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## Nickel Subsulfide Can Protect Human Fibroblasts from the Mutagenic Effect of Benzo[A]Pyrene Diolepoxide

Samir M. Hamdan

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NICKEL SUBSULFIDE CAN PROTECT HUMAN FIBROBLASTS FROM THE  
MUTAGENIC EFFECT OF BENZO[A]PYRENE DIOLEPOXIDE

by

Samir M. Hamdan

A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Master of Arts  
Department of Chemistry

Western Michigan University  
Kalamazoo, Michigan  
December 1998

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Finally I would like to dedicate this thesis to my family, especially my brother Wael Hamdan, since without his support and encouragement I would never have been able to accomplish this thesis.

Samir M. Hamdan

## NICKEL SUBSULFIDE CAN PROTECT HUMAN FIBROBLASTS FROM THE MUTAGENIC EFFECT OF BENZO[A]PYRENE DIOLEPOXIDE

Samir M. Hamdan, M.A.

Western Michigan University, 1998

Cancer researchers are interested in identifying the environmental causes and synergistic influences of different carcinogens. It is important to examine the combined effect of multiple carcinogens since this is a more realistic representation of the true environmental situation in which we are exposed daily. Our study has reported that pretreating human fibroblasts with nickel subsulfide followed by the simultaneous treatment with both nickel subsulfide and benzo[a]pyrene diolepoxide (BPDE) can protect human fibroblasts from the mutagenic effects of BPDE. These results correlate well with our previous chromium-BPDE co-treatment work, suggesting a transition metal dependent protective effect. This protective effect was dependent on the 46 hours preincubation with nickel subsulfide since the effect was not seen when cells were simply treated for 2 hours with both carcinogens. This antagonistic effect was also shown to be dependent upon the concentration of nickel subsulfide. Addition of vitamin E was able to reverse protective effect suggesting an oxidative stress dependent mechanism. The antagonistic effect on mutant frequency appeared to be species specific since it was not observed when Chinese hamster fibroblasts replaced normal human fibroblasts in the protocol described above.

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## CHAPTER I

### INTRODUCTION

#### Cancer and Carcinogenesis

Cancer is a disease in which the cells grow in an uncontrolled way to invade surrounding tissues and to metastasize to other areas. In molecular terms, cancer is a disease of abnormal gene expression. This altered gene expression can occur through a number of mechanisms including direct insult to DNA resulting in gene mutations, or amplifications, and abnormal gene transcription. These changes might result from external factors (e.g., environmental chemicals, ionizing radiation, ultraviolet radiation, viruses), internal factors (e.g., chromosomal abnormalities, hormonal dysfunction, immune system defects), or other factors including age and diet (Oppenheimer, 1985; and Ruddon, 1995).

For more than 200 years it has been recognized that long-term exposure to certain substances increases the probability of cancer. In fact, it is estimated that 90% of all human cancers are caused by exposure to environmental agents or carcinogens (Oppenheimer, 1985).

Carcinogens are divided into three groups including, direct, indirect and nongenotoxic or epigenetic carcinogens. Direct acting carcinogens are those that have structures permitting them to interact with cellular constituents, including proteins,

lipids, and nucleic acids. Examples of such carcinogens are methyl methane sulfonate, chloromethyl ether, and nitrogen mustard derivatives. Indirect acting carcinogens are stable in the environment, and thus, are more likely to be contacted by the general population, and are often called precarcinogens since they require metabolic activation before they can interact with the cellular components. One of the most important and common example of these type of carcinogen is polycyclic aromatic hydrocarbons. The third type of carcinogen is the nongenotoxic or epigenetic carcinogen which do not appear to bind to DNA, although they may form adducts with other cellular constituents, and have various mechanism of actions. Some examples of this group are hypolipidemic drugs, phthalate ester plasticizers, and trichloroacetic acid (Waalkes, et al., 1994; Arcos, et al., 1995).

Carcinogenesis or transformation is a complex multistep process which involves initiation, promotion, progression and metastasis, and usually takes a long period (20 years or more) to complete. This complex, long term process indicates that cancer does not result from a single molecular change in a cell but requires several distinct alterations, each of which can occur at very different times. Initiation of malignant transformation of normal cells by a carcinogenic agent involves a permanent, heritable change in the gene expression of the transformed cell. Promotion is a clonable expansion period of the initiated cells under the influence of promoting agents that act as a mitogen for the transformed cell type. The progression phase is the stage in which gradual evolution of the genotypically and phenotypically altered cells occurs because of the genetic instability of the progression cells. Metastasis is the last

phase of carcinogenesis that involves the spread of tumor cells to distant sites not directly connected to the original cancer (Mishra, et al., 1981).

Cancer researchers are interested in identifying the environmental causes of cancer and the mechanism by which different carcinogens induce DNA damage, as well as the synergistic influences of different carcinogens, in order to decrease the risk of cancer. Thus understanding of the molecular basis of chemical carcinogens has important implications in the prevention, diagnosis and treatment of human cancer.

