CELLULAR AND MUTAGENIC EFFECTS OF FORMALDEHYDE IN MAMMALIAN CELLS

By

Yun Xin Lim

A THESIS

Presented to the Department of Cell & Developmental Biology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Master of Science

May 2011

CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

Yun Xin Lim

Has been approved

Amanda K. McCullough, Ph.D.

Mathew J. Thayer, Ph.D.

Susan B. Olson, Ph.D.

Acknowledgements

I would like to thank all people who have helped and inspired me during my postgraduate study.

I am wholeheartedly thankful to my thesis adviser, Dr. Amanda K. McCullough, whose guidance and support throughout my postgraduate career at OHSU enabled me to grow professionally as well as to develop an understanding of formaldehyde mutagenesis and the significance of DNA repair in cellular response to environmental stress. I am privileged to have the opportunity to learn under the guidance of Dr. McCullough and Dr. R. Stephen Lloyd.

I would like to thank my thesis committee – Dr. Susan B. Olson and Dr. Mathew J. Thayer – for their encouragement as well as insightful comments.

I would like to extend my gratitude to Dr. Anuradha Kumari for her patience and willingness to guide me through the laboratory techniques step by step. I remain thankful to Dr. Kumari and Kinrin Yamanaka for being good friends and surmounting my obstacles in optimizing experimental conditions and getting the best presentable results. I am thankful to Dr. Aaron C. Jacobs for his constructive criticism and careful scrutiny of my thesis. I would like to thank all of my colleagues from the McCullough and Lloyd laboratories for providing invaluable comments about my thesis and for creating a pleasant working atmosphere.

I am grateful to Dr. Mitchell S. Turker for providing the *Aprt* cell line and sharing his expertise on mutagenesis. I also owe my gratitude to Cristian Dan for helping me familiarize myself with the

assays, and for making his assistance readily available.

Dr. Cheryl L. Maslen, the Director of the Program in Molecular and Cellular Biosciences, has been a great source of inspiration. Her encouragement and genuine kindness has helped me come this far. I am greatly indebted to her for all these. I sincerely thank Dr. Maslen and Dr. Alison D. Fryer for their constant support throughout my training at OHSU.

I am thankful to Dr. Rachel Dresbeck for honing my writing skills in scientific discourse. I am also thankful to Elaine Offield for her administrative support and her reassurance which unfailingly restores my confidence.

I would like to thank Dr. Tom Frederick, my former academic adviser at Rochester Institute of Technology, who has aspired me to pursue my passion in science at the postgraduate level.

Furthermore, I remain indebted to my good old friends Eric Fu and Kenny Tang, who have also matriculated at colleges and graduate schools in the U.S., for their constant motivation and encouragement. I would like to give special thanks to Eric for teaching me the LaTeX typesetting system and thus easing my thesis-writing process. I would also like to thank Ashley Kamimae-Lanning, Samaneth Zhian, and Maya Culbertson for their support, pushing me to achieve greater heights.

My deepest gratitude goes to my family for their infinite love and support throughout my life. My parents have been tremendously supportive in all my pursuits; my brother Qiheng has helped me dispel my doubts. I am equally thankful to the Deishers for their unconditional love and help since my first day in Portland. I am especially thankful to Gim Deisher who not only sets many aspiring examples, but also makes me feel as part of the family.

I am grateful for God's provision of joys, challenges, and grace for growth. Lastly, I offer my regards to all of those who supported me in any respect during the completion of the project.

Yun Xin Lim
Oregon Health & Science University
May 2011

Abstract

The Occupational Safety and Health Administration has estimated that two million U.S. workers in the health care, embalming, textile, resin, and plastic industries are exposed to formaldehyde. Formaldehyde is one of the most reactive aldehydes found naturally as an endogenous substance in the human body and in environmental sources such as automobile emissions and tobacco smoke. Increased rates of nasopharyngeal cancer and increased relative risk of myeloid leukemia in workers exposed to formaldehyde have prompted the International Agency for Research on Cancer to classify formaldehyde as a human carcinogen. Even though previous reports have shown a correlation between formaldehyde and cancer, the cellular and mutagenic effects of formaldehyde are not well understood.

This is the first report demonstrating altered nuclear content and increased mutant frequency as the consequences of formaldehyde exposure. We show that cells presented centrosome and microtubule defects following formaldehyde exposure, indicating a possibility that formaldehyde compromises mitosis and subsequently gives rise to cells with an altered DNA content. Additionally, a five-fold increase in mutant frequency was observed following formaldehyde exposure. Further analyses suggest an increase in mutational events contributed to the mutagenicity of formaldehyde.

Collectively, our findings highlight the potential of formaldehyde in increasing genomic instability by distorting ploidy status and mutant frequency.

Contents

List of Figures

List of Tables

List of Abbreviations

(In order of appearance)

ppm	parts per million
UFFI	urea-formaldehyde foam insulation
EPA	Environmental Protection Agency
OEL	Occupational Exposure Limit
OSHA	Occupational Safety and Health Administration
PEL	permissible exposure limit
STEL	short-term exposure limit
DPC	DNA-protein crosslink
NER	nucleotide excision repair
HR	homologous recombination
MN	micronuclei
SCE	sister chromatid exchange
CA	chromosomal aberration
HPRT	hypoxanthine phosphoribosyltransferase
ATCC	American Type Culture Collection
CE	cloning efficiency
FACS	Fluorescence Activated Cell Sorting
DAPI	4,6-diamidino-2-phenylindole
DAP	2,6-diaminopurine
MF	mutant frequency
PCR	polymerase chain reaction
LOH	loss of heterozygosity
HEX	hexachloro-fluorescein
FAM	carboxyfluorescein
TK	thymidine kinase
KO	knockout
BLK	C57BL/6
WT	wildtype
DBA	DBA/2
HET	heterozygote
IE	intragenic events
CL	chromosome loss
MR	mitotic recombination
DEL	deletion
DLOH	discontinuous loss of heterozygosity
ENU	N-ethyl-N-nitrosourea
FISH	Fluorescence *in situ* Hybridization

Chapter 1

Introduction

1.1 Properties of formaldehyde

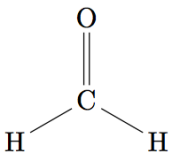


Figure 1.1: Structural formula of formaldehyde.

Formaldehyde is the simplest and the most reactive of all aldehydes, with a chemical formula of HCHO (Figure 1.1). Formaldehyde is colorless and is often characterized by its pungent smell. In 1867, August von Hofmann first identified it as the product formed when methanol and air were passed over heated platinum, a method which evolved into the basis of formaldehyde production today (http://www.encyclopedia.com/topic/August_Wilhelm_von_Hofmann.aspx). Formaldehyde is also produced as a metabolic byproduct in many organisms, including humans, and is ubiquitously present in all cells and tissues (NTP, 2005). It can be metabolically derived from several sources such as serine, glycine, choline, and a variety of xenobiotic compounds (Nelson et al., 1986). The endogenous level of formaldehyde in the blood is similar in humans, monkeys, and rats (Casanova et al., 1988).

In healthy cells, formaldehyde is oxidized to become formic acid, a key intermediate in the one-carbon pool that contributes to the biosynthesis of thymine, serine, purine, or methionine (Ridpath et al., 2007). Due to its electrophilicity, formaldehyde is highly prone to attack by nucleophilic compounds to form stable crosslinks, which is critical for its utilization as a one-carbon unit in biosynthetic reactions. Inhaled or ingested formaldehyde is quickly and almost completely absorbed within the respiratory or gastrointestinal tracts because it is rapidly metabolized by glutathione-dependent formaldehyde dehydrogenase before it reaches the systemic circulation. The end product, formic acid, is eventually exhaled as carbon dioxide or excreted in the urine (NTP, 2010).

1.2 Economic importance of formaldehyde

Formaldehyde is an economically important chemical, with an annual global production of approximately 46 billion pounds and a United States investment of over $500 billion annually. Over 55% of national formaldehyde consumption is used in the production of industrial resins (mainly in the form of urea-formaldehyde) to manufacture various domestic and industrial products such as plastics, synthetic fibers, paper, and particleboard products (NTP, 2005). Formaldehyde is also regularly used as an intermediate in the synthesis of other chemicals. Aqueous solutions of formaldehyde (37% by weight) are also used as preservatives and embalming agents in medical laboratories and mortuaries, respectively.

1.3 Prevalence of formaldehyde exposure

Formaldehyde is found in environmental sources such as automobile emissions, tobacco smoke, and photochemical smog (Nelson et al., 1986). Ambient formaldehyde levels usually range between 0.0008 and 0.02 parts per million (ppm) (WHO 2001) but are elevated in large cities such as Houston, Cairo, and Northern Savonia, where traffic volume is high (Zhang et al., 2009). The highest formaldehyde

exposure level is usually found in occupational environments with an average level of 0.74 ppm (WHO 2001). The main source of exposure, both environmentally and occupationally, is from inhalation of formaldehyde fumes in an indoor setting.

The abovementioned consumer products that contain formaldehyde could introduce greater than 0.03 ppm formaldehyde into an indoor setting (CSPC, 1997). The primary source of indoor formaldehyde outgassing is pressed wood products such as plywood paneling, particleboard underlays, and fiberboard furniture. Urea-formaldehyde foam insulation (UFFI), once a widely used insulating material in many homes in North America in the 1970s, was banned by the U.S. Consumer Product Safety Commission in 1982 when homes with UFFI installed were found to contain up to 0.07 ppm of formaldehyde (http://www.epa.gov/ttnatw01/hlthef/formalde.html; The Commonwealth of Massachusetts, 1986). More recent examples of formaldehyde exposure have also generated significant attention. Health issues reported by the residents of trailers and mobile homes provided to the victims of Hurricane Katrina prompted an assessment of indoor air quality of these trailers. The evaluation presented a dangerous level of up to 0.6 ppm formaldehyde. In 2010, stylists from a salon in Portland, Oregon, presented symptoms with difficulty breathing, nosebleeds, and eye irritation after using a hair straightening product as directed. Further testing demonstrated that the product contained 6.3% to 10.6% formaldehyde (approximately 4×10^9 fold greater than ambient formaldehyde concentration in air) even though the container was labeled formaldehyde-free. Furthermore, the product is applied to the hair with heat, increasing potential for exposure through inhalation (http://www.ohsu.edu/xd/research/centers-institutes/croet/emerging-issues-and-alerts.cfm). Despite various attempts to limit formaldehyde exposure from consumer products, the incidences of formaldehyde exposure are still occurring.

A comprehensive review of formaldehyde toxicity conducted by the Environmental Protection Agency (EPA) expressed a cancer risk of 1 in 1,000,000 in the general population when the levels of formaldehyde exceed 8 mg/m^3 in drinking water or 6.5 ppm in air breathed (http://www.epa.gov/iris/subst/0419.htm). In addition, to regulate occupational exposure, many countries have begun to decrease

the Occupational Exposure Limit (OEL) of formaldehyde, including the United States, Australia, Canada, and China. In the United States specifically, the Occupational Safety and Health Administration (OSHA) estimated that about two million workers in the health care, embalming, textile, resin, and plastic industries are occupationally exposed to formaldehyde. To minimize the occupational hazard, OSHA has established the permissible exposure limit (PEL) as 0.75 ppm in air measured as an 8-hour time-weighted average and the short-term exposure limit (STEL) as 2 ppm with a maximum exposure period of 15 minutes. Additionally, employers are also required to provide annual training to employees exposed to airbone concentrations of formaldehyde above 0.1 ppm (OHSA Fact Sheets, 1995).

1.4 Formaldehyde as a human carcinogen

Animal toxicity studies have consistently demonstrated a concentration-dependent increase in nasal epithelial cell proliferation and squamous cell carcinoma (Kerns et al., 1983; Monticello et al., 1996). The effects of formaldehyde on human respiratory tracts are also evidenced by epidemiology studies showing an increased risk of developing childhood and adult asthma (Rumchev et al., 2002; Wieslander et al, 1997) as well as acute respiratory illness (Tuthill, 1984). Based on animal studies, adverse health effects, and scarce evidence on human carcinogenicity (Hauptmann et al., 2003; Hayes et al., 1990; Monticello et al., 1996), formaldehyde has long been categorized as a probable human carcinogen (Group 2A).

