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Mitochondrial oxidative stress elicits chromosomal instability after exposure to isocyanates in human kidney epithelial cells

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Abstract
The role of oxidative stress is often attributed in environmental renal diseases. Isocyanates, a ubiquitous chemical group with diverse industrial applications, are known to undergo bio-transformation reactions upon accidental and occupational exposure. This study delineates the role of isocyanate-mediated mitochondrial oxidative stress in eliciting chromosomal instability in cultured human kidney epithelial cells. Cells treated with 0.005 μM concentration of methyl isocyanate displayed morphological transformation and stress-induced senescence. Along the time course, an increase in DCF fluorescence indicative of oxidative stress, depletion of superoxide dismutase (SOD) and glutathione reductase (GR) and consistent accumulation of 8-oxo-dG were noticed. Thus, endogenous oxidative stress resulted in aberrant expression of p53, p21, cyclin E and CDK2 proteins, suggestive of deregulated cell cycle, chromosomal aberrations, centromeric amplification, aneuploidy and genomic instability.

Keywords: *Free radicals, mitochondria, oxidative stress, genomic instability, carcinogenesis, isocyanates*

Abbreviations: *8-oxo-dG, 8-hydroxy-2′deoxy guanosine; CIN, Chromosomal instability; CDK, Cyclin-dependent kinase; CMH2DCFDA, 2′-7′- dichlorodihydrofluorescein diacetate; EMEM, Eagle's Minimal Essential Medium; FISH, Fluorescence in-situ hybridization; GR, Glutathione reductase; GCRs, Gross chromosomal rearrangements; GSH, Glutathione; HEK-293, human kidney epithelial cell; MIC, Methyl isocyanate; ROS, Reactive oxygen species; SKY, Spectral karyotyping; SOD, Superoxide dismutase.*

Introduction

The production of reactive oxygen species (ROS) in mammalian cells is tightly regulated because of their potential to damage macromolecules, including DNA. Over-produced ROS within cells act as secondary messengers in intracellular signalling cascades that induce and sustain the oncogenic phenotype of cancer cells [1]. Also, it has been speculated that oxidative damage, because of its mutagenic effects, has a pro-tumoural role of senescence in epithelial cells [2]. In cancer, genomic damage has been demonstrated to be of high patho-genetic relevance. DNA lesions may induce mutations of oncogenes and tumour-suppressor genes which, in the long-run, may lead to malignancies until and unless mitigated by repair mechanisms [3]. The quintessence of phenotypic transformation is associated with neoplasticity. Accumulation of genetic changes appearing at chromosome and/or gene levels results in the disturbance of the balance between cell proliferation and differentiation [4]. Acquisition of these genetic alterations is thought to involve 'genomic instability', a process which occurs with varying efficiency due to defective genome maintenance programmes. These include the

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accumulation of oxidative damage to DNA and other cellular constituents, flaws in mitochondrion function that promote oxidative stress [5]. Chromosomal instability, an important component in the process of carcinogenesis, can be caused by exposure to agents, which appear to act through induction of stress-response pathways related to oxidative stress [6]. In addition, oxygen metabolism and ROS are important sources of DNA damage that leads to gross chromosomal rearrangements (GCRs) [7].

Cells respond to oxygen-induced damage by expressing the tumour suppressor p53 and the cyclin-dependent kinase inhibitor p21 (Cip1/WAF1/Sdi1) (p21), which limits proliferation by blocking entry into S phase [8]. Cellular insults that alter mitochondrial function have important consequences on p53 and, incidentally, ROS generated at mitochondria are thought to regulate p53 activity [9]. Tumour-associated cell cycle defects are often mediated by alterations in cyclin-dependent kinase (CDK) activity, in turn inducing unscheduled proliferation and chromosomal instability [10].

Environmental stressors (cytotoxic agents, pollutants or toxicants) are well known to contribute to a variety of pathological conditions and oxidative stress seems to be the central element [11]. Isocyanates belong to a ubiquitous chemical group that has diverse industrial applications. Despite their wide spectrum usage, isocyanates have been shown to cause damage to DNA by forming DNA cross-links/adduct which in turn contribute to cytotoxicity and detrimental effects [12]. Earlier, we have demonstrated that methyl isocyanate (MIC), one of the most toxic isocyanates, is capable of undergoing biotransformation reactions, i.e. DNA damage, oxidative stress and inflammation in cultured human lymphocytes [13]. The kidney is the target of numerous xenobiotic toxicants, including environmental mutagens. Several anatomical, physiological and biochemical features of the kidney make it particularly sensitive to many environmental compounds [14]. Predominantly, the epithelial cell surface of the kidney is continuously exposed to large amounts of exogenous and endogenous agents, thereby making it prone to injuries. One of the factors of injury is mediated by ROS [15]. *In-vitro* incubation of renal tubulus cells to a multitude of toxic factors/chemicals induces DNA damage via enhanced generation of ROS [16]. Also, it has been demonstrated that mitochondria, under stressful conditions, generates nitric oxide and oxygen radicals, which further deteriorates kidney function [17]. Although preliminary evidence available in the literature suggests that exposure to isocyanates may have deleterious health effects including renal dysfunction [18,19], mechanistic insights pertaining to such an effect have never gained significant attention.

The aim of our study was to ascertain the molecular mechanisms underlying the isocyanate mediated mitochondrial oxidative stress in eliciting chromosomal instability in cultured human kidney epithelial (HEK-293) cell line using *N*-succinimidyl *N*-methylcarbamate, a surrogate chemical to MIC [20]. Our study provides evidence that MIC exposure leads to chromosomal instability mediated by mitochondrial oxidative stress in cultured human kidney epithelial-HEK-293 cells. We also predict that increasing knowledge on redox signalling leading to genome unsteadiness could provide new strategies for investigating the effects of oxidative stress response following xenobiotic exposure and predisposition to oncogenesis in the renal milieu.