### Metal Carcinogenesis

Several inorganic metals have been shown to be carcinogenic in humans and/or animals. Metals which are known as potential carcinogens include nickel, chromium, cadmium, arsenic, cobalt and beryllium (Hartwig, 1995). Humans are exposed to metals in considerable amounts through environmental and occupational exposure, as well as, diet. The mechanisms by which metals induce tumor formation are still unclear, since most metal compounds reveal weak mutagenic responses in mammalian cells (Christie, and Katasifis, 1990; Costa, et al., 1997 a).

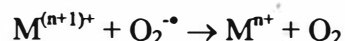
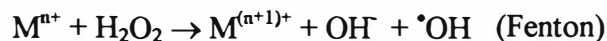
Because carcinogenesis is in itself a complex process, and metals are diverse groups, there is unlikely to be one unified mechanism for metal carcinogenesis. Even within compounds of the same metal, several mechanisms of mutagenicity can occur. This variety of cellular toxicity between different metals and within different compounds of one metal, is due to differences in bioavailability, uptake, and affinity toward amino acids and nucleotides (Hartwig, 1995).

The most important chemical properties of metals, however, are their ability to form complexes and to have more than one oxidation state. One of the general hypotheses of metal carcinogenicity involves a direct interaction between the metal and cellular components. This possibility is due to the abundance of ideal metal partners in DNA such as phosphate anions, and nitrogen and oxygen donor groups. However, the binding of metals cations by the DNA through ionic and coordination bonds is reversible and, as such, cannot produce all the lesions observed in chromatin of cells expose to carcinogenic metal compounds.

On the protein level, the structure and function of several enzymes and proteins depend on bound essential metal (Kasprzak, 1995). Relevant proteins include DNA polymerase (Leonard, 1986), zinc finger proteins (Breg, 1986), and some DNA repair enzymes (Mo, et al., 1992). Metal interactions with the biological molecules might interfere with their biological function and contribute to the metal carcinogenicity. One of the most important interactions between metal and protein is illustrated with the DNA repair machinery. Metals were shown to inhibit DNA repair, and it is believed that the inhibition of the DNA repair by carcinogenic metals might be associated with structural changes of DNA or modification of different repair proteins by competing with essential metals (Hartwig, 1995). Failure to repair genetic damage results in loss of integrity of genetic information, which in turn, can give rise to altered gene expression.

Metals are known to have the ability to carry out an oxidative stress dependent mechanism leading to the generation of reactive oxygen species (ROS). Since damage

in nuclear chromatin caused by carcinogenic metal compounds can be produced by oxygen radicals (Breimer, 1990), another hypothesis is that the ROS produced by the metal leads to oxidative DNA damage. This hypothesis is the most widely discussed and accepted mechanism in metal carcinogenicity. The most important mechanism of oxygen activation by transition metals involves Fenton/Haber Weiss chemistry and autoxidation. The reaction involves the conversion of  $\text{H}_2\text{O}_2$  and superoxide ( $\text{O}_2^{\cdot-}$ ), two common metabolic products (Imlay and Linn, 1988), into a powerful DNA damaging  $\cdot\text{OH}$  radical as described below



The sum of the two reactions is



Metals that can carry out the above reaction in the absence of chelators include Cu(I), Fe(II), Co(II), Ti(III) and Cr(III) ions (Walling, 1975). Other metal ions become reactive only after proper chelation, and in other cases, chelating might deactivate the electron transfer. Transition metal compounds interacting with  $\text{H}_2\text{O}_2$  produce not only free  $\cdot\text{OH}$  radicals, but also other strong oxidants, such as singlet oxygen  $^1\text{O}_2$  (Kawanishi, et al., 1989), and oxo and peroxy species, all capable of damaging DNA and proteins in a site specific manner (Kawanishi and Yamamoto, 1991 a). Some of the damage caused by metal generated oxygen species includes DNA base damage

(Kasprzak, and Hernansez, 1989), and DNA-DNA and DNA-protein cross-linking (Werdrychowski, et al., 1986).

The current study was designed to investigate the potential role of nickel compounds in the carcinogenic process. Nickel is both an environmental toxicant and an essential trace element. Nickel deficiency clearly leads to a variety of effects in several animals, consistent with an important role for this metal ion (Anke, et al., 1984). A major site of action for nickel appears to be the nickel containing enzyme urease, and an additional role for nickel might be through other nickel-containing enzymes such as hydrogenase, carbon monoxide dehydrogenase, and methyl coenzyme reductase (Hausinger, 1993).

