of apoptosis include chromatin marginalization along the nuclear membrane, nuclear condensation, budding, karyorrhexis, cell shrinkage, and fragmentation. The integrity of cellular organelles and plasma membrane is maintained until late in the process. DNA is often the target of toxicants, and DNA damage is currently thought to be the leading candidate for initiating the process of apoptosis[32].

Indeed, the key molecular event in apoptosis is the internucleosomal fragmentation of DNA, assumed to be caused by a specific endogenous endonuclease induced or activated due to a pre-existing inactive state[33]. Recent studies found that cadmium has the ability to replace calcium[34]. Moreover, cadmium can activate Ca^{2+}-dependent endonuclease, protein kinase C, and many other proteins in Ca^{2+}-dependent signaling pathways closely-related to the apoptotic process. One possibility is that cadmium promotes apoptosis in liver cells *via* direct stimulation of endonuclease. Lemarie *et al*.[35] showed that cadmium induces Hep3B cell apoptosis mainly by calcium- and oxidative stress-related impairment of mitochondria, which probably favors release of apoptosis-inducing factors and endonuclease G.

Caspases are crucial mediators of apoptosis. Among various caspases, caspase-3 appears to be particularly important due to its critical role in inducing characteristic apoptotic changes, including chromatin condensation, DNA fragmentation, and formation of apoptotic bodies, and its close association with many other mediators in apoptosis such as caspase-9, cytochrome c and PARP[36]. Shen *et al*.[34] reported that upon cadmium exposure, there is a rapid and sustained intracellular Ca^{2+} elevation, followed by caspase-3 activation and PARP cleavage, all of which precede the characteristic DNA fragmentation. BAPTA, a specific intracellular Ca^{2+} chelator, abolishes Cd-induced Ca^{2+} overloading and subsequently inhibits caspase-3 activation, PARP cleavage and apoptosis. These findings suggest that intracellular Ca^{2+} elevation may directly trigger caspase-3 activation through either mitochondria or activation of Ca^{2+}-dependent protease. Caspases can be broadly divided into two groups: initiator caspases, such as caspase-8, -9, and -12, whose main function is to activate downstream caspases, and executor caspases, such as caspase-3, -6, and -7, which are responsible for degradation of cellular proteins. A hallmark of apoptosis is the proteolytic inactivation of poly (ADP-ribose) polymerase (PARP) and several caspases such as caspase-3-degrade PARP in apoptosis. Kondoh *et al*.[36] found that cadmium induces activation of caspase-9 prior to DNA fragmentation. Caspase-3 is a caspase downstream of caspase-9. Therefore, it is suggested that the pathway of cadmium-induced apoptosis is partly dependent on the mitochondria pathway and that it is accompanied with caspase activation[36]. Several mechanisms underlying ROS induction of apoptosis have been proposed, however an integrated model is yet to be established. H_2O_2 acts upon mitochondria, causing a disruption of mitochondrial membrane potential and the release of cytochrome C. Once cytochrome C is in the cytosol, it binds to Apaf-1 and becomes an essential component of the apoptosome. Assembly of the apoptosome complex initiates the caspase cascade by first activating caspase-9[32].

In conclusion, cadmium at the doses of 5-20 μmol/kg can induce hepatocellular DNA damage, expression of proto-oncogenes c-myc, c-fos, and c-jun as well as apoptosis in rats. Further studies are needed to display the exact mechanisms of cadmium-induced carcinogenesis in humans and animals.

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