Materials and methods

Reagents

N-succinimidyl *N*-methylcarbamate [CAS No. 18342–66–0] (Sigma Aldrich Laboratories, St. Louis, MO) dissolved in 2 mM DMSO at a final concentration of 0.005 μM (1 μg/μl) was used for investigations. The culture petri-dishes were procured from Nalgene-Nunc Inc. (Roskilde, Denmark). Foetal bovine serum was obtained from HyClone Labs (Logan, Utah). Eagle's Minimal Essential Medium (EMEM) growth medium was procured from Gibco/BRL Life Technologies, Inc. (New York). Evaluation of ROS was performed by CMH2DCFDA from Molecular Probes, Invitrogen Co. (Carlsbad, CA). Formation of 8-hydroxy-2'deoxyguanosine (8-oxodG) and depletion of superoxide dismutase (SOD) glutathione reductase (GR), markers of oxidative stress, were evaluated by using ELISA kits from Trevigen Inc. (Gaithersburg, MD). For assessing the cell cycle regulatory proteins p53, p21, cyclin E and CDK2 and immunocytochemical analysis of centrosomal protein pericentrin, antibodies procured from Abcam (Cambridge, UK) and Calbiochem (Nottingham, UK) were used with appropriate dilutions. For FISH analysis, human pancentromeric probes (FITC green labelled) procured from ChromBios GmbH (Muhlenstr, Raubling, Germany) and SKY analysis by SKY DNA kit obtained from Applied Spectral Imaging GmbH (Edingen, Neckarhausen, Germany) were used. Cellular senescence assay was performed using senescence associated β-gal staining (SA-β gal) kit procured from Chemicon International (Temecula, CA).

Cell culture

Human kidney epithelial HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were seeded at 2×10^5 cells/60 mm culture dishes in EMEM supplemented with 10% foetal bovine serum, 1.0 mM

sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate at 37°C in the humidified atmosphere of 5% CO_2 in air according to ATCC catalogue instructions. After optimum confluency, the cells were treated with the experimental agent, *N*-succinimidyl *N*-methylcarbamate. At the onset of the experiments, the cells were growing exponential and asynchronously.

Study design

Different sampling intervals ($n = 5$) ranging from 0–180 h were chosen. The cells were treated using a constant 0.005 μM concentration of *N*-succinimidyl *N*-methylcarbamate. The selection of the dose was performed on the basis of authors' previous observations in which this concentration was found optimum to induce DNA damage, oxidative stress and inflammation in cultured mammalian cells [13]. Controls were untreated normal HEK-293 cells.

Oxidative stress and antioxidant defense system

Assay of intracellular ROS. A fresh stock solution of CMH2DCFDA (5 mM) was prepared in DMSO and diluted to a final concentration of 1 μM in 1×PBS. The cells were washed with 1×PBS followed by incubation with 50 μl of working solution of fluorochrome marker CMH2DCFDA (final working concentration adjusted to 2.5 μg/50 μl) for 2 h. The cells were harvested, washed in PBS and cell-associated fluorescence was measured by flow cytometry in FL1 channel [13].

Evaluation of oxidative stress by 8-oxo-dG. 8-Oxo-dG is a modified nucleoside base, which is the most commonly studied and detected byproduct of DNA damage that is excreted upon DNA repair. ELISA for quantification of 8-oxo-dG in culture supernatant was performed according to the manufacturer instructions and optical density was measured at 450 nm on an ELISA reader (Tecan Sunrise, Austria) [13].

Estimation of SOD activity. Superoxide dismutases (SOD, EC 1.15.1.1) are a group of isozymes functioning as superoxide radical scavenger in the living organisms. Superoxide dismutases (SODs) catalyse the dismutation of the superoxide radical ($O_2^{\cdot -}$) into H_2O_2 and elemental oxygen (O_2) which diffuses into the intermembrane space or mitochondrial matrix. Levels of antioxidant defense system enzyme, SOD, were measured using an instructions manual provided by the manufacturer and absorbance kinetics were measured at 450 nm through an ELISA reader [13].

Estimation of GR activity. Glutathione reductase plays an essential role in maintaining the appropriate levels of intracellular reduced GSH. Levels of antioxidant defense system enzyme, GR, were measured using instructions as supplied by the manufacturer and absorbance kinetics were measured at 340 nm through an ELISA reader [13].

Cell cycle regulation

Western blot analysis. Briefly, cells were rinsed twice in PBS and lysed in the buffer (10% SDS, 1 M Tris pH-7.6, 5 mM EDTA). The obtained cell lysates were centrifuged at 12 000 rpm for 10 min at 4°C and the supernatant was collected. Protein concentrations were determined from the supernatant by Bradford assay. An amount of 100 μg protein was analysed through 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane in 25 mM Tris, 194 mM glycine and 20% methanol at 4°C with semi-dryer transfer unit of Hoefer (Holliston, MA). Membranes were blocked with 5% non-fat milk in 0.1% PBST and incubated overnight at 4°C with primary monoclonal antibody specific for cyclin E, CDK-2, p53, p21 (1:1000 dilution). Membranes were washed two-to-three times for 20 min with 0.1% PBST and incubated for 2 h at room temperature with alkaline phosphatase conjugated secondary antibody (1:2500 dilution). Membranes were washed and bound antibodies were analysed visually.