All animals, including humans, are constantly exposed to nickel ion and particulate nickel compounds through the food we eat and the air we breathe. The total dietary exposure levels of human is estimated to be 300-600 ug/day (Sunderman, 1977), but the level can vary widely depending on the diet. The amount of nickel that enters the body by inhalation is usually negligible (estimated at 0.16 ug/day) (Neiboer, 1988), however, in certain work places or environmental conditions, inhalation of nickel-containing dust or gaseous nickel compounds can be significant. Epidemiological studies of refinery workers exposed to nickel by inhalation have demonstrated a high incidence of lung, nasal, and pharyngeal cancer (Costa, et al., 1989; Sunderman, 1984). Despite the fact that nickel refinery workers are exposed to other carcinogenic metals, there is a general agreement that insoluble nickel oxides and

sulfides are the principal carcinogens for these workers (Cristie and Katasifis, 1990; Costa, et al., 1997 a).

Insoluble nickel subsulfide has been shown to induce tumors in experimental animals. Water soluble nickel compounds, however, do not induce tumors (Costa, et al. 1997 a; Fletcher, et al., 1994). In cell culture, both crystalline and soluble nickel compounds induce transformation, though soluble compounds must be present at much higher levels (Fletcher, et al., 1994). The carcinogenicity of different nickel compounds is believed to occur via a similar mechanism, but the carcinogenic potencies of these different nickel compounds appear to be related to their bioavailability. Particulate nickel compounds can enter the cell via facultative phagocytosis which deliver large quantities of metal into the cell. The phagocytosized particles enter the lysosomes leading to solubilization of the nickel. Nickel then enters the nucleus via fusion of the vacuoles with the nuclear membrane (Costa, 1991 b). This may explain the high potency of the insoluble nickel subsulfide due to the route of entry into the cell, and its capability of maintaining an elevated concentration of nickel ions in the nucleus over a prolonged period (Costa, 1991 b).

Although the carcinogenic effect of nickel seems to be well documented, the exact mechanism by which nickel causes cellular transformation is not clear since, unlike many heavy metals, nickel ion is relatively innocuous to most tissues unless exposure is at very high level (Costa, 1991 b). Several hypothesis have been postulated in an attempt to explain the mechanism of nickel carcinogenicity. These hypothesis are summarized in Figure (1) and discussed in more detail below.



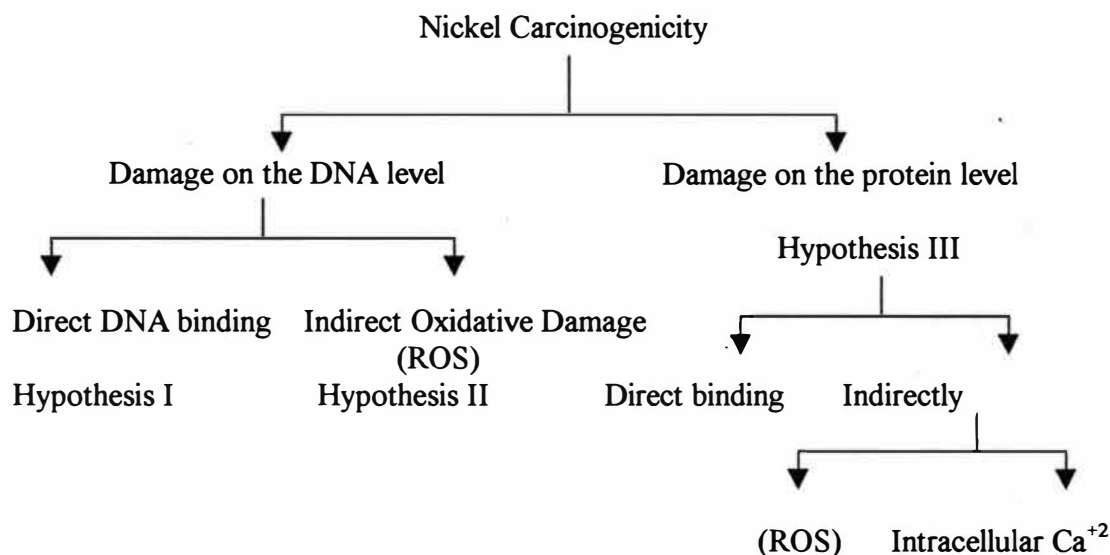


Figure 1. Summary of the Hypothesis of Nickel Carcinogenicity.

One hypothesis, is that nickel can cause direct damage or modification at the DNA level by directly binding to the DNA. The damage caused by nickel binding at the DNA level can cause changes in transcriptional regulation. For example, nickel was been shown to bind to histone proteins resulting in enhanced condensation and DNA methylation (Costa, et al., 1997 b, Costa, et al., 1995 a and Costa, 1995 b). This could be critical to cellular transformation if the methylation takes place in the regulatory region of tumor suppressor genes. Nickel can also cause direct damage at the DNA level via carrying out direct DNA oxidation. Evidence for such direct oxidative damage include the fact that Ni (II) was been shown to be able to cause DNA damage in vitro, in the absence of H<sub>2</sub>O<sub>2</sub>, which indicates that Ni (II) bound to chromatine can activate ambient O<sub>2</sub> (Nakerdien, et al., 1991). Also, it has been shown that nickel-DNA-protein bridging links are dominated by weak bonding

(Wedrychowski, et al., 1986), which is characteristic of direct metal bonds, and not free radicals which are characterized by covalent bonds.