In June 2004, the International Agency for Research on Cancer reclassified formaldehyde as a known human carcinogen (Group 1) based on six major cohort studies (Cogliano et al., 2005). Specifically, the studies of embalmers showed an increased mortality rate from nasopharyngeal cancer following formaldehyde exposure (Hayes et al., 1990). The largest cohort studies of industrial workers from ten different formaldehyde-using and -producing facilities further substantiated this by demonstrating an increased relative risk for nasopharyngeal cancer with average exposure intensity, cumulative

exposure, highest peak exposure, and duration of exposure to formaldehyde (Hauptmann et al., 2004). Increased relative risk of myeloid leukemia in workers exposed to formaldehyde also suggests a causal relationship between formaldehyde exposure and leukemia (Hauptmann et al., 2003). In 2010, Zhang and colleagues described the hematotoxic property of formaldehyde based on the evidence that formaldehyde-exposed workers in five different plants presented significantly lower peripheral blood counts and an increased level of monosomy 7 and trisomy 8 which are often associated with myelodysplasia (Zhang et al., 2009), although several experimental setup shortcomings were noted (Speit et al., 2010). Collectively, this evidence suggests a correlative relationship between formaldehyde exposure and cancer risk.

1.5 Formaldehyde induces DNA-protein crosslinks

DNA-protein crosslinks (DPCs) are produced endogenously as intermediates during normal DNA metabolism and as byproducts of abortive base excision repair (Reardon et al., 2006). DPCs are also produced upon exposure to exogenous DNA-damaging agents such as ionizing radiation, metal compounds, x-rays, oxygen radicals, and reactive aldehydes (Fornace and Little, 1977; Fornace and Seres, 1982; Izzotti et al., 1999; Kuykendall and Bogdanffy, 1992; Merk et al., 2000; Olinski et al., 1992). It is well established that formaldehyde generates DPCs as its major form of DNA damage (Casanova et al, 1994).

Formaldehyde induces the formation of DPCs by reacting with a protein amine, followed by a second reaction with a nucleobase to form the general structure of protein-NH-CH2-NH-DNA as illustrated in Figure 1.2. Formaldehyde induces DPCs by linking the DNA with proteins such as major histone proteins (H1, H2a, H2b, H3, and H4) (O'Connor and Fox, 1989) and vimentin (Tolstonog et al., 2001). The formation of DPCs is enhanced when the glutathione-dependent defense mechanism is suppressed (Nelson et al., 1986). Due to the steric hindrance established by immobilized proteins on DNA, DPCs are considered a threat to genomic integrity as they may block DNA and RNA polymerase

progression, compromising replication and transcription. However, the DNA repair mechanism for the removal of DPCs is still inadequately understood.

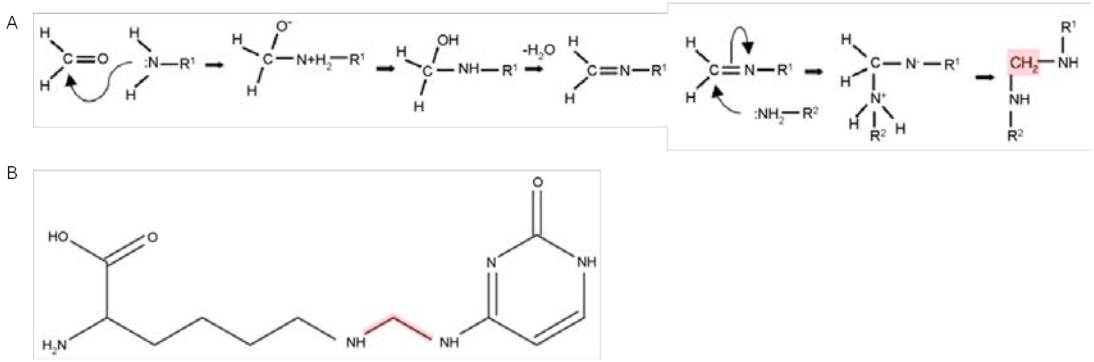


Figure 1.2: Formaldehyde crosslinking mechanism and structure of formaldehyde-induced DNA-protein crosslink. (A) A graphic representation of the reaction of formaldehyde with an amino group of a protein side chain to form a Schiff base which subsequently reacts with another amino group of a nucleobase to form the crosslink. (B) A formaldehyde-induced crosslink between cytosine and lysine. Reprinted with permission; Zhang et al., 2009.

1.6 Repair of formaldehyde-induced DNA damage

Several reports have implicated specific pathways in the repair and tolerance of formaldehyde-induced DNA lesions even though the relative contribution of each pathway is still to be described. Among these pathways, nucleotide excision repair (NER) and homologous recombination (HR) are most often invoked as the critical mechanisms in the repair or tolerance of formaldehyde-induced DPCs.

Biochemical and genetic studies using *Escherichia coli* demonstrated that both UvrA and RecA proteins (NER and HR pathways, respectively) contribute to the repair of formaldehyde-induced DPCs. The UvrABC nuclease complex incised a 16-kDa protein crosslinked to a 12-mer DNA, which suggested that NER plays a role in repairing this lesion (Minko et al., 2002). *In vivo* studies on wildtype cells showed increased incision efficiency with partially digested histone (1.8 – 4.5 kDa) rather than the intact histone (22 kDa) compared to that of the *uvrA* cells, further validating the role of NER in repairing crosslinked proteins of smaller sizes. In similar studies, homologous recombination processed DPCs with either small or large crosslinked proteins (Nakano et al., 2007).

Furthermore, the *Saccharomyces cerevisiae* non-essential gene deletion library screen indicated that HR limits formaldehyde-induced cytotoxicity following a chronic exposure; while NER protects against formaldehyde-induced DNA damage under an acute formaldehyde exposure (de Graaf et al., 2009). Similarly, the DT40 chicken B lymphocyte cell lines deficient in HR (FANCD2, BRCA2, and XRCC2) also showed significant reduction in the LC50 following a chronic formaldehyde exposure (Ridpath et al., 2007). Even though the human equivalents of the FANCD2 and BRCA2 deficient cells did not exhibit sensitivity similar to that of the DT40s, the HR mutants of Chinese hamster ovary cells (Rad51D and XRCC3) were sensitive to formaldehyde (Ide et al., 2010). Quievryn and Zhitkovich demonstrated that human cell lines deficient in NER (XPA and XPF) had very similar kinetics for the elimination of DPCs and thus indicated that the NER machinery has a minimal role in mitigating formaldehyde-induced DPCs, although the limited quantities of crosslinked DNA might not be detectable in the assays employed (Quievryn and Zhitkovich, 2000). Interestingly, the NER-deficient XPF cell lines were sensitive to formaldehyde (Quievryn and Zhitkovich, 2000), and micronuclei were induced in an NER-deficient XPA cell line (Speit et al., 2000), suggesting a possible role of NER in mitigating formaldehyde-induced chromosomal aberrations. Even though the relative involvement of HR and NER are still to be investigated, both of these repair pathways are critical in mitigating formaldehyde-induced DNA lesions.

1.7 Formaldehyde-induced chromosomal alterations

It is plausible that formaldehyde or formaldehyde-induced DPCs lead to other chromosomal events. Even though the underlying mechanisms are unclear, cellular studies demonstrated that formaldehyde induces chromosomal alterations such as micronuclei (MN) and sister chromatid exchange (SCE) in a dose-dependent fashion (Speit et al., 2007). Further, human subjects exposed to formaldehyde have consistently shown elevated frequencies of chromosomal aberration (CA), MN, and SCE (He et al., 1998; Jakab et al., 2010 ; Schmid et al., 1986; Shaham et al., 2002; Suruda et al., 1993; Ye et al., 2005). A significant increase of dicentrics and ring chromosomes was also reported in a

group of workers from a paper factory where formaldehyde was used for impregnation processing (Bauchinger and Schmid, 1985).

1.8 Mutagenic effects of formaldehyde

The genotoxicity of formaldehyde is indisputable in that it generates DPCs and induces various chromosomal alterations as previously described. Formaldehyde was also found to cause diverse histopathological changes such as loss of cytoplasm and hyperchromatic nuclei in livers (Cikmaz et al., 2010). Expression of genes associated with nucleic acid metabolism, apoptosis, and metabolism regulation was also altered following formaldehyde exposure (Li et al., 2007; Neuss et al., 2010).

The genotoxic and carcinogenic effects of formaldehyde naturally prompt questions on its potential to generate mutations. Identifying the mutational events caused by formaldehyde exposure not only provides clues as to which DNA damage tolerance and repair pathways may be involved, but also presents the basis for risk assessment of environmental agents and understanding of how carcinogens trigger cancer (Turker, 2003). Over the last two decades, there has been a significant interest in the mutagenicity of formaldehyde. Studies in *Escherichia coli* and the mammalian hypoxanthine phosphoribosyltransferase (*Hprt*) systems demonstrated that formaldehyde increased mutant frequency (Grafstrom et al., 1993; Graves et al., 1996; Wang et al., 2007); while other studies reported an unchanged mutant frequency (Jakab et al., 2010 ; Merk and Speit, 1998). Similarly, while some studies reported that formaldehyde generated point mutations and increased microsatellite instability in bacteria (Crosby et al., 1988; Wang et al., 2007) and deletions and single base transversions in mammalian cells (Crosby et al., 1988; Grafstrom et al., 1993; Wang et al., 2007), others have not observed these effects (Merk et al., 2000; Speit and Merk, 2002). It is apparent that these varying mutagenicity results necessitate further investigations.

1.9 Objectives

The purpose of this thesis project is to investigate the effects of formaldehyde at the cellular and DNA level. Based on our observations that formaldehyde increased the post-G2/M peak populations and the polyploidy populations as evidenced by cytogenetics and FACS analyses, respectively, we hypothesized that formaldehyde alters the ploidy status by impairing mitosis.

Since bacterial transgenes are the most commonly used mutational target, a majority of studies describing the types of mutations induced by genotoxins focus on small events such as single base pair changes (Gossen et al., 1994; Dean et al., 1999), even though large events that trigger loss of heterozigosity are very common in cancers (Lasko et al., 1991). As previously depicted, the mutagenicity of formaldehyde is still to be defined. In consideration of the carcinogenic and genotoxic potential of formaldehyde, we also hypothesized that formaldehyde induces a unique mutational signature.

Chapter 2

Materials and Methods

2.1 Cell lines and chemicals

Chemicals were purchased from Sigma unless noted otherwise. The wildtype AA8 Chinese hamster ovary cells were purchased from American Type Culture Collection (ATCC). The mouse kidney epithelial cells derived from clone 4a, designated as the 4a cells, were obtained from Dr. Mitchell Turker (Oregon Health and Science University) (Turker et al., 2009). Cells were maintained in standard growth medium DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1× antibiotic/antimycotic at 37°C in a 5% CO_2 incubator. Formaldehyde (CAS No. BP531-25, 37% by weight) was purchased from Fisher Scientific and diluted in DMEM immediately before use.

2.2 Cell survival assays

To assess the sensitivity of the 4a cells to formaldehyde exposure as measured by survival, cells were trypsinized, counted using the counting chamber hemacytometer, seeded at 600 cells/100-mm dish, and allowed to attach overnight at 37°C. The cells were then subjected to acute (transient, high

dose) exposures of formaldehyde (0 – 1.25 mM) for 4 to 24 hr, followed by two washes and media replenishment; or chronic exposures (0 – 60 μM formaldehyde) where formaldehyde was replenished every 3 – 4 days throughout the course of the experiments. After 2 – 3 weeks, surviving colonies were fixed, stained with crystal violet, and counted. Cloning efficiency (CE) was determined by the following equation:

$$CE = \frac{\text{Number of colonies}}{\text{Number of cells seeded}}. \tag{2.1}$$

2.3 Cell cycle analyses

Subconfluent cultures of 4a cells were exposed to various concentrations of formaldehyde for 4 hr and allowed a varying period of recovery before harvesting. The cells were harvested by trypsinization, fixed overnight at $-20°$C in 70% ethanol, and stained with propidium iodide (50 μg/mL) (Invitrogen) for Fluorescence Activated Cell Sorting (FACS) analyses. The DNA content of the cells was determined by using a FACS Calibur (Becton Dickinson) (Flow Cytometry Core, OHSU). The results were analyzed using FlowJo software (Tree Star). The histograms were generated using ungated cell populations to account for any changes in cellular properties following formaldehyde exposure.