Chromosomal instability

Structural and numerical chromosomal aberrations. Briefly, exponentially growing (50–80% confluence) HEK-293 cell cultures were exposed to 0.04 μg/ml colchicine (Sigma, St. Louis, MO) for 6 h at 37°C. Metaphase spreads were prepared and stained by conventional methods as described previously [21]. A minimum of 50 metaphases for control and treated cells was selected and photographed and analysed using spectral imaging 4.0 software (Applied Spectral Imaging, Germany) to determine chromosome frequency distribution and morphology.

Immunocytochemistry of pericentrin. Cells were grown overnight on slides in EMEM and subjected to *N*-succinimidyl *N*-methylcarbamate treatment for 6 h. The cells were fixed in 10% formaldehyde for 1 h and permeabilized with 0.1% Triton-X-100 for 30 min, blocked with 3% BSA for 3 h and then incubated with anti-pericentrin antibody (dilution 1:1000) for 3 h and FITC-conjugated secondary antibodies (dilution 1:200) for 1 h. For qualitative analysis, nuclear counterstaining with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) was performed and immediately mounted with antifade solution, cover slipped and stored in the dark at 4°C. The images were then acquired and analysed using

spectral imaging 4.0 software (Applied Spectral Imaging, Germany).

Centromeric FISH. Briefly, the cell suspension in fresh Carnoy's fixative was dropped onto the pre-chilled slide from an optimum height and left for ageing overnight at RT. The requisite amount of probe was denatured for 2 min at 80°C in the dark. Following denaturation in 70% formamide/2 × SSC at 70°C for 2 min and processing through a serial ethanol series (70%, 90%, 100%) at −20°C, slides were hybridized in the presence of probe, specific for α-satellite sequences of centromeric region, overnight at 37°C in a humidified chamber (in the dark). After hybridization, slides were then washed with 0.4×sodium saline citrate (SSC) and 2× SSC, three times each. Counter staining of metaphase spreads was done in 70 μl of DAPI and finally slides were mounted with 10 μl of antifade solution, cover-slipped and stored in dark at 4°C. Around 20 metaphases for control and treated cells were selected and photographed and analysed using spectral imaging 4.0 software (Applied Spectral Imaging, Germany).

SKY. SKY analysis was performed on metaphase preparations using the SKY DNA paints according to the manufacturer's instructions and at least 10 metaphase spreads were selected and photographed and analysed using 4.0 version software (Applied Spectral Imaging, Germany) to determine any chromosomal rearrangements. A gain of a chromosome was described when identified in at least two metaphase spreads, a loss when identified in three or more cells and a chromosomal rearrangement when identified in two or more cells.

Phenotypic transformation and senescence

Cellular senescence assay. Cellular senescence assay was performed through senescence associated β-gal staining (SA-β-gal) in compliance with the manufacturer's instruction manual and stained cells were examined under a phase contrast microscope.

Statistical analysis

Student's *t*-test was employed for statistical comparisons and $p < 0.001$ was considered as statistically significant.

Results

Oxidative stress and antioxidant defense system

Intracellular ROS. The production of intracellular ROS was measured by DCFH oxidation. The CMH2DCFDA reagent passively diffuses into cells wherein it is hydrolysed by intracellular esterase to liberate 2'-7'-dichlorofluoressein, which, during the reaction with oxidizing species, yields a highly fluorescent compound 2'-7'-dichlorofluorescein (DCF) that is trapped inside the cell [22]. A significant time-dependent increase in the ROS generation, as indicated by DCF fluorescence, was recorded in the treated cells as compared to the controls ($p < 0.001$). In cells treated with *N*-succinimidyl *N*-methylcarbamate with a fixed dose (0.005 μM) during different time courses, there was a consistent increase, which appeared early and persisted for at least 48 h following treatment, indicative of extent of H_2O_2 production after exposure (Figure 1A).

8-oxo-dG. Notably, a significant increase in oxidative stress was observed along time-course experiments with maximum accumulated concentration of 265 ng/ml of 8-oxo-dG after 24 h ($p < 0.001$) (Figure 1B).

SOD activity. As shown in Figure 1C, there was consistent diminution in SOD activity along the time gradient with maximum inhibition observed at 96 h ($p < 0.001$) in *N*-succinimidyl *N*-methylcarbamate treated cells.

GR activity. GSH, long known for its protective function against oxidative cell damage, is thought to play an important role in a multitude of cellular processes, including cell differentiation and proliferation, and, consequently, disarrayed GSH homeostasis are implicated in the aetiology and/or progression of a number of human diseases [23]. In our study, the maximum inhibition in the GR activity was observed at 96 h ($p < 0.001$) (Figure 1D).

Cell cycle regulation

Analyses of cell cycle regulatory proteins through Western blot unveiled interesting results. Both p53 and p21 proteins showed perturbed expression in *N*-succinimidyl *N*-methylcarbamate treated cells during the time course study for 24, 48, 72, 96 h, denoting their ineptness to regulate the cell cycle due to profused cellular ROS in comparison to controls cells (Figure 2A). On the other hand, overexpression of cyclin E and CDK2 proteins were observed after 120 h and 150 h, respectively, in contrast to controls (Figure 2B) implicating their role in tumourigenesis due to inactivation of cell cycle checkpoints that, in turn, prevent proliferation of abnormal cells. The modifications in cell cycle progression caused by *N*-succinimidyl *N*-methylcarbamate exposure might be due to ROS overproduction and subsequent oxidative stress.