A second hypothesis used to explain nickel damage at the DNA level is that nickel can cause DNA damage indirectly by facilitating the generation of reactive oxygen species through an oxidative stress dependent mechanism. Nickel oxidative effects depend on the electron transfer couple Ni(III)/Ni(II), which in the biological environment is formed only by chelation with certain amino acids, oligopeptides, and/or protein (Kasprzak, 1991 a). This specific chelation may explain the localized nickel oxidative damage in the protein-rich heterochromatin region of the X-chromosome, which suggests the possibility of mediation of nickel (II) complexes and ligands on the selectivity of DNA oxidation with various oxidants.

Hydroxyl radicals ( $\cdot\text{OH}$ ) are one of the generated reactive oxygen species produced by nickel and are believed to be the initiators of the primary indirect events in nickel mutagenicity (Costa, et al., 1994 a). The reactions produced via the Fenton/Harber-Weiss chemistry described above. Some of the other reactive oxygen species generated by nickel through its reaction with oligopeptides are superoxides, singlet oxygen, and nickel oxo and peroxo species (Kawanishi, et al., 1989; Cotelle, et al., 1992). Also nickel sulfur compounds were shown to enhance the generation of sulfur trioxide anion radical and sulfate anion radical in vitro (Kasprzak, et al., 1994) through the autoxidation of sulfite to sulfate. Sulfur trioxide anion radicals have been shown to cause several types of damage to biological molecules including oxidation of methionine (Klebanoff, 1961), and diphosphopyridine nucleotides (Lizada, and Yang,

1981), as well as modification of nucleic acids (Hayatsu, and Miller, 1972). Also sulfate radicals are believed to produce potentially genotoxic effects by deamination of DNA (Kasprzak, et al., 1991 b) and degradation of proteins (Kawanishi, and Ito, 1991 b). The autoxidation of sulfite was shown to enhance the generation of hydroxyl radicals in vitro, as well (Kasprzak, et al., 1994). This sulfite oxidation pathway might explain the potency of nickel subsulfide. Another possibility is the generation of free radicals via the inhibition of the antioxidant cellular defense machinery (Mirsa, et al., 1990). Due to the variability of the effects of nickel on antioxidants in different species, strains and tissues, however, no general conclusions can be drawn about the significance of antioxidant enzyme inhibition by nickel.

The reactive oxygen species generated by nickel, predominantly hydroxyl radicals, can cause several types of DNA damage including DNA base modification through the generation of 8-hydroxyl-2'-deoxyguanosine. This base alteration results primarily in G-C to T-A transversions (Kasprzak, and Hernansez, 1989). Other types of DNA damage caused by reactive oxygen species can include DNA-protein and DNA-DNA cross-linking (Oleinick and Ramakrishnan, 1992; Dizdarglu, 1992) and DNA depurination and strand scission (Dizdarglu, 1992).

A third postulated hypothesis is that nickel induces damage on the protein level. Nickel has been shown to inhibit the DNA repair machinery by inhibiting the polymerization and/or the ligation steps, and failure to repair genetic damage can give rise to altered gene expression (Hartwig, et al. 1998; Lynn, et al. 1997, Kasprzak, et al., 1997; Hartwig, et al. 1992; Hartwig and Beyersmann, 1989). Also, nickel has been

shown to induce an effect at the post translation level by interacting with metabolic enzymes or structural proteins (Holst and Nordlind, 1988). There are many examples of enzymes that are inhibited or altered in their function by reversible binding to nickel, including many kinases, which can lead to important cellular changes (Davis, et al., 1975, Holts and Nordlind, 1988, Sorge, et al., 1993, and Cole, et al, 1997). Recently, nickel has been shown to have an indirect effect on the protein level by sustaining the elevation of free intracellular calcium (Costa, et al, 1998), and such an alteration is critical due to the fact that calcium is involve in regulating several metabolic pathways. It has also been suggested that the reactive oxygen species produced by nickel may react with proteins to alter their function.

### Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are compounds which represent some of the most potent and thoroughly studied carcinogens (Ruddon, 1995). PAH were first recognized as environmental carcinogens when it was discovered in the eighteenth-century by Sir Percival Pott (1718-1788) that there was a high incidence of scrotal cancer among chimney sweeps caused by soot accumulating in their scrotal folds (Francis, B. M., 1994). PAH are particular interesting carcinogens because they are formed during the incomplete combustion of organic compounds (pyrosynthesis), and are found in crispy fried or broiled foods, as well as, in the smoke of incinerators, fireplaces, and cigarettes (Francis, B. M., 1994). PAH by themselves are not a mutagen, but usually require metabolic activation by the cytochrome P-450 system in

order to induce DNA damage in a variety of tissues and species (Errol, et al., 1995). This metabolic activation by the biological system takes place as a defense mechanism to detoxify the organic compound to a more polar, excretable species, as shown in Figure (2), (Errol, et al., 1995), since the accumulation of these organic compounds might interfere with the biological function of cellular membranes.