2.4 Cytogenetic analyses

Cells were exposed to formaldehyde for 4 hr and allowed a 48-hr recovery period at 37°C before harvesting. Colcemid (0.05 μg/mL) was added 3 hr prior to harvesting to enrich the metaphase populations. Cells were then treated with a hypotonic solution containing 0.075 M KCl and 5% fetal calf serum (GIBCO) for 10 min, fixed with 3:1 methanol:acetic acid, and dropped onto cytogenetic slides (Fisher Scientific). The chromosome spreads were stained with 0.03% Wright's stain in 5% pHydrion buffer (METAPAK) for 3 min. For each condition, 50 metaphases were analyzed for breaks and radials using a Nikon Eclipse E800 photoscope.

2.5 Immunofluorescence

Cells ($1 - 5\times\ 10^5$) were seeded on cover slips and allowed to attach overnight in DMEM. After formaldehyde treatment or a period of recovery after treatment, cells were washed with PBG (50 mM glycine in PBS) and fixed in ice-cold methanol:acetone 3:1 at $-20°$C for 10 min. Cells were washed with PBG and permeabilized with 0.2% Triton-X100 in PBS. Next, cells were rinsed three times with PBG, blocked with 0.5% BSA, and labeled with rabbit anti-γ-tubulin (1:10,000 Sigma T3559) and anti-β-tubulin (1:1,000 Sigma 2-33-28) antibodies in blocking reagent for one hour. After three washes, cells were incubated with Alexa Fluor 594-conjugated goat anti-rabbit (1:1,000 Invitrogen) and Alexa Fluor 488-conjugated anti-mouse (1:300 Invitrogen) secondary antibodies for 90 and 60 min, respectively. Finally, the cells were washed again and mounted on glass slides with Prolong Gold Antifade Reagent with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). All images were taken with an Axioskop 2 microscope (Zeiss) or an Olympus FW1000 confocal microscope using $40\times$ or $100\times$ objectives. The size and number of giant nuclei were verified using ImageJ software.

2.6 Determination of mutant frequency

To minimize spontaneously arising mutants, 300 4a cells were plated in individual wells within 24-well plates and expanded sequentially to 6-well plates, T25 and T75 flasks (Turker et al., 2009). Once expanded, cells were treated with various concentrations of formaldehyde (0 – 1 mM) and cultured for 5 – 7 days in fresh DMEM to allow phenotypic expression of the *Aprt* gene. After the expression period, cells were seeded at high density (10^5 cells/100-mm dish) in the presence of 2,6-diaminopurine (DAP) (80 μg/mL) to select for *Aprt* null cells. Concurrently, cells were also plated at low densities (600 cells/100-mm dish) in the absence of selection to determine the cloning efficiency. Cells were kept at 37°C and replenished with fresh DAP every week for 3 – 4 weeks. Selected and unselected colonies were fixed and stained with crystal violet after the incubation. Mutant frequency (MF)

was determined by the following equation (Kasameyer et al., 2008):

$$MF = \frac{\text{Number of DAP-resistant colonies}}{\text{Number of cells seeded}} \times \frac{1}{\text{Cloning efficiency}}. \tag{2.2}$$

2.7 Mutant selection and DNA extraction

Following 21 – 25 days of selection as described in Section 2.6, DAP-resistant (DAP^r) colonies were isolated for further characterization. Each DAP^r colony was isolated with a cloning cylinder, trypsinized, and the cells plated in a single well of a 24-well plate in DMEM. The cells were allowed sufficient time to become confluent and subcultured with a splitting ratio of 1:3. The cells were maintained to confluency, at which time the genomic DNA of each selected DAP^r colony was extracted using the salting-out method (Miller et al., 1988) where cellular proteins are salted out by dehydration and precipitation. Cells were lyzed with 500 μL of nuclei lysis buffer (10 mM Tris, 400 mM NaCl, and 2 mM EDTA, pH 8.0) and digested overnight at 37°C with 40 μL of 10% SDS and 10 μL of proteinase K solution. On the next day, 170 μL of saturated NaCl was added to each tube, shaken vigorously for 15 sec, and centrifuged for 10 min at 12,000 – 13,000 $\times$ g. The supernatant containing the DNA was transferred to a new 1.5 mL eppendorf tube and two volumes of room temperature absolute ethanol was added. The tube was inverted several times until DNA strings were visible. After centrifugation, the supernatant was removed and washed with 70% ethanol. The DNA was then dried, resuspended in 200 μL of TE buffer (10 mM Tris, 0.2 mM EDTA, pH 8.0), and incubated overnight at 37°C. On the following day, 20 μL of 7.5M NH_4OAc and 440 μL of absolute ethanol were added to precipitate the DNA. The tube was inverted until DNA strings precipitated and the DNA was pelleted. The DNA pellet was washed and allowed to dry until transparent before dissolving in 30 μL of TE buffer. Finally, the DNA was quantified using a NanoDrop® ND-1000 spectrophotometer.

2.8 Molecular characterization

The mutational events that led to the loss of *Aprt* expression in each of the resistant clones were identified by polymerase chain reaction (PCR) amplification of 13 polymorphic microsatellite loci on chromosome 8 as previously described (Turker et al., 2009). The loss of heterozygosity (LOH) of each marker was determined by the loss of the DBA/2 fragment harboring the wildtype *Aprt* allele. The fragment size for each marker from proximal to distal end on the C57BL/6 and DBA/2 chromosomes is shown below:

Markers	**C57BL/6 Fragment (bases)**	**DBA/2 Fragment (bases)**	**SV129 Fragment (bases)**	**Size difference (bases)**
124	125	131		6
3	172	178		6
125	128	141		13
190	134	98		36
100	108	100		8
75	154	130		24
106	144	113		31
312	86	78		8
166	115	119		4
Aprt	140	140	157	17
13	94	94	102	8
326	123	127		4
56	160	181		21

Table 2.1: Length of the CA dinucleotide repeats for C57BL/6 and DBA/2 marker fragments.

The forward primer of each marker was tagged with a fluorophore while the reverse primers were unlabeled. These primer sequences were ordered from Applied Biosystems and are listed as follows:

Primers	**Primer Sequence 5′ to 3′**
D8Mit124 F	**HEX**-CAACTGTGTATCATAAACTGGGAA
D8Mit124 R	GAAGAATCACTCAGCAGTGTATGG
D8Mit3 F	**FAM**-TCCCATTCTCGCATAAGTCC
D8Mit3 R	GATGGGAAGACAGGGTAGCA
D8Mit125 F	**HEX**-ATCGCTCTATCTACTCATCTATTCACA
D8Mit125 R	GACCCTGACTCTTAATCCTAGTGC
D8Mit190 F	**FAM**-CTTTGTTGCTGTTTCATTCTGG
D8Mit190 R	AGTCATATACAAGGTCAACCTGAGC
D8Mit100 F	**FAM**-AGCCTCAGGTGTATGGTTGC
D8Mit100 R	ATGAAGAGAATAAAGGACTGTGGG
D8Mit75 F	**FAM**-TGGTGACTATGGTTGCCTGA
D8Mit75 R	GCCTTTTGGAGAGCAACACT
D8Mit106 F	**HEX**-TGTCACATACCCATGCGTG
D8Mit106 R	AGCAAACGAGGGTGCAAG
D8Mit312 F	**FAM**-ATTGAGACTTGAGACTGTCTTTAAACA
D8Mit312 R	GTTGGTCTGGTCTCTCAGTGC
D8Mit166 F	**HEX**-AGAAGGGAAAAACTAACTCCCG
D8Mit166 R	ATTGGAGATGGTGCATGTAGG
Aprt F	**FAM**-TTCATAACGGAGCTTCCCTTTAGT
Aprt R	GGACCTTCCTGTGAGCCCGTG
D8Mit13 F	**HEX**-CCTCTCTCCAGCCCTGTAAG
D8Mit13 R	AACGTTTGTGCTAAGTGGCC
D8Mit326 F	**HEX**-TCTTGTACTCCATGTAGGTTTTGC
D8Mit326 R	ATATTTTGCTTACTAGCACCTGGG
D8Mit56 F	**HEX**-ACACTCAGAGACCATGAGTACACC
D8Mit56 R	GAGTTCACTACCCACAAGTCTCC

Table 2.2: Primer sequences for PCR amplification of 13 polymorphic loci on chromosome 8. Bold lettering represents the fluorophores that were attached at the 5′ end of the forward primers. HEX: hexachloro-fluorescein; FAM: carboxyfluorescein; F: forward; R: reverse.

Using the primers listed in Table 2.2, a master mix was made resulting in the following for each reaction:

PCR Reaction Mix	**1 × (12 μL reaction)**
10× PCR buffer	1 μL
dNTPs (1.25 mM/base)	1.6 μL
$MgCl_2$ (50 mM)	0.3 μL
Sterile deionized water	6.155 μL
Forward primer (10 μM)	0.3 μL
Reverse primer (10 μM)	0.3 μL
Taq DNA polymerase (5 U/μL)	0.045 μL
Template (10 μL)	2 μL

Table 2.3: Recipe for a single PCR reaction mix.

The following PCR program was then used to amplify each template:

Temperature	**Time (sec)**	**Cycles (×)**
96°C	120	1
94°C	45	
57°C	45	30
72°C	60	
72°C	420	1

Table 2.4: Configuration of the PCR program.

To amplify each DNA sample with 13 primer sets, the following arrangements were used for PCR in 96-well plates:

Marker 106			**Marker 312**			**Marker 13**			**Marker 125**		
1	9	17	1	9	17	1	9	17	1	9	17
2	10	18	2	10	18	2	10	18	2	10	18
3	11	19	3	11	19	3	11	19	3	11	19
4	12	20	4	12	20	4	12	20	4	12	20
5	13	Het	5	13	Het	5	13	Het	5	13	Het
6	14	Blk	6	14	Blk	6	14	Blk	6	14	Blk
7	15	Dba	7	15	Dba	7	15	Dba	7	15	Dba
8	16	H_2O	8	16	H_2O	8	16	H_2O	8	16	H_2O

Table 2.5: Sample arrangement for PCR amplification. 1 – 20: individual DNA clone samples; Het: *Aprt* heterozygote; Blk: C57BL/6; Dba: DBA/2.

Following the completion of PCR, the PCR products were multiplexed into a thermocycler-compatible 96-well plate. For instance, multiplexing was performed such that Multiplex 1 contains PCR products

amplified using markers 106, 312, 13, and Aprt as shown in the following table:

Multiplex 1	Markers 106, 312, 13, Aprt
Multiplex 2	Markers 3, 125, 190
Multiplex 3	Markers 100, 75, 166
Multiplex 4	Markers 326, 56, 124

Table 2.6: Multiplexing arrangement.

The multiplexed PCR products were sent to the Plant-Microbe Genomics Facility at Ohio State University for microsatellite fragment analyses. The facility used an ABI Prism 3700 DNA analyzer to separate fluorescently labeled PCR products. Electropherograms from microsatellite analyses were generated using the GeneMapper® v4.0 software.

Chapter 3

Formaldehyde Induces Genomic Instability

Anuradha Kumari[1], Yun Xin Lim[1,2], Amy H. Newell[3], Susan B. Olson[3], Amanda K. McCullough[1,3]

[1]Center for Research on Occupational and Environmental Toxicology (CROET),

[2]Department of Cell & Developmental Biology,

[3]Department of Molecular & Medical Genetics,

Oregon Health & Science University, Portland, OR 97239

The manuscript has been submitted for publication.

3.1 Preface

This work has been submitted for publication.

The author's contributions to the manuscript include performance of the immunoblotting assays, immunofluorescence assays, data analyses, the construction of Figures 2C, 2D, 4, and 5, and the writing of the manuscript.

Anuradha Kumari contributed to the conception, design, and performance of experiments such as the survival assays and flow cytometry analyses. She also prepared Figures 1, 2A, 2B, 3, 6, and Table I, compiled data for analyses, and contributed to the writing of the manuscript.

Amy H. Newell contributed to the design of the cytogenetic experiments, performed the cytogenetic analyses, constructed Figure S1, and contributed to the writing of the manuscript.

Susan B. Olson contributed to the design, supervision of the cytogenetics studies, data analyses and contributed to the writing of the manuscript.

Amanda K. McCullough contributed to the conception of this project, experimental design, data analyses, writing of the manuscript, and provided funding and lab space for the execution of this work.

A fraction of the manuscript is presented here as part of this thesis.