Chromosomal instability

Chromosomal aberrations. The ability of *N*-succinimidyl *N*-methylcarbamate to induce chromosomal

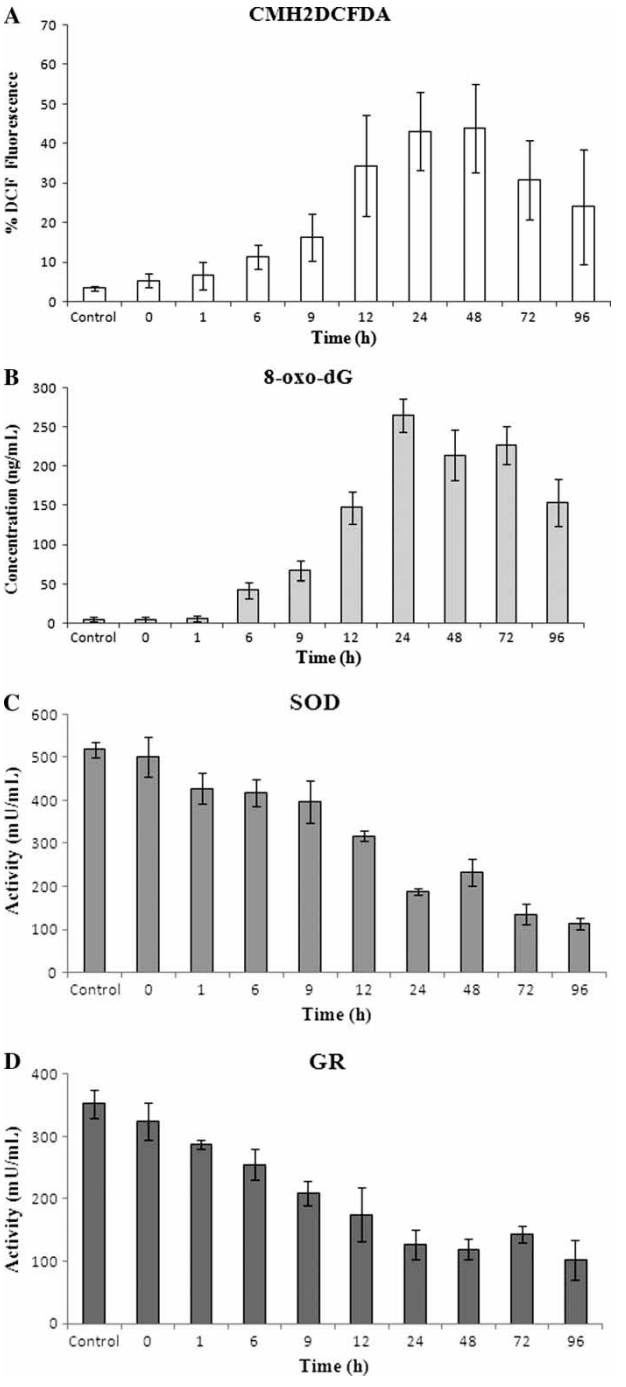

Figure 1. (A) Intracellular ROS production. Effect of 0.005 μM of *N*-succinimidyl *N*-methylcarbamate along the time course at 0–96 h in cultured HEK-293 cells leading to oxidative stress through generation of ROS (CM-H_2DCFDA). (B) 8-oxo-dG accumulation. Effect of 0.005 μM of *N*-succinimidyl *N*-methylcarbamate along the time course at 0–96 h in cultured HEK-293 cells showing oxidative damage through formation of 8-hydroxy-2'deoxyguanosine. (C) Depletion in SOD activity. Effect of 0.005 μM of *N*-succinimidyl *N*-methylcarbamate along the time course at 0–96 h in cultured HEK-293 cells displaying diminution of antioxidant defense enzyme activity of SOD. (D) Decrease in GR activity. Effect of 0.005 μM of *N*-succinimidyl *N*-methylcarbamate along the time course at 0–96 h in cultured HEK-293 cells causing depletion of antioxidant defense enzyme activity GR. $^{\star}p<0.001$.

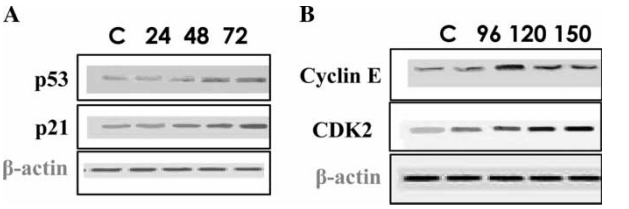

Figure 2. Status of cell cycle regulatory proteins. Western blot analysis of p53, p21, Cyclin E and CDK2 proteins in control (C) and following treatment with 0.005 μM *N*-succinimidyl *N*-methylcarbamate at 24, 48, 72, 96, 120, 150, 180 h in HEK-293 cells. Blots were probed with *β*-actin as loading control. The blots represent one of five reproducible experiments. (A) Expression of p53 and p21 proteins at 24, 48, 72, 96 h in treated cells was altered with respect to control. (B) Over-expression of cyclin E and progressive expression of CDK2 proteins at 120 and 150 h observed after treatment in comparison to control.

instability was evident from the results obtained with cytogenetic analysis. Structural aberrations at the chromosomal level, including premature centromeric separation (PCS), dicentrics (dic), chromatid breaks, end-to-end fusions, iso-chromatid lesions and, notably, numerical polyploidization of chromosomes at 96–180 h ($p< 0.001$), were observed on cytogenetic examination of the cells following treatment (Figure 3A–F and Figure 4).