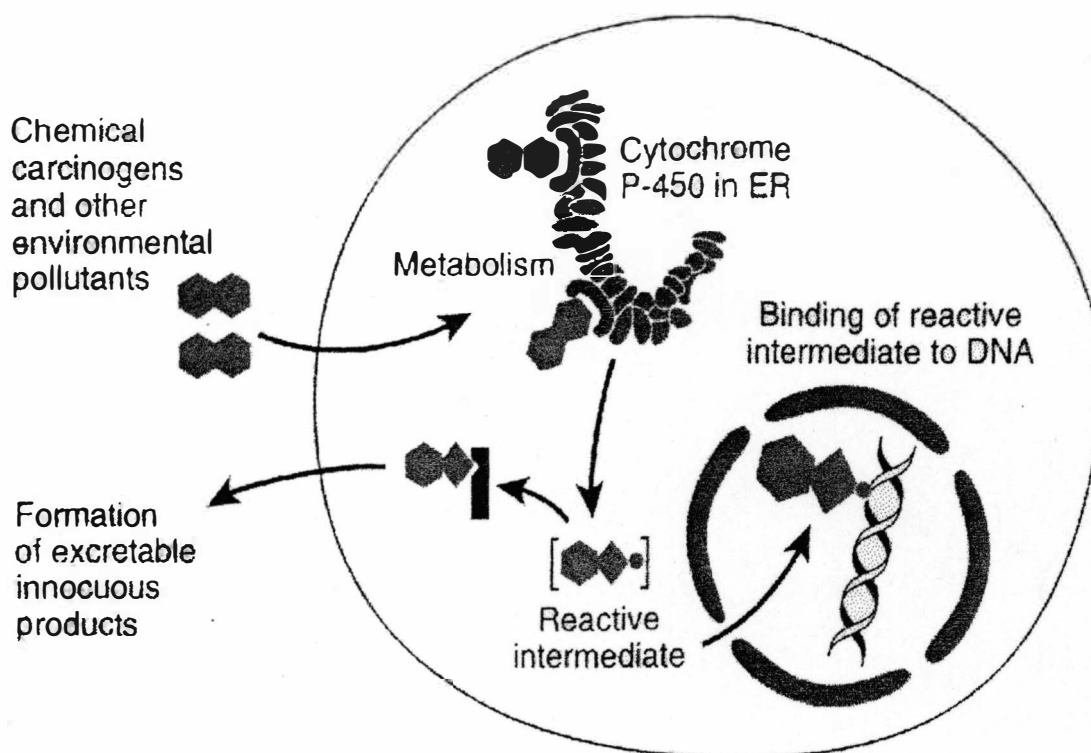


Figure 2. Metabolism of PAH by Cytochrome P-450.

Source. Errol C. Friedberg, Graham C. Walker, and Wolfram siede, (1995). DNA Repair and Mutagenesis. Used with the permission of the pulisher American Society for Microbiology at Washington, DC 20005

The ultimate carcinogenic form of many PAH are the dihydrodiol epoxides. These epoxides are strong electrophilic reactants that combine with nucleophilic sites in target cells including DNA, RNA and protein (Lloyd, et al., 1995).

Benzo[a]pyrene (B[a]P) is one of the most thoroughly studied PAH with potent carcinogenic properties. It is one of the most common carcinogens found in the environment resulting from the incomplete combustion of organic materials, and has been identified in cigarette smoke, atmospheric pollutants, and a variety of broiled food (Francis, B. M., 1994). B[a]P was shown to be carcinogenic in a number of animal systems (Cornney, 1982; and Meehan, et al., 1982). Similar to many other PAH compounds, B[a]P by itself is not a carcinogen, but when taken into the body, gets metabolized by the cytochrome P-450 system to polar compounds including quinones, phenols, and diols. Diols further metabolize to form benzo[a]pyrene diolepoxide (BPDE), (Figure (3)), (Jeffrey, et al., 1977; Roilides, 1988; Anderson, and Lambert, 1990; Zaleski, et al., 1991). BPDE can bind covalently to DNA through its epoxide ring (Cornney, 1982; and Meehan, et al., 1982) to form adducts primarily at the 2-amino group of deoxyguanosine, (Figure (4)), (Jeffrey, et al., 1978; Roilides, 1988), with minor adducts formed at the N<sup>7</sup> position of guanine, N<sup>6</sup> position of adenine, and N<sup>3</sup> position of cytosine (Jeffrey, 1977; Osborne, et al., 1981; and Altaf, et al., 1995).