3.2 Rationale

While chromosomal changes are a well-known consequence of formaldehyde exposure, little is known about the effects of formaldehyde at the cellular level. Cell cycle analyses of the wildtype AA8 Chinese hamster ovary cells displayed a G2/M arrest following formaldehyde treatment, where the effects were more extensive in cells that went through a 24- to 48-hr recovery period. Cytogenetic data also demonstrated an escalation in the number of cells with abnormal ploidy status. To delineate possible mechanisms leading to the polyploidy phenotype, this chapter addresses the effects of formaldehyde on nuclear morphology and centrosome and microtubule distributions.

3.3 Results

3.3.1 Formaldehyde induces the enlargement of nuclei

A nuclear counterstaining with DAPI revealed a high percentage of cells with enlarged nuclei following a 4 hr formaldehyde treatment (Figure 3.1). The untreated cells had an average nuclear size of 119 μm^2. Relative to untreated cells, a significantly high number of cells with giant nuclei ($\geq$150 μm^2) were observed in the cell population that were processed immediately after the 4 hr treatment (average size of giant nuclei: 185 μm^2) as well as those that had undergone a 48 hr recovery following formaldehyde treatment (average size of giant nuclei: 210 μm^2) (Figure 3.1 A and B). In accord with antecedent studies, micronuclei were also observed following formaldehyde treatment (Figure 3.1 A, arrow).

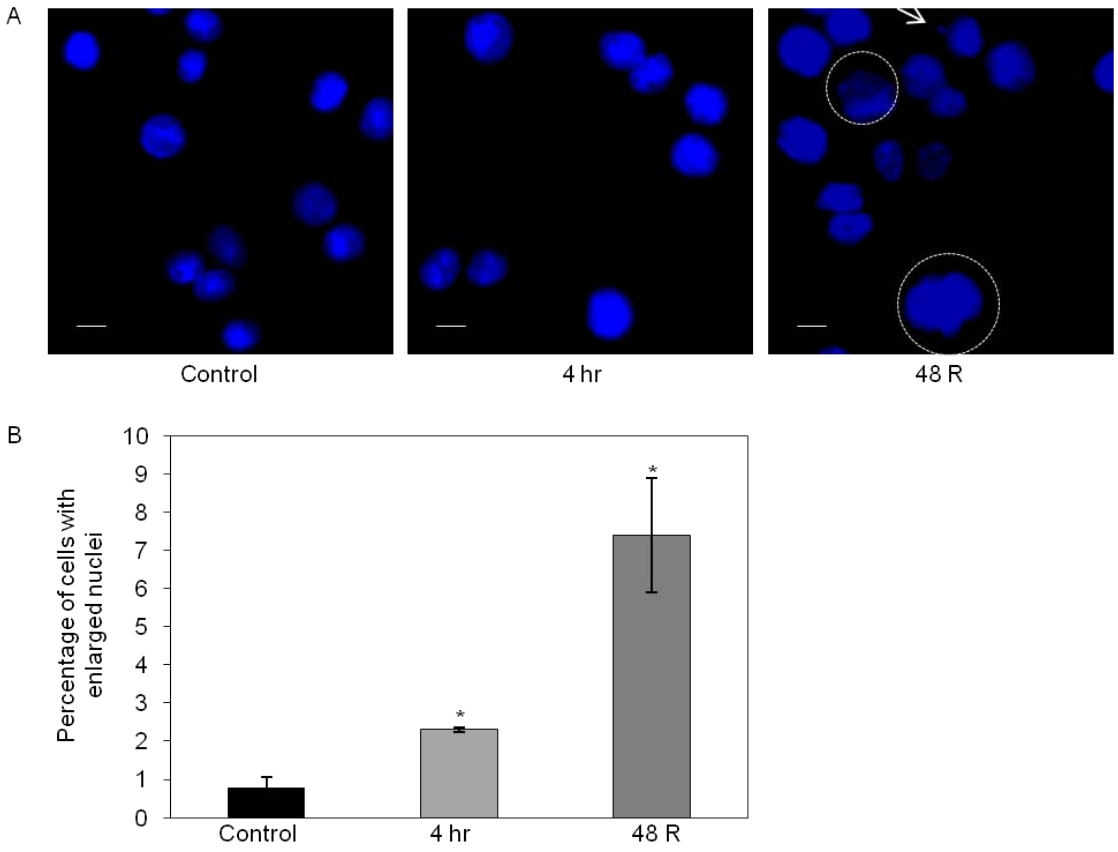
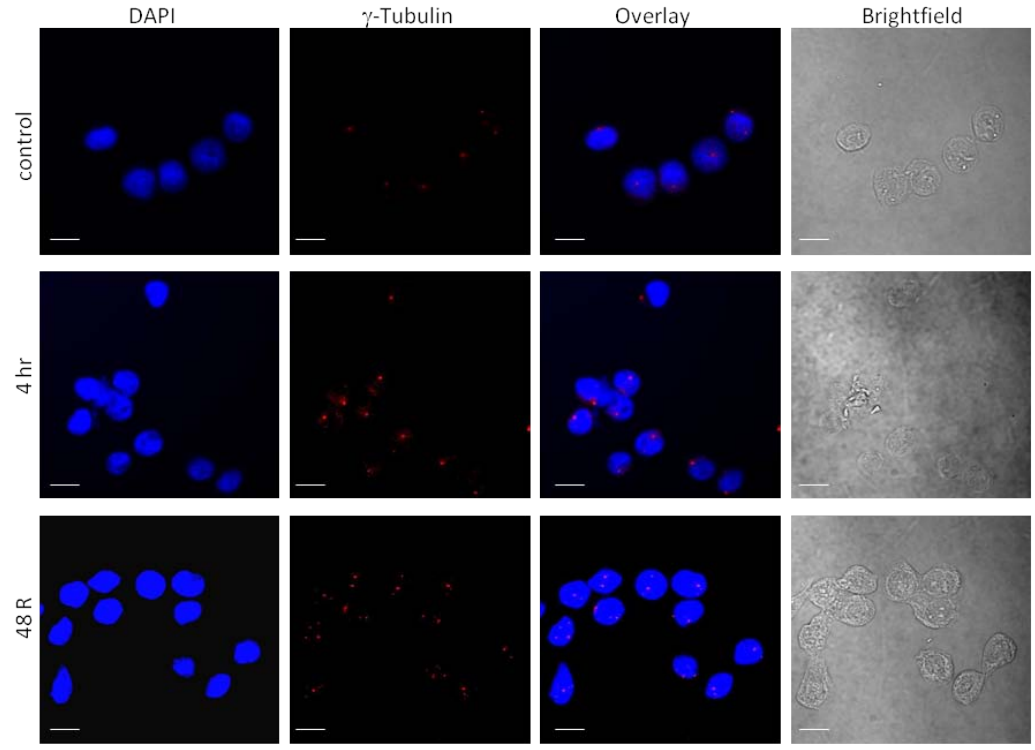


Figure 3.1: Formaldehyde induces the enlargement of nuclei. Cells were treated with a sublethal dose of formaldehyde (300 μM) for 4 hr and fixed immediately or allowed a 48-hr recovery period (48 R). (A) Cells were fixed and counterstained with DAPI following formaldehyde exposure. Giant nuclei were encircled; micronucleus was indicated with an arrow. (B) Approximately 400 cells from three independent experiments were analyzed for each condition. Images were captured with an Axioskop 2 microscope (Zeiss) (40×/0.75 Plan Neofluar); error bars represent standard deviations; * P<0.05. Scale bars = 25 μm.

3.3.2 Formaldehyde induces centrosomal defects

Considering the heightened post-G2 peaks from the FACS analyses and the enhanced polyploidy populations from the cytogenetic studies (Kumari et al., submitted), cells were immunostained with an anti-γ-tubulin antibody to visualize centrosomes. Relative to the untreated control, formaldehyde-treated cells have aberrant numbers and sizes of centrosomes, where the defects were already discernible in cells that were processed immediately after the 4 hr treatment (Figure 3.2 Top and Bottom). Unlike the nuclear morphology defects that appear much later, centrosomal defects were

detected immediately following formaldehyde exposure, suggesting that the centrosomal defects are precedable or causative of the giant nuclei phenotype.



Cells with	Control (%)	4 hr (%)	48 R (%)
$\leq$ **2 centrosomes**	98	88	90
$>$ **2 centrosomes**	0.8	4	3
large centrosomes	0.5	1	3
<1, large centrosomes	0.7	7	4

Figure 3.2: Formaldehyde induces centrosomal defects. (Top) Cells were treated with formaldehyde and immunostained with an anti-γ-tubulin antibody to visualize centrosomes. (Bottom) The percentage of cells with various numbers and sizes of centrosome. Approximately 600 cells from three independent experiments were analyzed for each condition. Images were captured with an Olympus FW1000 confocal microscope (40×/1.3 Oil Plan Fluorite). 48 R: 48 hr recovery; scale bars = 25 μm.

3.3.3 Formaldehyde induces microtubule defects

During mitosis, duplicated centrosomes function as major microtubule organizing centers (MTOC), coordinating the mitotic spindle apparatus to prepare for proper chromosome segregation. To ex-

amine the possibility of extra centrosomes correlating with microtubule defects, cells were immunostained with an anti-β-tubulin antibody to detect microtubules. Errors in microtubule polarization were observed in formaldehyde-treated cells with centrosomal defects (Figure 3.3). These cells were found to be able to undergo both bipolar and multipolar cell divisions. Taken together, these observations suggest that following formaldehyde exposure, cells undergo errors in centrosome duplication, leading to progenies with missegregated chromosomes after cell division, hence the aneuploidy populations or the polyploidy populations when the progenies go through endoreduplication.

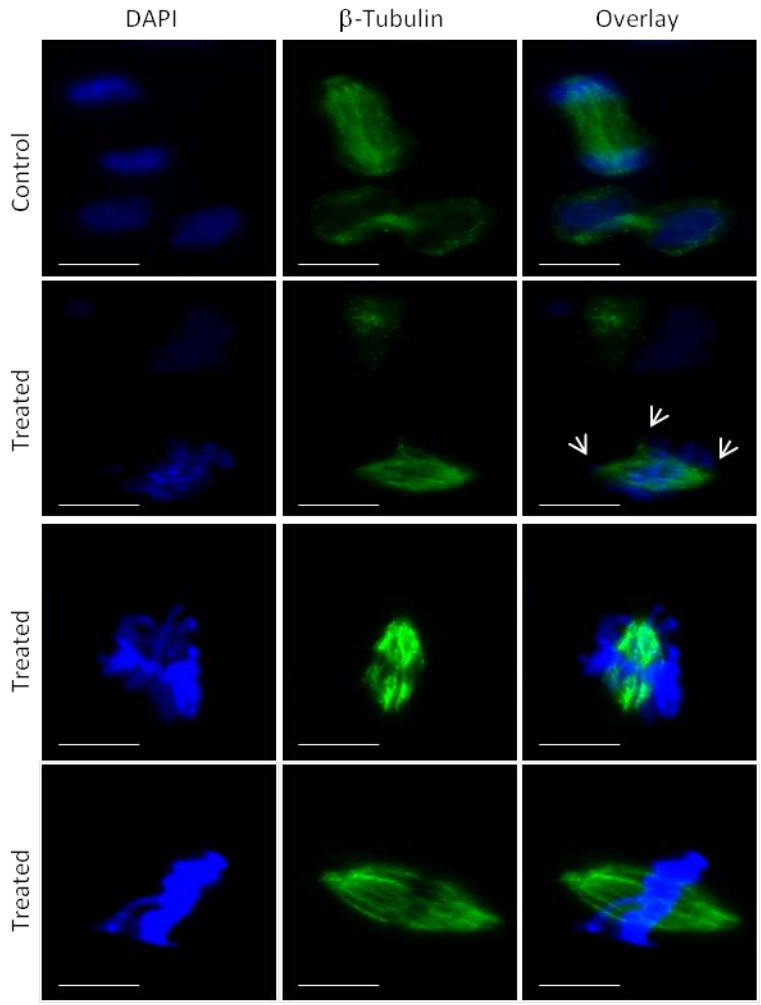


Figure 3.3: Formaldehyde induces microtubule defects. Cells were treated with formaldehyde and immunostained with an anti-β-tubulin antibody to visualize microtubules. Arrows indicate a cell undergoing the tripolar cell division. Images were captured with an Axioskop 2 microscope (Zeiss) (100×/1.30 Oil Plan Neofluar). Scale bars = 10 μm.