Pericentrin. Immunocytochemical analysis of the pericentrin in treated cells indicated an abnormal centrosome functioning, which displayed a near 2-fold increase in the levels of pericentriolar protein pericentrin in contrast to control after 120 h (Figure 5).

Centromeric FISH. FISH with centromeric probes on treated metaphase spreads after 96 h revealed increased binding of the probes in the centromeric region of chromosomes suggestive of amplification of centromeric *α*-satellite repeats in the chromosomes and possible incidence of instability at centromeres (Figure 6).

SKY. SKY with DNA painting probes on treated metaphases displayed multiple translocations at 120 h which, in turn, corroborated with 'end-to-end fusions' and 'dicentrics' observed in the conventional giemsa stained chromosomal analysis for aberrations, indicating loss of telomeric function and instability at this part of the genome (Figure 7).

Phenotypic transformation and senescence

Cellular senescence assay performed in *N*-succinimidyl *N*-methylcarbamate-treated cells unveiled phenotypic transformations at 120 h as evident from increased cell size, distinct flat morphology of cells. The absorption of blue stain by these cells is indicative of accumulation of lipofuscein granules

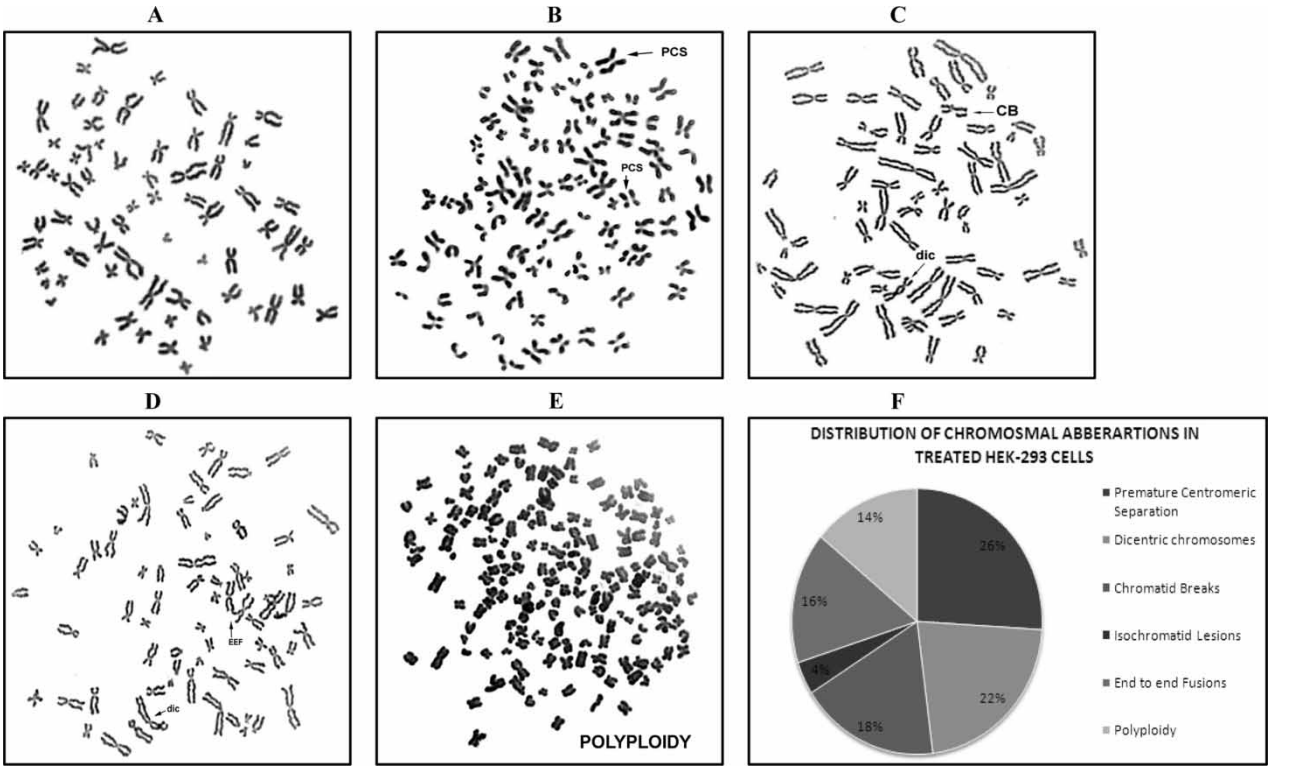

Figure 3. Structural chromosomal aberrations. Representative metaphase spreads of HEK-293 cells showing (A) Control karyotype; (B–E) Cells treated with 0.005 μM of *N*-succinimidyl *N*-methylcarbamate showing premature centromeric separation, dicentric chromosomes, chromatid breaks, end-to-end fusion and polyploid chromosomes from 96–180 h; (F) Pie diagram illustrating distribution of chromosomal abnormalities observed in treated cells.

and the senescence associated β-galactosidase activity at 150 h (Figure 8).