The DNA-BPDE adduct is repaired via the nucleotide excision repair system which specializes in repairing bulky groups. When the DNA polymerase reaches a BPDE-guanine adduct, during DNA replication, it does not recognize the adduct, and may insert a wrong base in inducing a point mutation (Errol, et al., 1995). When these

adducts are left unrepaired, they can result in many types of mutations including base substitution (Eisenstadt, et al., 1982), frame shifts (Mizusawa, et al., 1981) and deletions (Wei, et al., 1984). Several studies have shown that mutations induced by BPDE are primarily point mutations involving G-C to T-A transversions (Anderson and Lambert, 1990; Roilides, et al, 1988). If these mutations take place in a critical amino acid of a protein, such as a proto-oncogene, they may contribute to the development of a tumor.

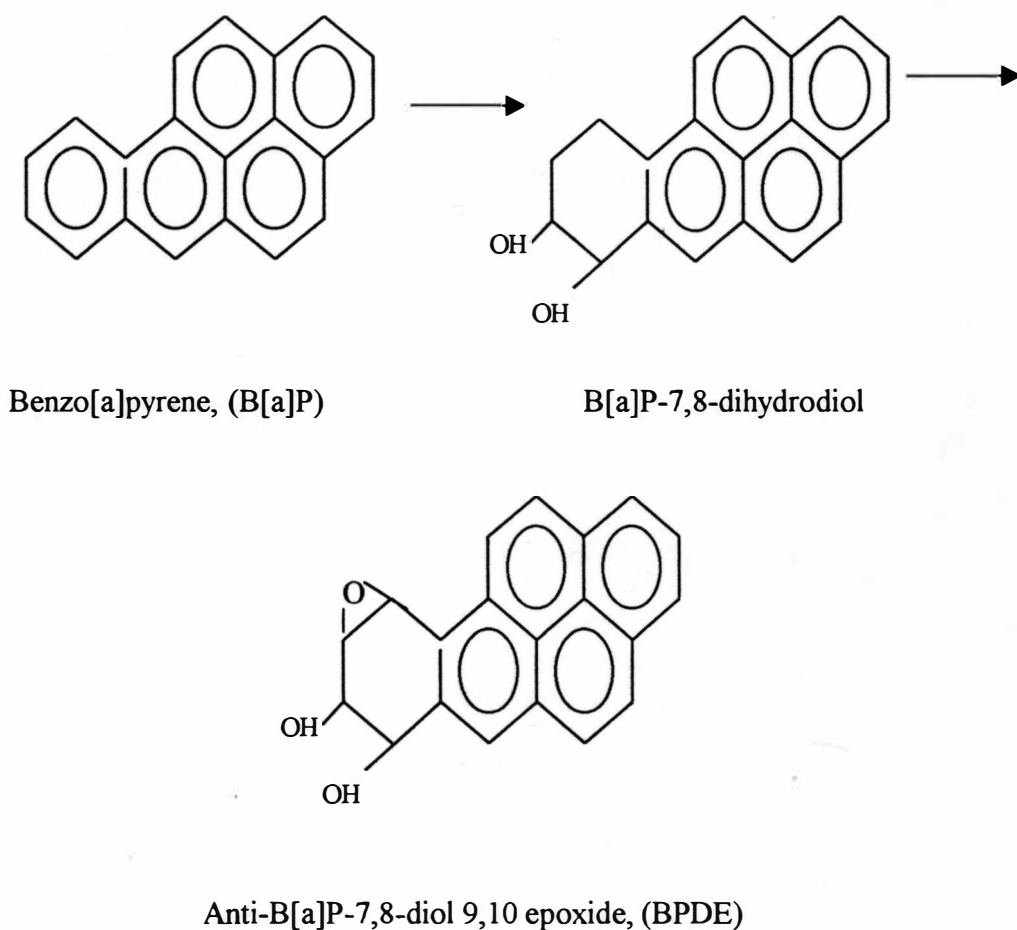
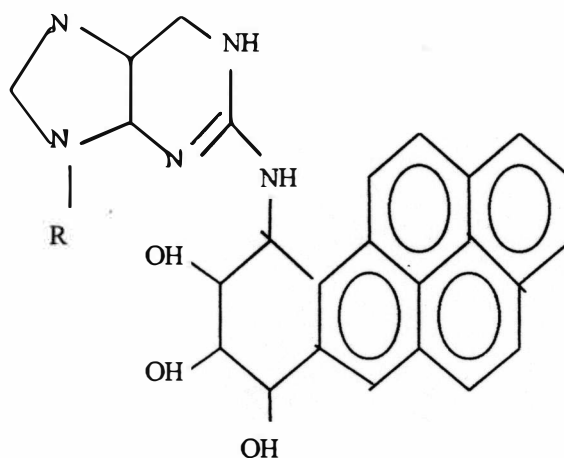


Figure 3. Metabolism of Benzo[a]pyrene to Benzo[a]pyrene Di-epoxide.



BPDE-N-2-deoxyguanosine adduct

Figure 4. BPDE-N-2-deoxyguanosine Adduct.