3.4 Discussion

One of the hallmarks of cancer is gross alterations in nuclear morphology or DNA content. This is the fundamental feature in the identification of tumor grade along with an assessment of tissue differentiation (Lingle et al., 2005). Near-diploid or aneuploid karyotypes dominate across cancer types, including 75% of hematopoietic cancers and over 90% of solid tumors (Weaver and Cleveland, 2006). The studies herein show an altered DNA content as a possible consequence of compromised mitoses following formaldehyde treatment, and is the first report that demonstrates the potential of formaldehyde in altering ploidy status. Therefore, the observation that formaldehyde altered DNA content verifies its carcinogenicity.

This study clearly demonstrates that cells develop amplified centrosomes and aggregated centrosomes within 4 hr of formaldehyde treatment. This observation precludes failure of cytokinesis or tetraploidization as the mechanisms for the generation of supernumerary centrosomes given that the Chinese hamster ovary cells have a 16-hr doubling time. Centrosome amplification is an early event in breast, bladder, prostate, and cervix cancer. This event is found to precede ploidy changes in bladder cancer and is a signature of a low-grade tumor (Lingle et al., 2005). Apropos to the correlation between centrosome instability and cancer, our studies indicate a possibility that centrosome amplification occurs prior to chromosomal instability and manifestation of formaldehyde-induced cancers and that cells harboring supernumerary centrosomes might generate further instability through unequal and multipolar mitoses. Cells undergoing multipolar cell division (Figure 3.3, second row) are predicted to give rise to inviable progenies; while segregation of genetic material containing a lagging chromosome (Figure 3.3, last row) to opposing poles will give rise to aneuploidy (following cytokinesis) or polyploidy (in the absence of cytokinesis) if viable progenies endoreduplicate (Ganem et al., 2009). Several mechanisms such as cell-cell fusion, mitotic slippage, and cytokinesis failure have been postulated to give rise to polyploidy and aneuploidy (Lingle et al., 2005; Storchova and Kuffer, 2008). This study indicates both persistent errors in mitotic checkpoint leading to premature mitosis exit (mitotic slippage) and abnormal spindle positioning or lagging chromosome trapped in the cleavage

furrow resulting in failure in cytokinesis (cytokinesis failure) as possible causes for aberrant ploidy status following formaldehyde exposure. Collectively, this study shows that following formaldehyde exposure, supernumerary centrosomes aggregate at opposite poles, giving rise to daughter cells with missegregated chromosomes following bipolar cell division, a possible mechanism that contributes to formaldehyde-induced cancer development.

Chapter 4

The Genotoxic and Mutagenic Effects of Formaldehyde in Mammalian Cells

Yun Xin Lim[1,2], Cristian Dan[1], Mitchell S. Turker[1,3], Amanda K. McCullough[1,3]

[1]Center for Research on Occupational and Environmental Toxicology (CROET),

[2]Department of Cell & Developmental Biology,

[3]Department of Molecular & Medical Genetics,

Oregon Health & Science University, Portland, OR 97239

Manuscript is currently in preparation.

4.1 Preface

This work is currently in preparation for submission.

The author's contributions to the manuscript include the conception, design, performance of experiments, data analyses, and composition of the manuscript currently in preparation for submission.

Cristian Dan contributed technical assistance, supply of reagents, data analyses, and discussion of the project.

Mitchell S. Turker provided the *Aprt* heterozygous cell line, contributed to the experimental designs, data analyses, and gave us valuable comments on the project.

Amanda K. McCullough contributed to the conception, design, data analyses of this project, provided funding and lab space for the execution of this work.

Cytogenetics experiments were performed as a fee-for-service by Oregon Health & Science University Cytogenetics Research Core Service.

4.2 Rationale

Even though the *Hprt* system is widely used in mutagenesis experiments, detection of mutagenic events at the functionally hemizygous *Hprt* is limited to point mutations and deletions, due to its location on the X-chromosome (Van Sloun et al., 1998); because of the absence of the *Hprt* homolog, chromosome loss and recombination cannot be scored. Unlike *Hprt* ,*Aprt* (adenine phosphoribosyltransferase) is located on an autosome and is not flanked by critical genes so the size of selectable deletions is not constrained (Turker et al., 1997). *Aprt* is therefore a more suitable selectable target for the detection of a wide range of mutations and thus is a more appropriate system for the determination of formaldehyde-induced mutations. Moreover, since working with inherently heterozygous *TK* (thymidine kinase) or *Aprt* cell lines can be challenging unless there is molecular heterozygosity for the syntenic markers, it is preferred to use an *Aprt* cell model system that is derived from gene targeting and subsequent backcrossing into mice with a different background to score for loss of heterozygosity (Engle et al., 1996; Turker et al., 1995).

The *Aprt* heterozygous cells are usually used to score for second-step mutations where both copies of the *Aprt* allele are inactivated (Ponomareva et al., 2002). *Aprt* null cells can be easily selected in vitro on the basis of their resistance to the toxic purine analog DAP (Van Sloun et al., 1998). APRT enzyme functions in the adenine salvage pathway where it rescues free adenines by converting them into adenosine monophosphates. In the presence of DAP, APRT converts DAP into DAP-monophosphate, which is eventually converted into DAP-deoxyribonucleotide. DAP-deoxyribonucleotide feedback inhibition of the ribonucleotide reductase prevents NTP to dNTP conversion, limiting the dNTP pool for DNA synthesis and eventually rendering cell death (Weckbecker and Cory, 1987). As discussed in Chapter 1.8, due to conflicting reports regarding the mutagenic effect of formaldehyde, this chapter includes experiments designed to decipher the mutagenic potential of formaldehyde using the 4a *Aprt* cell line.

4.3 Results

4.3.1 The 4a cells exhibit a dose- and time-dependent sensitivity to formaldehyde treatment

Based on the cytotoxicity range of formaldehyde in various mammalian cells tested in our laboratory, the effects of acute (transient, high dose) and chronic (continuous, low dose) formaldehyde exposure on 4a cell viability were examined. Figure 4.1 A demonstrates the dose-dependent sensitivity of 4a cells following chronic exposures from 0 – 60 μM throughout the course of experiment. The 4a cells were also subjected to acute exposures from 0 – 400 μM for varying periods of time (Figure 4.1 B) or at increasing doses for 4 hr (Figure 4.1 C). The dose that confers 10% survival was used in order to generate maximum mutational events without killing all of the cells. The results demonstrate that a 200 μM formaldehyde exposure for 12 hr, 300 μM for 24 hr (Figure 4.1 B), and 1 mM for 4 hr (Figure 4.1 C) confer the 10% survival. The following experiments were designed to examine cellular and mutagenic effects following acute exposures of formaldehyde in the 4a cells.

4.3.2 Formaldehyde impairs cell cycle progression and induces chromosome breaks and radials

To determine the effects of formaldehyde on cell cycle progression, cells were treated with 1 mM formaldehyde and allowed to recover prior to staining with propidium iodide (PI) for flow cytometric analyses. Treated cells started to accumulated in G2/M phases 2 days after the treatment removal and continued to stay arrested up to 5 days (Figure 4.2), consistent with our observations in other mammalian cells. It is worth mentioning that the cells displayed the greatest polyploidy population (post-G2/M peak) 2 days after the treatment removal, but this population diminished overtime and was almost comparable to that of the control 7 days after formaldehyde removal.

In addition, formaldehyde induces breaks and radials in the 4a cells (Figure 4.3), where the effects

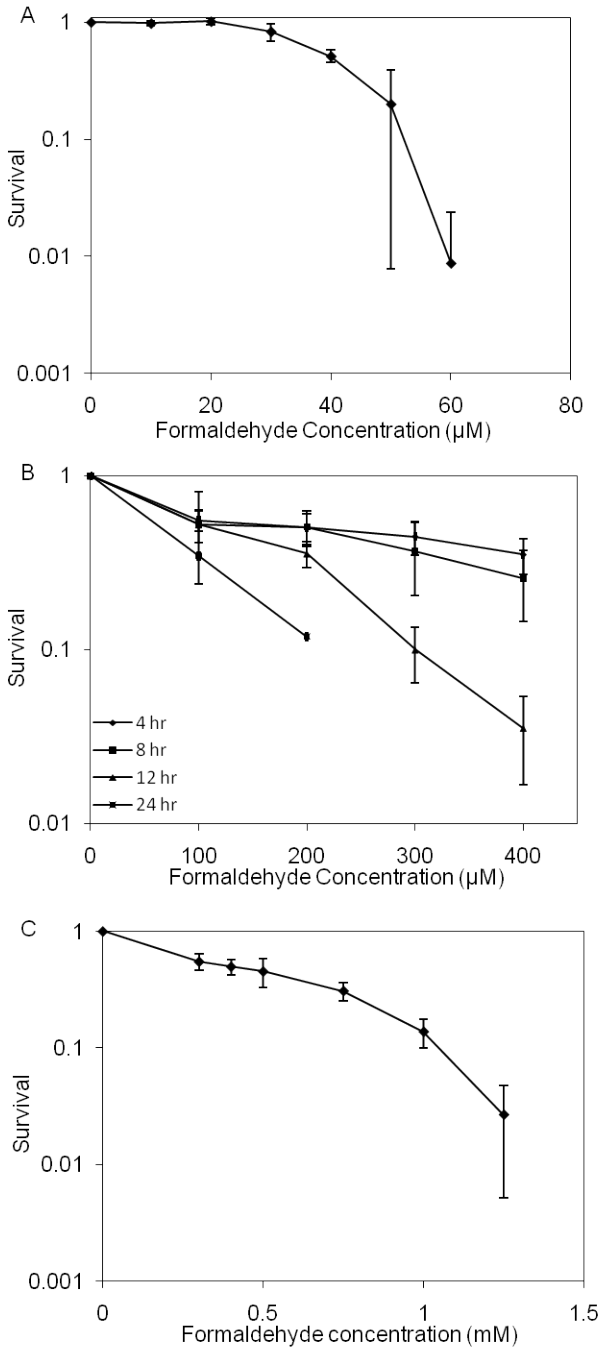


Figure 4.1: Sensitivity of 4a cells to formaldehyde. Chronic (continuous, low dose) exposure of formaldehyde (A) and acute (transient, high dose) exposure of formaldehyde for various durations (B) and concentrations for 4 hr (C). The standard deviations were derived from three or more independent experiments.

are more apparent when the treated cells were given 48 hr to recover. Taken together, these analyses justify the 4a cells as a good model system and verify the general effects of formaldehyde on chromosome and ploidy status across species and cell types.

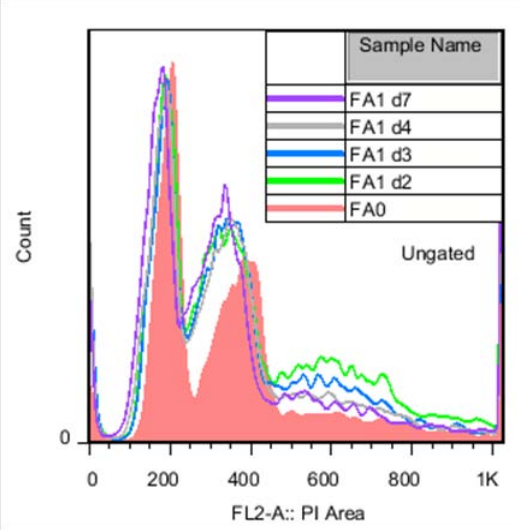


Figure 4.2: Cell cycle progression of formaldehyde-treated cells. Treated cells were allowed 2 – 7 days of recovery (d2 – d7) prior to PI staining for flow cytometry analyses. FA0: control; FA1: 1 mM formaldehyde.

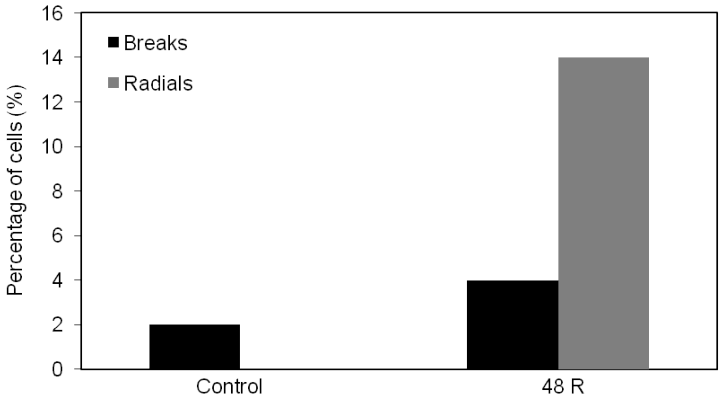


Figure 4.3: Formaldehyde induces breaks and radials. Percentage of cells containing chromosome breaks and radials is graphically presented. Presented is a representative of a single experiment where 50 cells were analyzed for each condition. 48R: 48 hr recovery.