Discussion

Human cells rely on ATP for growth, differentiation and response to physiological stimuli and environmental challenge. The biological mechanisms responsible for the initiation and progression of diseases resulting from exposure to environmental mutagens are not fully understood. Isocyanates, the highly reactive ubiquitous chemicals, are turning out to be of prominent interest in the field of genetic toxicology due to their ability to modulate biomolecules through biotransformation [24]. MIC is used as a chemical intermediate for the production of carbamate insecticides and herbicides. MIC, in spite of high hydrolytic instability, has been shown to exert mutagenic response, *per se*, in the absence of exogenous activation [25,26]. MIC can traverse cell membranes and reach distant organs, which may explain its systemic effects [27]. In addition, MIC has also been shown to induce hyperoxia through its inhibitory effects on mitochondrial respiration [28]. Recently, isocyanates have been implicated to induce oxidative stress through excessive ROS production in human lymphocytes [13]. Because mitochondria are a major natural cellular source of ROS, we hypothesized that mitochondrial oxidative stress induced in our MIC-treated human kidney epithelial cells has a role in causing chromosomal instability. Cellular response to mitochondrial oxidative stress, cell cycle regulation, chromosomal unsteadiness, phenotypic transformation and senescence in recipient cells were evaluated using *N*-succinimidyl *N*-methylcarbamate, a MIC substitute.

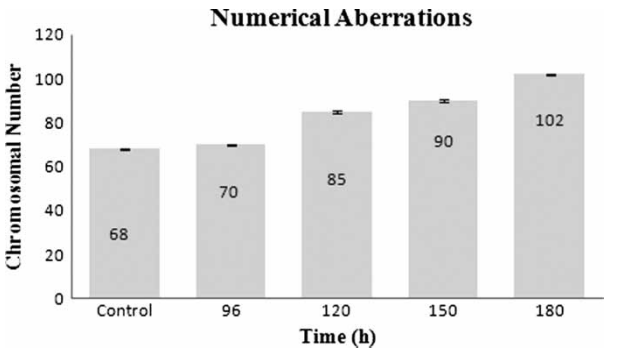

Figure 4. Numerical chromosomal aberrations. Histogram demonstrating changes in chromosome numbers in HEK-293 cells at 96, 120, 150, 180 h after treatment with 0.005 μM *N*-succinimidyl *N*-methylcarbamate. $^{*}p < 0.001$.

Mitochondria are often susceptible to damage due to various exogenous and endogenous ROS-producing stresses that compromise cells' future ability to produce energy owing to impaired electron transport chain [29,30]. ROS are also produced by NADPH oxidases, xanthine oxidase, cyclooxygenase,

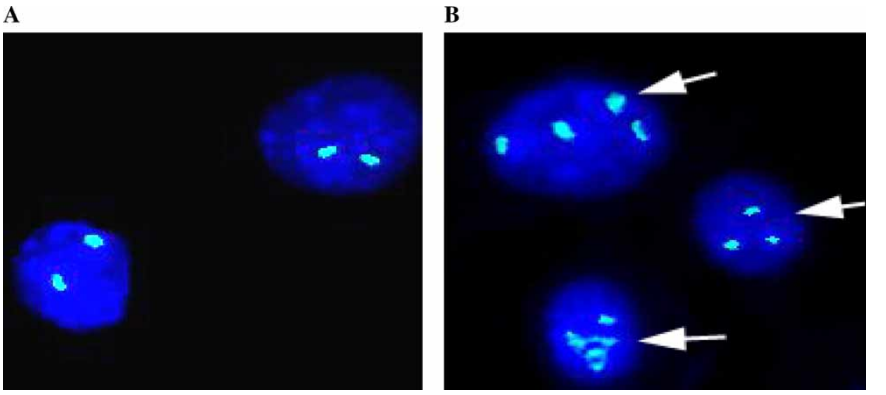

Figure 5. Immuno-localization of pericentrin. Immuno-fluorescent photomicrographs (×200) showing near 2-fold over-expression of pericentrin protein (white arrows) in HEK-293 cells treated with *N*-succinimidyl *N*-methylcarbamate (0.005 μM) in comparison to control. (A) Control; (B) treated cells at 120 h.

lipoxygenase and uncoupled nitric oxide synthase [31]. Extensive oxygen free radicals generated from many nephrotoxic chemicals concentrated in renal tubular cells and H_2O_2 generated through oxidant stress have been shown to bring about renal injury via participation of mitochondria [15,32,33]. In the present study, a time-dependent increase in DCF fluorescence observed in the *N*-succinimidyl *N*-methylcarbamate treated cells indicated surfacing of oxidative stress condition through excessive H_2O_2 production (Figure 1A).

Furthermore, over-production of ROS challenges the antioxidant defense system. The antioxidant enzymes-superoxide dismutase as well as glutathione (GSH) are the most important intracellular antioxidants in the metabolism of ROS and have a vital antioxidant role in human health, conferred by their scavenging of one of the ROS, superoxide anion. While SOD is among the first line of defense in the detoxification of products resulting from oxidative stress, GR plays a vital role in defense against the toxicity of superoxide radical and previous studies have documented that any depletion in both the activities of SOD and GR can result in the breakdown of antioxidant defense system and indirectly increases the incidences of free radicals' diseases [34–36]. Upon toxic exposure to MIC and its metabolites, GR is susceptible to inhibition due to loss of its enzymatic activity [37–39]. Correspondingly, the present study herein demonstrated the inhibition of SOD and GR activities in *N*-succinimidyl *N*-methylcarbamate exposed cells in a time-dependent manner (Figure 1C and D), suggesting the break-down of antioxidant defense system owing to dysfunction of mitochondrial system and indirectly act as contributory factors for genomic instability and mitochondrial diseases [6].

Endogenous or xenobiotic-enhanced formation of ROS like hydroxyl radicals may adversely alter development by oxidatively damaging cellular lipids, proteins and DNA [40]. One of the best biomarkers for oxidative stress is 8-oxo-dG, a modified nucleoside base, which is detected as a byproduct of DNA damage that is excreted upon DNA repair [41]. We, in our study, found that along the time course there was consistent accumulation of 8-oxo-dG in *N*-succinimidyl *N*-methylcarbamate-treated culture supernatant, indicating the coupling of reactive species to DNA bases due to oxidative damage (Figure 1B).