#### Multiple Carcinogen Treatment and Objective of the Current Study

There have been several studies examining the mechanism by which individual carcinogens transform cells, but there have been relatively fewer studies that examine the cellular effect of multiple carcinogen treatments. Since humans are in an environment in which they are exposed to multiple carcinogens, studies that examine the effect of multiple carcinogen treatments contribute more to our understanding of the transformation process during real environmental conditions. The current study is designed to look at the effects of nickel subsulfide on BPDE induced cytotoxicity and mutant frequency in normal human fibroblasts.

This is not, however, the first study that has examined the effect of metals, particularly nickel compounds, and organic compounds in mammalian systems. Nickel-



B[a]P co-treatment is of particular interest since some data in literature indicates that cigarette smoking potentiates the carcinogenic effect of nickel in lung cancer (McEvan, 1976; and Kreyberg, 1978). Sunderman et al. (Sunderman, et al., 1971) have shown that the interval between carcinogen administration and development of sarcomas was significantly shorter in male Fisher rats that received injections of benzpyrene and nickel than those receiving only one of the carcinogens. Sanner and Rivedal (Sanner, and Rivedal, 1980) have shown a synergistic effect on ouabain resistance when nickel and B[a]P were combined together. The same authors (Sanner and Rivedal, 1981) have also shown a similar synergistic effect using a cellular transformation assay in which nickel, chromium, or cadmium was combined with B[a]P. Uziel et al. (Uziel, et al., 1986) have also reported a synergistic effect when the combined treatment of nickel with B[a]P was examined using a nucleoside excretion assay. Using a cellular transformation assay, Christie (Christie, 1989) has reported a synergistic response between nickel and B[a]P when cells were post or pre-treated with nickel.

Synergistic and additive effects have also been observed when nickel co-treatment has been used with carcinogens other than benzo[a]pyrene. Hartwig and Beyersmann (Hartwig and Beyersmann, 1989) have shown, using the HGPRT mutation assay, that combined nickel and ultraviolet light (UV) treatment produces synergistic effect. Christie (Christie, 1989) has shown similar synergistic effects with nickel combined with UV and X-ray co-treatment.

Many of the reported synergistic and additive effects were specific not a general phenomena. For example, Sanner and Rivedal (Sanner and Rivedal, 1980), who showed a synergistic effect when cells were treated with nickel and B[a]P, observed no synergistic effect when nickel treatment was combined with methylcholanthrene. Also, Hartwig and Beyersmann (Hartwig and Beyersmann, 1989), who reported synergistic effects of UV-nickel treatment, reported no synergistic influences when the methylating agent methyl methanesulfonate (MMS) was used in place of UV light. The above mentioned studies all used rodent cell lines, but three studies have been reported using human cell lines. Christie et al. (Christie, et al., 1996), using sister chromatid exchange, have shown, that treating human lymphocytes with nickel and UV, chromium or X-ray has less than additive or antagonistic effect. Au, et al., (Au, et al., 1994), using chromosomal aberration and sister chromatid exchange, have shown that deletion type chromosomal aberration is unchanged after exposing human lymphocytes to different combination of nickel and  $\gamma$ -ray. Tesfai, et al., (Tesfai, et al., 1998) using a human fibroblasts cell line and selecting for HGPRT mutants, have shown an antagonistic effect with nickel and BPDE treatment that depends on the order of treatment.

The current study is an extension of the work done in our lab (Tesfai, et al., 1998), which showed that pretreatment with potassium dichromate can protect normal human fibroblasts from the mutagenic effects of BPDE. As a result, this study was designed to investigate the following objectives:

1. Can nickel subsulfide produce the same protective effect?

2. Is the pretreatment with nickel subsulfide important to see the protective effect?
3. Does the protective effect depend on the concentration of nickel subsulfide?
4. Can we resolve the differences between previously reported results and ours?
5. Does the protective effect involve an oxidative stress dependent mechanism?

## CHAPTER II

### MATERIALS AND EXPERIMENTAL METHODS

#### Cells and Cell Culture

A normal human fibroblast cell line (L266-C) derived from human foreskin tissue, supplied by Dr. Justin McCormick at Michigan State University, as well as, a Chinese hamster lung fibroblast (V79-4) cell line obtained from American Type Culture Collection, were used in this study. Eagle's Minimum Essential Media (DMEM) supplemented with 1.0 mM sodium pyruvate, 0.2 mM serine, 0.2 mM aspartate, 10 % supplemented calf serum (Summit Biotechnology), penicillin (100 units/ml), streptomycin (100 µg/ml) and hydrocortizone (10 µg/ml) was used as a complete medium for cell growth. Cells were grown in humidified incubator maintained at 37°C and 5.0% CO<sub>2</sub>.