4.3.3 Formaldehyde exposure results in an increased mutant frequency

To examine how formaldehyde alters mutant frequency, cells were exposed to 1 mM of formaldehyde for 4 hr and mutants that lost *Aprt* expression were selected by growth on DAP-containing media. After adjusting for the cloning efficiency (Equation 2.2), formaldehyde was shown to increase mutant frequency (MF) in a dose-dependent fashion (Figure 4.4 A). Relative to the spontaneous mutation background, a five-fold increase of MF was achieved following a 1 mM of formaldehyde treatment. It is noted that the rise in MF did not occur at the cost of reduced cloning efficiency (Figure 4.4 B) given the fact that formaldehyde exposure generated more DAP^r clones (Figure 4.4 C).

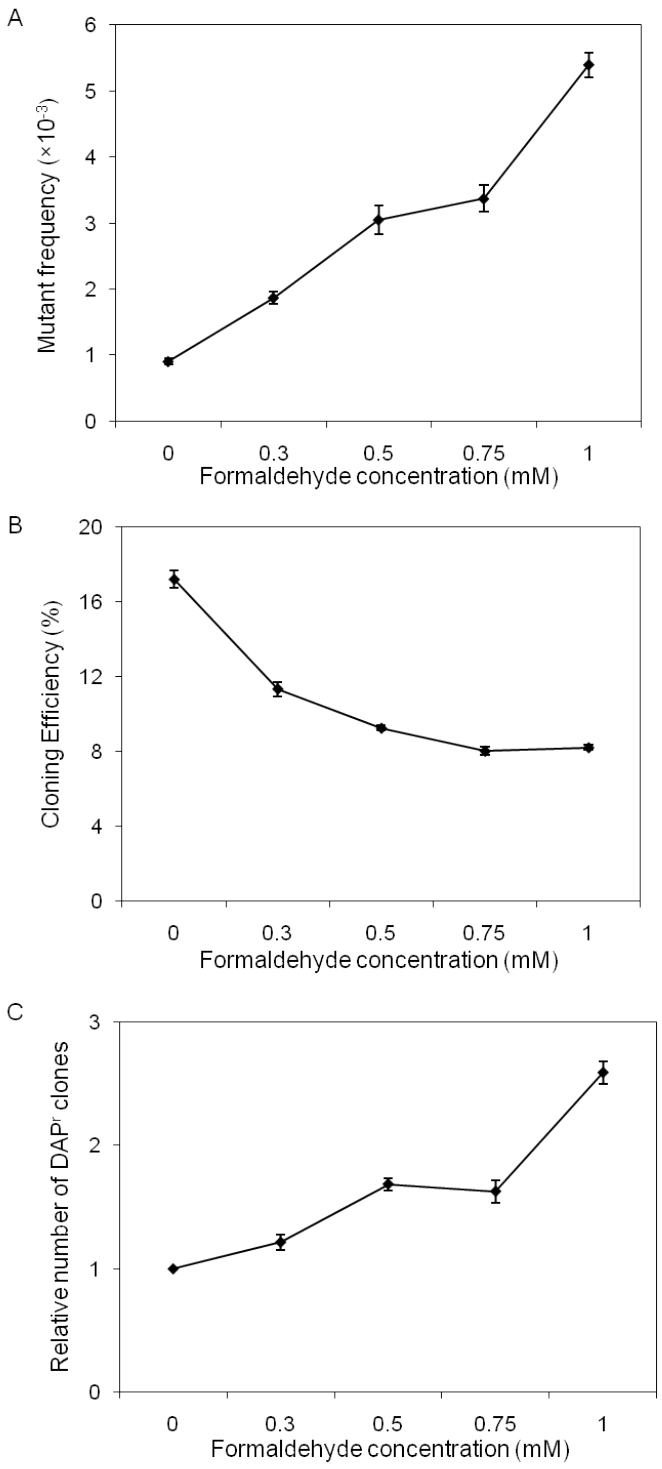


Figure 4.4: Dose-dependent mutant frequency of formaldehyde. Mutant frequency of formaldehyde (A) was determined by adjusting the number of DAP^r clones (B) to corresponding cloning efficiency (C). The standard errors of the mean were derived from three independent experiments.

4.3.4 Increased mutational events following formaldehyde exposure

Since the mutational spectrum of formaldehyde has not been described in mammalian cells, individual *Aprt* mutant clones were isolated for DNA extraction, followed by the determination of the mutational events. All DNA preparations were examined for loss of heterozygosity (LOH) for 13 proportionately distributed polymorphic CA repeat loci on mouse chromosome 8 (Figure 4.5 A). The LOH patterns, namely all the recoverable mutational events, were used to determine the mutational fingerprint of formaldehyde. All four controls and examples for each LOH pattern are depicted on Figure 4.5 B and C, respectively.

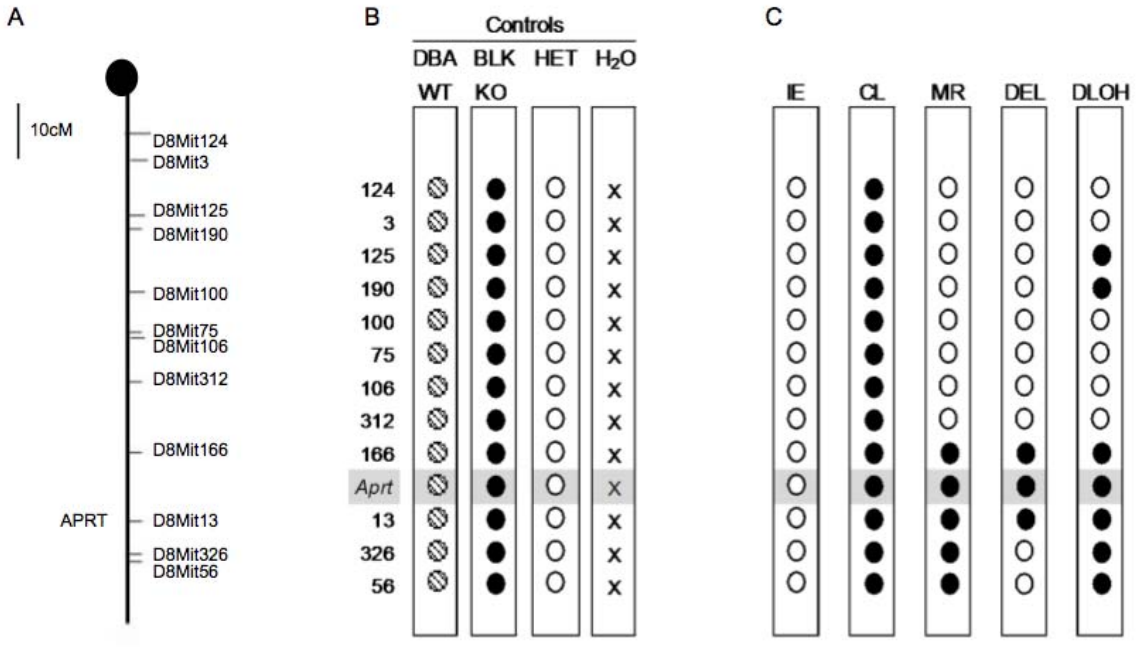


Figure 4.5: All mutational events recoverable by loss of heterozygosity (LOH) patterns. All 13 chromosome 8 microsatellite loci that were examined in this study and their relative positions are presented in (A); cM: centimorgan. The controls and all recoverable mutational events are illustrated in (B) and (C), respectively. The 4a cells have an *Aprt* knockout allele (KO) inherited from the C57BL/6 (BLK) parents (filled circles). LOH is defined as the loss of the wildtype (WT) *Aprt* allele inherited from the DBA/2 (DBA) parent (stippled circles). *Aprt* heterozygous cells (HET) are indicated with open circles. The PCR-based molecular analyses yield mutational events that can be classified into one of five categories: intragenic events (IE), chromosome loss (CL), mitotic recombination (MR), deletion (DEL), and discontinuous loss of heterozygosity (DLOH). DLOH is defined as an LOH event that is apparently unlinked to the LOH event that causes the loss of *Aprt* expression (Ponomareva et al., 2002). The LOH patterns for each event shown in this figure are representative of the actual patterns observed.

The spontaneous and formaldehyde-induced mutational spectra were determined by examining approximately 60 independent *Aprt* mutants for each condition (Table 4.1). The most common spontaneous mutations observed were mitotic recombination (40%) and chromosome loss (47%). Intragenic events (5%), deletion (3%), and discontinuous LOH (5%) constitute the remaining events.

Mitotic recombination (47%) is the major event observed in *Aprt* mutants isolated from formaldehyde-exposed cells. A notable decrease in chromosome loss was observed concurrently with a slight increase in intragenic events (12%), deletions (7%), and discontinuous LOH (7%). It can be inferred that an overall increase in estimated MF across all mutational events accounts for the mutagenicity of formaldehyde (Figure 4.4). Specifically, taking estimated MF into consideration, chromosome loss and intragenic events are likely candidates that contribute to the mutagenic effects of formaldehyde.

		IE	**MR**	**DEL**	**CL**	**DLOH**	**Total**
Spontaneous	N	3	23	2	27	3	58
	%	5	40	3	47	5	100
	MF	5	36	3	42	5	92
Formaldehyde	N	7	28	4	16	4	59
	%	12	47	7	27	7	100
	MF	64	256	37	146	37	540

Table 4.1: Mutational spectrum of formaldehyde. IE: intragenic events; MR: mitotic recombination; DEL: deletion; CL: chromosome loss; DLOH: discontinuous loss of heterozygosity. N represents the number of mutations examined in each category. MF is the estimated mutant frequency ($\times 10^{-5}$) for each mutational event.

4.4 Discussion

The formaldehyde mutagenesis studies conducted thus far either employed strategies that analyze small mutations such as single base substitutions, or used functionally hemizygous model systems where the number of recoverable mutational events are limited. The observations that the second-step loss of gene expression is accompanied by large mutational events (Van Sloun et al., 1998; Ponomareva et al., 2001) is one of the main reasons to use the 4a cells in this investigation. This is the first report that examines the mutagenic effects of formaldehyde using a model system that

registers an extensive spectrum ranging from point mutation to chromosome loss. Our findings show that formaldehyde increased mutant frequency by raising the mutational events. To ensure the overall mutational spectrum reflects the actual effects of formaldehyde, only 2 – 4 independent clones were picked from individual dishes to minimize the chance of selecting sibling clones. In addition, the high throughput DNA analyzer has a one-base-pair resolution, hence microsatellite analysis errors are unlikely. Given that the 4a cell line has 66% heterozygosity on chromosome 8 and that it tolerates events resulting in LOH, it is safe to assume that most mutations can be recovered.

Our studies demonstrate that formaldehyde significantly increased MF from 0.9×10^{-3} to 5.4×10^{-3} ($P<0.0001$). In contrast to ionizing irradiation which increased MF by three fold (Turker et al., 2009), formaldehyde raised the MF by five fold, further attesting to its mutagenic potential. Even though the Fisher's Exact Test failed to compute a statistical significance by comparing the spectrum of events recovered from spontaneously-arising mutants and that from the formaldehyde-induced mutants ($P<0.271$), it is plausible that the general increase in all mutational events across the spectrum account for the mutagenicity of formaldehyde. (Table 4.1, estimated MF). Although the overall shift in percentage of mutational events was marginally significant, taking the estimated MF into consideration, formaldehyde seemed to cause an increase in intragenic events while decreasing chromosome loss events on mouse chromosome 8. This might be the unique trend of events following formaldehyde exposure. It is noted that the mutational fingerprints reported herein only represent the events on chromosome 8. A genome-wide examination could provide a more informative and impartial sets of events on each chromosome (Dan et al., 2011).