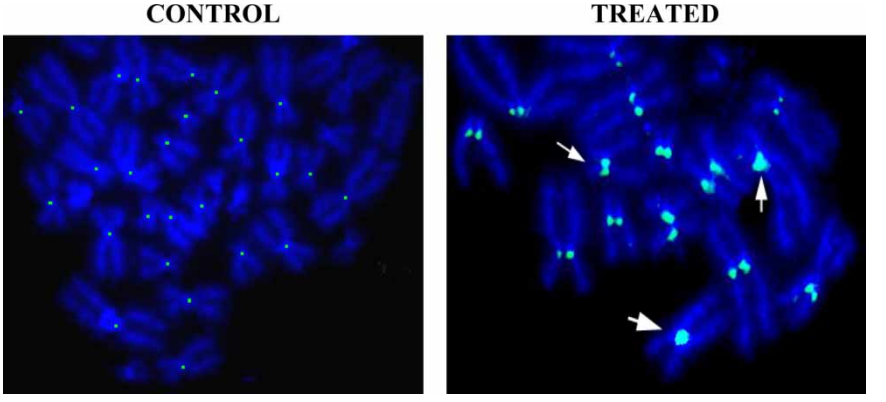

Figure 6. Centromeric FISH. Partial metaphase spreads of control and 0.005 μM *N*-succinimidyl *N*-methylcarbamate treated HEK-293 cells upon centromeric FISH (×6300). Cells showing amplification of α-satellite repeats of centromeric region (white arrows) after 96 h of treatment as compared to control.

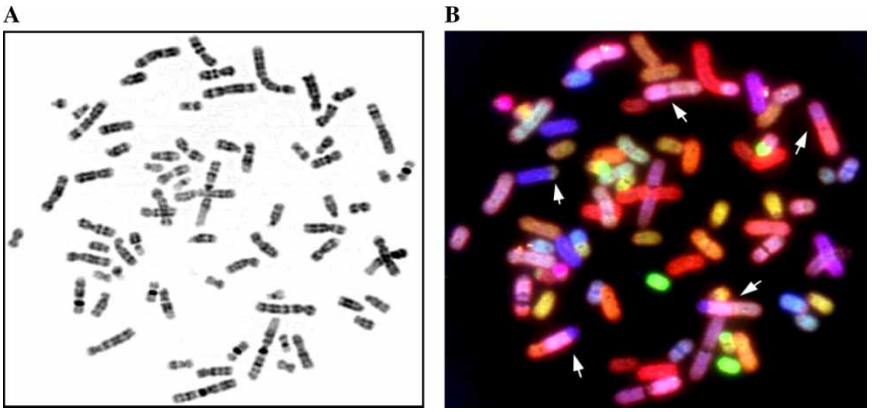

Figure 7. SKY analysis. Photomicrographs showing multiple translocations after treatment with 0.005 μM *N*-succinimidyl *N*-methylcarbamate in HEK-293 cells. (A) Inverted DAPI image; (B) SKY view image at 150 h (white arrows).

It has been indicated that ROS are capable to act as signalling substances in the cell regulatory network, which determines the mode of cellular response to disturbance: proliferation pace, a course of differentiation [42]. Cyclin E, an activator of cyclin-dependent kinase (CDK)-2, accumulates at the G1/S boundary of the cell cycle, where it stimulates functions associated with entry into and progressions through the S phase [43]. However, the over-expression of cyclin E has been observed in a broad spectrum of human malignancies [44] and deregulated cyclin E is thought to promote early loss of heterozygosity of p53 and tumourigenesis [45]. Also, loss or mutational inactivation of p53 leads to uncontrolled activation/expression of CDK2 in cells arrested for cell cycling due to physiological stress as well as damaged DNA [46]. We, in our present study, have demonstrated the alteration in the progression of cell cycle due to the disturbed expression of p53 and p21 proteins along 24, 48, 72 and 96 h and over-expression of cyclin E and CDK2 proteins at 120 and 150 h, respectively, in treated cells (Figure 2), lending evidence to the propositions that ROS are capable of modifying many intracellular signalling pathways including protein phosphatases, protein kinases and transcription factors [47] and, parallelly, over-expression of cyclin E stimulates cellular proliferation during tumourigenesis and chromosome instability and polyploidy [48,49].

Besides, it is well documented that the normal functioning of centrosomes, including organization of the cytoskeleton, mitotic spindle and cell cycle progression, is supported by pericentrin, a protein present in it [50,51]. Interestingly, prior studies have reported that perturbed pericentrin function leads to defective mitotic spindle assembly and multi-polar mitosis [52] and oxidative stress can trigger hyper-amplification of centrosome and multinucleated cell formation [53]. In accordance, there was a near 2-fold increase in pericentrin levels in the cells following treatment at 120 h, suggestive of a defective centrosome functioning as a consequence of oxidative stress, thereby generating supernumerary centrioles and improper segregation of chromosomes during cell division (Figure 5).

Moreover, it has been shown that surpassing of the cellular capacity to aptly manage oxidative DNA damage due to defective cell cycle checkpoints results in a 'gain of chromosomal-instable phenotype' and leads to profound karyotypic instability [54].

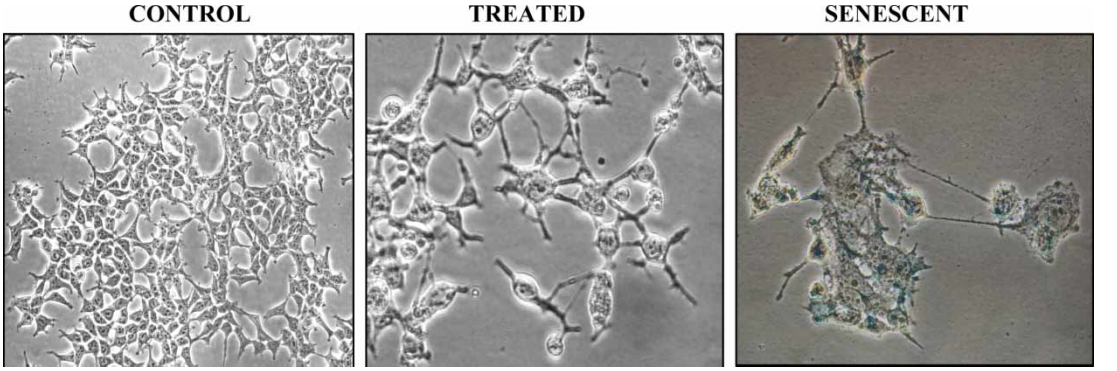

Figure 8. Phenotypic transformation and senescence. Representative phase contrast microphotographs (×200) of HEK-293 cells prior to and after treatment with 0.005 μM *N*-succinimidyl *N*-methylcarbamate at 120 h and senescent cells after 150 h. Treated cells showing noticeable decrease in density with apparent morphological transformation at 120 h.

Also, mitochondrial dysfunction has been illustrated to result in chromosomal instability, which could explain its role in tumour development [55]. In this regard, isocyanates and their derivative compounds (*N*-methylcarbamate) have been demonstrated to cause chromosomal aberrations in mammalian cells [56–58]. Premature (early) centromere division (PCD, i.e. the separation of centromeres during the pro-metaphase/metaphase of the mitotic cycle) has been considered to be a plausible manifestation of chromosome instability observed in human chromosome-breakage syndromes, neoplastic and transformed cells. Thus, it is suggested as a potential exposure-related cytogenetic biomarker for cancer risk assessment [59,60]. The relatively higher frequency of PCS and dicentric chromosomes in treated cells noted in our study during 96–180 h further support this data and hint at a rise of precariousness of the centromeres due to isocyanate exposure (Figure 3A–F). In addition, the results observed on cytogenetic analysis of HEK-293 cells following exposure to *N*-succinimidyl *N*-methylcarbamate collectively confirmed that there is a synergistic instigation of instability in the genome, as indicated by various structural chromosomal abnormalities and numerical discrepancy in chromosome number all through 96–180 h (Figure 4).

Chromosomal instability in the form of centromeric instability and aneuploidy are considered to be defining characteristics of human kidney cancers [61]. The centromere is essential for the proper segregation and inheritance of genetic information and requires the presence of alpha satellite DNA in all humans [62]. It has been shown that centromeres are the primary target for destabilization in cases of genomic instability that contribute in the formation of novel chromosome rearrangements due to amplification of satellite repeats [63]. Centromeric amplification could be confronted probably by repeated unequal exchanges in heterochromatic regions of centromere; which, owing to their repetitive nature, are highly susceptible to such amplification [64]. Concurrently, the centromeric FISH analysis at 96 h specific for α-satellite repeats of pancentromeric region of the humans revealed centromeric amplification in *N*-succinimidyl *N*-methylcarbamate treated cells (Figure 6) suggesting that this portion of the genome may be destabilized and rapidly altered and in turn might lead to 'neo-centromerization'.

Moreover, genetic instability might also result from gross chromosomal changes, such as translocations or amplifications that lead to chromosomal instability [65]. Earlier investigations have postulated that extensive DNA amplification, cell-cycle checkpoint deficiency, sister chromatid fusion and prolonged breakage/fusion/bridge (B/F/B) cycles are the initial lesions causing destabilizing chromatin bridges in proliferative cells, ultimately leading to loss of telomere function [66] and exposure to toxic chemicals can cause telomeric dysfunction [67]. Concurrently, multiple translocations observed in the present study upon SKY analysis in the treated cells at 150 h (Figure 7) imply the susceptibility of telomeres to isocyanate exposure, which, consecutively, might play a part in transfer of instability from one chromosome to another through abnormal telomeres and generate many types of rearrangements commonly associated with chromosomal instability [68].

Progressive DNA damage in live cells by oxidants is the key factor contributing to cell ageing and preconditioning to neoplastic transformation. Recently, it has been stated that ROS are potent enough to induce oxidative DNA damage through endogenous processes and redox-cycling of xenobiotic compounds that eventually may contribute to cell transformation and tumour initiation [1,69]. Also, a significant amount of evidence suggests the entry of cells into senescence triggered by oncogene expression that, in part, mediated by a rise in cellular ROS levels and perturbed antioxidants states [70]. Our study further substantiates these observations by means of the morphological variations observed in *N*-succinimidyl *N*-methylcarbamate-treated cells at 120 h and increased uptake of β-gal stain by these, probably transformed, senescent cells at 150 h (Figure 8). We presume that one of the causal reasons might be the redox imbalance which in turn may be related to oncogenic stimulation.

In conclusion, our results indicate that isocyanates cause chromosomal instability through mitochondrial mediated oxidative stress, deregulated cell cycle progression and pro-tumoural instigation of senescence in the HEK-293 human kidney epithelial cells. We anticipate that our findings would substantiate the causal relationship between mitochondrial oxidative stress in elicitation of chromosomal instability and genomic heterogeneity during renal carcinogenesis.

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