Chapter 5

Discourse

Exposure-induced cancers often present increased rates of mutation. Mutability is attained by increasing the sensitivity to mutagenic agents, while compromising the surveillance systems that normally oversee genomic integrity and drive damaged cells into senescence or apoptosis (Hanahan and Weinberg, 2011). In addition to the epidemiology studies described in Chapter 1, the carcinogenic potential of formaldehyde has also been implicated through network analyses of formaldehyde-responsive genes where significant associations with cancer, inflammation, and endocrine system regulation were revealed (Li et al., 2007; Rager et al., 2010). Interestingly, alteration of signaling pathways associated with inflammation is in accordance with recent recognition of the role of the innate immune system on neoplastic progression (Hanahan and Weinberg, 2011). Taking these reports and epidemiology studies into consideration, it is difficult to rebut the carcinogenicity of formaldehyde. Our studies clearly verify the mutability, hence the carcinogenic potential of formaldehyde by inducing pronounced centrosome and microtubule alterations, causing aberrant chromosome segregation, and by increasing mutant frequency.

While the mechanisms for polyploidy and aneuploidy have yet to be elucidated, it has long been considered a consequence of unequal chromosome segregation. In addition to the change in ploidy

status, centrosomal abnormalities are also found in many cancer cells. As the primary role of centrosomes as the microtubule organizing centers is to organize microtubules, an essential apparatus during mitosis, centrosomal alterations could potentially promote multipolar cell division, leading to missegregated chromosomes (Shimada and Komatsu, 2009). This theory is revisited by recent studies that showed that multiple centrosome-containing cells could undergo multipolar cell division but failed to produce viable progenies. However, multiple centrosomes can also aggregate at opposing spindle poles in an effort to promote bipolar cell division but give rise to viable progenies with missegragated chromosomes (Ganem et al., 2009). Taking this piece of data into account, our studies suggest that formaldehyde induces multiple centrosomes, promoting bipolar cell divisions to give rise to daughter cells with missegregated chromosomes, an observation that is corroborated with ploidy changes revealed by flow cytometry and cytogenetic analyses. Another interesting observation is the latent effects of formaldehyde exposure; the giant nuclei phenotype was not apparent until the recovery period, suggesting its latent genotoxic potential long after the acute exposure. Using live-cell imaging experiments to follow the fate of formaldehyde-treated cells would be a powerful way to visualize the development of aneuploidy and polyploidy populations and the extent of their survival.

The *Hprt* and *Aprt* model systems are often used to assess mutagenic potential in mammalian cells. Using the *Hprt* system, it was shown that the alkylating anticancer drug, thiotepa, induced 80% G:C to T:A transversions while *N*-ethyl-*N*-nitrosourea (ENU) generated 88% base pair substitutions at the A:T base pairs (Casciano et al., 1999). Utilizing the *Aprt* reporter gene, Turker et al. established mitotic recombination and discontinuous loss of heterozygosity as the signatures of ionizing radiation and oxidative stress, respectively (Turker et al., 1999; Turker et al., 2009). A dose-dependent increase in MF was also observed in *Aprt* heterozygous mice following ionizing radiation exposure (Liang et al., 2007).

Less comprehensive and limited numbers of studies on formaldehyde mutagenesis challenge the mutagenicity of formaldehyde. Different treatment regimens used by different research groups further

contribute to the complexity for mutagenicity assessment. For example, the dispute between two studies using the same V79 *Hprt* cell line where contradicting effects of formaldehyde on mutant frequency were reported could be due to the treatment regimens – Grafstrom et al. exposed the cells with 0 – 1 mM formaldehyde for one hr; Merk and Speit treated the cells for 4 hr with up to 0.5 mM formaldehyde (Grafstrom et al., 1993; Merk and Speit, 1998). Furthermore, Grafstrom et al. allowed a 14-day incubation period for the colonies to form; while Merk and Speit recovered the colonies in 7 days. As formaldehyde treatment and the presence of DAP can possibly slow the growth of the mutant cells, the short incubation period might explain the absence of mutagenic effects of formaldeyde as observed by Merk and Speit. Another study using the *Hprt* human lymphoblasts found 47% deletions following formaldehyde exposure (Crosby et al., 1988). However, the cytotoxicity and MF data were now shown, putting into question the possibility of repetitive exposure (given every 4 days for a total of eight exposures) at a single concentration (150 μM for 2 hr) biasing toward the recovery of more deletions. Only small mutations were examined in the field of formaldehyde mutagenesis to date since the events recoverable in the *Hprt* system are limited as previously described. The argument that formaldehyde does not cause gene mutations (Merk and Speit, 1998) is also simply based on unchanged mutant frequencies; DNA sequencing or LOH analyses were not performed.

Our studies demonstrate a formaldehyde-induced dose-dependent increase in mutant frequency and a possible formaldehyde-induced mutational signature. Our studies also suggest that formaldehyde has an effect on small intragenic events such as small-scale deletions, as well as large events such as chromosome loss. Rather than the suppression of event, chromosome loss is likely to be dampened by the increase in intragenic events following formaldehyde exposure. Chromosome loss can be accompanied by duplication of the remaining homolog, an event that cannot be determined by the LOH analysis. As mitotic recombination or chromosome loss can be followed by duplication of the homolog harboring the *Aprt* knockout allele or duplication of the remaining homolog, the Fluorescence *in situ* Hybridization (FISH) analyses (Van Sloun et al., 1998) can dissect the consequences of these events. The identity of small events, in contrast, can be identified using Southern Blotting or DNA sequence analyses on the *Aprt* locus.

The structure of different mouse chromosomes is similar in that the chromosomes are acrocentric and condense in unison in preparation for mitosis. In contrast, at any given time, sets of genes that are expressed or inactivated, and distribution of methylation patterns will differ among chromosomes. Accordingly, not only can a genome-wide LOH analysis reveal the mutational fingerprint of formaldehyde, chromosomes that are more prone to mutations following formaldehyde exposure, if any, can also be identified. Using FISH, in combination with the genome-wide analysis, we would be better informed on the mutational spectrum of formaldehyde.

The siRNA knockdown technology could be used as a strategy to screen for the repair and tolerance pathways that mitigate formaldehyde-induced damage in cultured cells. However, since cultured cells lack tissue microenvironment, mouse inhalation studies might be a better model to score for mutations. It is possible that mutant cells gradually accumulate in mouse tissues due to the low turn over rate. Another advantage for using the mouse model is that the degree of exposure can be easily matched to human exposures. It is also possible to breed DNA repair-deficient mice to *Aprt* heterozygous mice and examine the effects of formaldehyde under the DNA repair-deficient background to delineate the repair pathways that are responsible for the repair of formaldehyde-induced DNA damage.

In conclusion, our studies demonstrate that both the elevation of mutant frequency and centrosome aberrations contribute to the carcinogenicity of formaldehyde. The increase in mutations can possibly increase the likelihood of inactivating mutations in tumor suppressor genes and activating mutations in oncogenes, driving the initiation events for formaldehyde-induced cancer development. Our observations on chromosome instability as the consequence of compromised mitoses following formaldehyde treatment further corroborate the carcinogenic potential of formaldeyde.

Bibliography

Bauchinger, M. and Schmid, E. (1985) Cytogenetic effects in lymphocytes of formaldehyde workers of a paper factory. Mutat Res, 158, 195-199.

Casanova, M., Heck, H.D., Everitt, J.I., Harrington, W.W., Jr. and Popp, J.A. (1988) Formaldehyde concentrations in the blood of rhesus monkeys after inhalation exposure. Food Chem Toxicol, 26, 715-716.

Casanova, M., Morgan, K.T., Gross, E.A., Moss, O.R. and Heck, H.A. (1994) DNA-protein cross-links and cell replication at specific sites in the nose of F344 rats exposed subchronically to formaldehyde. Fundam Appl Toxicol, 23, 525-536.

Casciano, D.A., Aidoo, A., Chen, T., Mittelstaedt, R.A., Manjanatha, M.G. and Heflich, R.H. (1999) Hprt mutant frequency and molecular analysis of Hprt mutations in rats treated with mutagenic carcinogens. Mutat Res, 431, 389-395.

Cikmaz, S., Kutoglu, T., Kanter, M. and Mesut, R. (2010) Effect of formaldehyde inhalation on rat livers: a light and electron microscopic study. Toxicol Ind Health, 26, 113-119.

Consumer Product Safety Commission. (1997) An Update On Formaldehyde.
http://www.cpsc.gov/CPSCPUB/PUBS/725.html.

Crosby, R.M., Richardson, K.K., Craft, T.R., Benforado, K.B., Liber, H.L. and Skopek, T.R. (1988) Molecular analysis of formaldehyde-induced mutations in human lymphoblasts and E. coli. Environ Mol Mutagen, 12, 155-166.

Dan, C., Grygoryev, D., Sandfort, K., Connolly, M., Cross, B., Lasarev, M., Kronenberg, A. and Turker, M.S. (2011) Marked aneuploidy and loss of multiple chromosomes are common in autosomal mutants isolated from normal mouse kidney epithelium. Genes Chromosomes Cancer, 50, 239-249.

de Graaf, B., Clore, A. and McCullough, A.K. (2009) Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks. DNA Repair (Amst), 8, 1207-1214.

Dean, S.W., Brooks, T.M., Burlinson, B., Mirsalis, J., Myhr, B., Recio, L. and Thybaud, V. (1999) Transgenic mouse mutation assay systems can play an important role in regulatory mutagenicity testing in vivo for the detection of site-of-contact mutagens. Mutagenesis, 14, 141-151.

Engle, S.J., Stockelman, M.G., Chen, J., Boivin, G., Yum, M.N., Davies, P.M., Ying, M.Y., Sahota, A., Simmonds, H.A., Stambrook, P.J. and Tischfield, J.A. (1996) Adenine phosphoribosyltransferase-deficient mice develop 2,8-dihydroxyadenine nephrolithiasis. Proc Natl Acad Sci USA, 93, 5307-5312.

Fornace, A.J., Jr. and Little, J.B. (1977) DNA crosslinking induced by x-rays and chemical agents. Biochim Biophys Acta, 477, 343-355.

Fornace, A.J., Jr. and Seres, D.S. (1982) Repair of trans-Pt(II) diamminedichloride DNA-protein crosslinks in normal and excision-deficient human cells. Mutat Res, 94, 277-284.

Ganem, N.J., Godinho, S.A. and Pellman, D. (2009) A mechanism linking extra centrosomes to chromosomal instability. Nature, 460, 278-282.

Gossen, J.A., de Leeuw, W.J. and Vijg, J. (1994) LacZ transgenic mouse models: their application in genetic toxicology. Mutat Res, 307, 451-459.

Grafstrom, R.C., Hsu, I.C. and Harris, C.C. (1993) Mutagenicity of formaldehyde in Chinese hamster lung fibroblasts: synergy with ionizing radiation and N-nitroso-N-methylurea. Chem Biol Interact, 86, 41-49.

Graves, R.J., Trueman, P., Jones, S. and Green, T. (1996) DNA sequence analysis of methylene chloride-induced HPRT mutations in Chinese hamster ovary cells: comparison with the mutation spectrum obtained for 1,2-dibromoethane and formaldehyde. Mutagenesis, 11, 229-233.

Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. Cell, 144, 646-674.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B. and Blair, A. (2003) Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries. J Natl Cancer Inst, 95, 1615-1623.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B. and Blair, A. (2004) Mortality from solid cancers among workers in formaldehyde industries. Am J Epidemiol, 159, 1117-1130.

Hayes, R.B., Blair, A., Stewart, P.A., Herrick, R.F. and Mahar, H. (1990) Mortality of U.S. embalmers and funeral directors. Am J Ind Med, 18, 641-652.

He, J.L., Jin, L.F. and Jin, H.Y. (1998) Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. Biomed Environ Sci, 11, 87-92.

Ide, H., Shoulkamy, M.I., Nakano, T., Miyamoto-Matsubara, M. and Salem, A.M. (2010) Repair and biochemical effects of DNA-protein crosslinks. Mutat Res.

Izzotti, A., Cartiglia, C., Taningher, M., De Flora, S. and Balansky, R. (1999) Age-related increases of 8-hydroxy-2'-deoxyguanosine and DNA-protein crosslinks in mouse organs. Mutat Res, 446, 215-223.

Jakab, M.G., Klupp, T., Besenyei, K., Biro, A., Major, J. and Tompa, A. (2010) Formaldehyde-induced chromosomal aberrations and apoptosis in peripheral blood lymphocytes of personnel working in pathology departments. Mutat Res, 698, 11-17.

Kerns, W.D., Pavkov, K.L., Donofrio, D.J., Gralla, E.J. and Swenberg, J.A. (1983) Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res, 43, 4382-4392.

Kuykendall, J.R. and Bogdanffy, M.S. (1992) Efficiency of DNA-histone crosslinking induced by saturated and unsaturated aldehydes in vitro. Mutat Res, 283, 131-136.

Lasko, D., Cavenee, W. and Nordenskjold, M. (1991) Loss of constitutional heterozygosity in human cancer. Annu Rev Genet, 25, 281-314.

Li, G.Y., Lee, H.Y., Shin, H.S., Kim, H.Y., Lim, C.H. and Lee, B.H. (2007) Identification of gene markers for formaldehyde exposure in humans. Environ Health Perspect, 115, 1460-1466.

Liang, L., Mendonca, M.S., Deng, L., Nguyen, S.C., Shao, C. and Tischfield, J.A. (2007) Reduced apoptosis and increased deletion mutations at Aprt locus in vivo in mice exposed to repeated ionizing radiation. Cancer Res, 67, 1910-1917.

Lingle, W.L., Lukasiewicz, K. and Salisbury, J.L. (2005) Deregulation of the centrosome cycle and the origin of chromosomal instability in cancer. Adv Exp Med Biol, 570, 393-421.

Merk, O., Reiser, K. and Speit, G. (2000) Analysis of chromate-induced DNA-protein crosslinks with the comet assay. Mutat Res, 471, 71-80.

Merk, O. and Speit, G. (1998) Significance of formaldehyde-induced DNA-protein crosslinks for mutagenesis. Environ Mol Mutagen, 32, 260-268.

Minko, I.G., Zou, Y. and Lloyd, R.S. (2002) Incision of DNA-protein crosslinks by UvrABC nuclease suggests a potential repair pathway involving nucleotide excision repair. Proc Natl Acad Sci U S A, 99, 1905-1909.

Monticello, T.M., Swenberg, J.A., Gross, E.A., Leininger, J.R., Kimbell, J.S., Seilkop, S., Starr, T.B., Gibson, J.E. and Morgan, K.T. (1996) Correlation of regional and nonlinear formaldehyde-induced nasal cancer with proliferating populations of cells. Cancer Res, 56, 1012-1022.

Nakano, T., Morishita, S., Katafuchi, A., Matsubara, M., Horikawa, Y., Terato, H., Salem, A.M., Izumi, S., Pack, S.P., Makino, K. and Ide, H. (2007) Nucleotide excision repair and homologous recombination systems commit differentially to the repair of DNA-protein crosslinks. Mol Cell, 28, 147-158.

National Toxicology Program. (2005) National Toxicology Program: Formaldehyde (Gas) CAS no. 50-00-0. http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s089form.pdf.

National Toxicology Program. (2010) Final Report on Carcinogens Background Document for Formaldehyde. Rep Carcinog Backgr Doc, i-512.

Nelson, N., Levine, R.J., Albert, R.E., Blair, A.E., Griesemer, R.A., Landrigan, P.J., Stayner, L.T. and Swenberg, J.A. (1986) Contribution of formaldehyde to respiratory cancer. Environ Health Perspect, 70, 23-35.

Neuss, S., Holzmann, K. and Speit, G. (2010) Gene expression changes in primary human nasal epithelial cells exposed to formaldehyde in vitro. Toxicol Lett, 198, 289-295.

O'Connor, P.M. and Fox, B.W. (1989) Isolation and characterization of proteins cross-linked to DNA by the antitumor agent methylene dimethanesulfonate and its hydrolytic product formaldehyde. J Biol Chem, 264, 6391-6397.

Olinski, R., Nackerdien, Z. and Dizdaroglu, M. (1992) DNA-protein cross-linking between thymine and tyrosine in chromatin of gamma-irradiated or H2O2-treated cultured human cells. Arch Biochem Biophys, 297, 139-143.

OSHA. (1995).OccupationalExposuretoFormaldehydeFactSheet.

Ponomareva, O.N., Rose, J.A., Lasarev, M., Rasey, J. and Turker, M.S. (2002) Tissue-specific deletion and discontinuous loss of heterozygosity are signatures for the mutagenic effects of ionizing radiation in solid tissues. Cancer Res, 62, 1518-1523.

Quievryn, G. and Zhitkovich, A. (2000) Loss of DNA-protein crosslinks from formaldehyde-exposed cells occurs through spontaneous hydrolysis and an active repair process linked to proteosome function. Carcinogenesis, 21, 1573-1580.

Rager, J.E., Smeester, L., Jaspers, I., Sexton, K.G. and Fry, R.C. (2010) Epigenetic Changes Induced by Air Toxics: Formaldehyde Exposure Alters miRNA Expression Profiles in Human Lung Cells. Environ Health Perspect, 119, 494-500.

Reardon, J.T., Cheng, Y. and Sancar, A. (2006) Repair of DNA-protein cross-links in mammalian cells. Cell Cycle, 5, 1366-1370.

Ridpath, J.R., Nakamura, A., Tano, K., Luke, A.M., Sonoda, E., Arakawa, H., Buerstedde, J.M., Gillespie, D.A., Sale, J.E., Yamazoe, M., Bishop, D.K., Takata, M., Takeda, S., Watanabe, M., Swen-

berg, J.A. and Nakamura, J. (2007) Cells deficient in the FANC/BRCA pathway are hypersensitive to plasma levels of formaldehyde. Cancer Res, 67, 11117-11122.

Rumchev, K.B., Spickett, J.T., Bulsara, M.K., Phillips, M.R. and Stick, S.M. (2002) Domestic exposure to formaldehyde significantly increases the risk of asthma in young children. Eur Respir J, 20, 403-408.

Schmid, E., Goggelmann, W. and Bauchinger, M. (1986) Formaldehyde-induced cytotoxic, genotoxic and mutagenic response in human lymphocytes and Salmonella typhimurium. Mutagenesis, 1, 427-431.

Shaham, J., Gurvich, R. and Kaufman, Z. (2002) Sister chromatid exchange in pathology staff occupationally exposed to formaldehyde. Mutat Res, 514, 115-123.

Shimada, M. and Komatsu, K. (2009) Emerging connection between centrosome and DNA repair machinery. J Radiat Res (Tokyo), 50, 295-301.

Speit, G., Gelbke, H.P., Pallapies, D. and Morfeld, P. (2010) Occupational exposure to formaldehyde, hematotoxicity and leukemia-specific chromosome changes in cultured myeloid progenitor cells. Cancer Epidemiol Biomarkers Prev, 19, 1882-1884; author reply 1884-1885.

Speit, G. and Merk, O. (2002) Evaluation of mutagenic effects of formaldehyde in vitro: detection of crosslinks and mutations in mouse lymphoma cells. Mutagenesis, 17, 183-187.

Speit, G., Schmid, O., Neuss, S. and Schutz, P. (2008) Genotoxic effects of formaldehyde in the human lung cell line A549 and in primary human nasal epithelial cells. Environ Mol Mutagen, 49, 300-307.

Speit, G., Schutz, P., Hogel, J. and Schmid, O. (2007) Characterization of the genotoxic potential of formaldehyde in V79 cells. Mutagenesis, 22, 387-394.

Speit, G., Schutz, P. and Merk, O. (2000) Induction and repair of formaldehyde-induced DNA-protein crosslinks in repair-deficient human cell lines. Mutagenesis, 15, 85-90.

Storchova, Z. and Kuffer, C. (2008) The consequences of tetraploidy and aneuploidy. J Cell Sci, 121, 3859-3866.

Suruda, A., Schulte, P., Boeniger, M., Hayes, R.B., Livingston, G.K., Steenland, K., Stewart, P., Herrick, R., Douthit, D. and Fingerhut, M.A. (1993) Cytogenetic effects of formaldehyde exposure in students of mortuary science. Cancer Epidemiol Biomarkers Prev, 2, 453-460.

The Commonwealth of Massachusetts. (1986) UFFI Information Sheet.
http://www.mass.gov/Eeohhs2/docs/dph/environmental/sanitation/uff_information_sheet.pdf

Tolstonog, G.V., Mothes, E., Shoeman, R.L. and Traub, P. (2001) Isolation of SDS-stable complexes of the intermediate filament protein vimentin with repetitive, mobile, nuclear matrix attachment

region, and mitochondrial DNA sequence elements from cultured mouse and human fibroblasts. DNA Cell Biol, 20, 531-554.

Turker, M., Walker, K.A., Jennings, C.D., Mellon, I., Yusufji, A. and Urano, M. (1995) Spontaneous and ionizing radiation induced mutations involve large events when selecting for loss of an autosomal locus. Mutat Res, 329, 97-105.

Turker, M.S. (2003) Autosomal mutation in somatic cells of the mouse. Mutagenesis, 18, 1-6.

Turker, M.S., Connolly, L., Dan, C., Lasarev, M., Gauny, S., Kwoh, E. and Kronenberg, A. (2009) Comparison of autosomal mutations in mouse kidney epithelial cells exposed to iron ions in situ or in culture. Radiat Res, 172, 558-566.

Turker, M.S., Gage, B.M., Rose, J.A., Elroy, D., Ponomareva, O.N., Stambrook, P.J. and Tischfield, J.A. (1999) A novel signature mutation for oxidative damage resembles a mutational pattern found commonly in human cancers. Cancer Res, 59, 1837-1839.

Turker, M.S., Pieretti, M. and Kumar, S. (1997) Molecular evidence for the induction of large interstitial deletions on mouse chromosome 8 by ionizing radiation. Mutat Res, 374, 201-208.

Tuthill, R.W. (1984) Woodstoves, formaldehyde, and respiratory disease. Am J Epidemiol, 120, 952-955.

Van Sloun, P.P., Wijnhoven, S.W., Kool, H.J., Slater, R., Weeda, G., van Zeeland, A.A., Lohman, P.H. and Vrieling, H. (1998) Determination of spontaneous loss of heterozygosity mutations in Aprt heterozygous mice. Nucleic Acids Res, 26, 4888-4894.

Wang, W., Xu, J., Xu, L., Yue, B. and Zou, F. (2007) The instability of (GpT)n and (ApC)n microsatellites induced by formaldehyde in Escherichia coli. Mutagenesis, 22, 353-357.

Weaver, B.A. and Cleveland, D.W. (2006) Does aneuploidy cause cancer? Curr Opin Cell Biol, 18, 658-667.

Weckbecker, G. and Cory, J.G. (1987) 2,6-Diaminopurinedeoxyriboside as a prodrug of deoxyguanosine in L1210 cells. Cancer Res, 47, 2218-2223.

Wieslander, G., Norback, D., Bjornsson, E., Janson, C. and Boman, G. (1997) Asthma and the indoor environment: the significance of emission of formaldehyde and volatile organic compounds from newly painted indoor surfaces. Int Arch Occup Environ Health, 69, 115-124.

Ye, X., Yan, W., Xie, H., Zhao, M. and Ying, C. (2005) Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. Mutat Res, 588, 22-27.

Zhang, L., Steinmaus, C., Eastmond, D.A., Xin, X.K. and Smith, M.T. (2009) Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. Mutat Res, 681, 150-168.

Zhang, L., Tang, X., Rothman, N., Vermeulen, R., Ji, Z., Shen, M., Qiu, C., Guo, W., Liu, S., Reiss, B., Freeman, L.B., Ge, Y., Hubbard, A.E., Hua, M., Blair, A., Galvan, N., Ruan, X., Alter, B.P., Xin, K.X., Li, S., Moore, L.E., Kim, S., Xie, Y., Hayes, R.B., Azuma, M., Hauptmann, M., Xiong, J., Stewart, P., Li, L., Rappaport, S.M., Huang, H., Fraumeni, J.F., Jr., Smith, M.T. and Lan, Q. (2010) Occupational exposure to formaldehyde, hematotoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells. Cancer Epidemiol Biomarkers Prev, 19, 80-88.

ELSEVIER LICENSE TERMS AND CONDITIONS April 11, 2011

This is a License Agreement between Yun Xin Lim ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

License number	2646171161544
License date	Apr 11, 2011
Licensed content publisher	Elsevier
Licensed content publication	Mutation Research/Reviews in Mutation Research
Licensed content title	Formaldehyde exposure and leukemia: A new meta-analysis and potential mechanisms
Licensed content author	Luoping Zhang, Craig Steinmaus, David A. Eastmond, Xianjun K. Xin, Martyn T. Smith
Licensed content date	March-June 2009
Licensed content volume number	681
Licensed content issue number	2-3
Number of pages	19
Type of Use	reuse in a thesis/dissertation
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Cellular and Mutagenic Effects of Formaldehyde in the Mammalian Cells
Expected completion date	May 2011
Estimated size (number of pages)	1
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD / 0.0 GBP
Total	0.00 USD