#### Cell Harvesting and Counting

Cells were washed with phosphate buffered saline (PBS), followed by treatment with a 0.25% trypsin solution for 3-4 minutes, and harvested using 10 ml of complete medium. An aliquot of the cell suspension was counted using a hemocytometer. The cells counts were used for plating dishes at the appropriate cell density.

## Test Chemicals

Nickel subsulfide ( $\text{Ni}_3\text{S}_2$ ) was purchased from Aldrich Chemical Company. Nickel subsulfide was ground and dissolved in acetone, then it was filtered through a  $5.0\ \mu\text{m}$  polycarbonate membrane filter and left overnight to dry. The filtered, nickel subsulfide was weighed and dissolved in acetone to give 10 mM stock solution. The stock solutions were diluted with the complete medium to the desired final concentration shortly before being used. BPDE was purchased from ChemSyn Science Laboratories and dissolved in DMSO just prior to use to give a 1.0 mM stock solution. The stock solution was diluted with the complete medium to the desired final concentration. The final concentration of the DMSO used was consistently less than 0.20%. 6-Thioguanine was purchased from Sigma Chemical Company and dissolved in 1.0 N NaOH to give 40 mM stock solution. The stock solution was diluted with the complete medium to give a final concentration of  $30\ \mu\text{M}$  shortly before being used. Following 6-thioguanine addition, the medium was filtered through a  $0.2\ \mu\text{m}$  cellulose membrane filter.

## Nickel and BPDE Treatment and Analysis

The experimental procedure was divided into two steps. First, the cloning ability of cells was measured by the ability of the cells to form colonies. Second, the mutant frequency was measured by the number of hypoxanthine guanine phosphoribosyltransferase (HGPRT) mutants per  $10^6$  clonable cells.

Four treatment conditions were used as listed here: (1) control; no treatment; (2) nickel subsulfide treated; (3) BPDE treated; (4) combination, nickel subsulfide and BPDE treated. Solvents being used to dissolve nickel (acetone) and BPDE (dimethyl sulfoxide) were added as controls to the appropriate treatment condition.

Initially, three hundred cells per 100 mm dish were plated for measuring the cloning ability, and 500,000-1000,000 cells were plated per 150 mm dish for measuring the mutant frequency. In measuring the cloning ability, the 100 mm tissue culture plates were used in order to prevent clones from overlapping. The 150 mm tissue culture plates were used in measuring the mutant frequency so that the more mutant number of cells could be treated in one dish. Cells were then placed in the humidified incubator overnight to allow for attachment to the dishes. Each of the four conditions had three 100 mm dishes for the cloning ability assay and one 150 mm dish for the mutant frequency determination. Following cell attachment, complete medium containing the desired final concentration of nickel subsulfide was prepared. Medium was aspirated off the dishes and replaced by the medium containing the desired final concentration of the metal. Cells were incubated for 46 hours before medium was changed to that containing the appropriate concentrations of nickel subsulfide and/or BPDE was added to the medium immediately, 1-2 minutes, before the treatment. The concentration of BPDE used throughout the experiment was  $0.3\mu\text{M}$  for the human fibroblast cell line and  $1.2\mu\text{M}$  for the Chinese hamster fibroblast cell line, while the concentration of nickel subsulfide used varied depending on the experimental condition. After the required time of treatment, the media was aspirated off, cells were

washed with (PBS), and supplemented with a freshly prepared complete medium. The 150 mm dishes received fresh medium every 3-4 days, and the cells were splitted once they neared confluence. The 100 mm dishes received a medium change every 6-7 days. The 100 mm dishes used to determine the cloning ability were stained with crystal violet, and clones counted after 13-18 days in the case of human fibroblasts. In the case of Chinese hamster fibroblasts, cloning dishes were stained after 5-8 days. The crystal violet used for staining contained 5% methanol to fix the cells. The relative cloning ability was calculated as the average number of colonies formed as a percentage of colonies formed in the control. The control was defined as 100% cloning ability.

$$\% \text{ Relative Cloning Ability} = \frac{\text{Average \# of colonies in treated dish}}{\text{Average \# of colonies in control dish}} \times 100\%$$

Cells in the 150 mm dishes were grown for 10-14 days to allow expression of mutants, and to obtain sufficient cells to start the second part of the experiment in which the mutant frequency of the nickel and BPDE treated cells was measured. The cells were then trypsinized and 200,000 cells per 100 mm dish were replated in complete medium containing 30.0  $\mu\text{M}$  6-thioguanine to select for HGPRT mutants. The supplemental calf bovine serum was replaced by fetal bovine serum in this selection medium. Ten dishes were used per condition and were referred to as selection dishes. To correct for the ability of the cells in each condition to clone, 300 cells were replated on 100 mm culture dishes in triplicate for each condition, and these dishes were referred to as cloning dishes. Again fetal bovine serum was used instead of

calf bovine serum in preparing the complete medium. The medium of the cloning dishes did not contain 6-thioguanine. Medium for the selection dishes that contain 6-thioguanine was changed every 4-5 days while medium over the cloning dishes that didn't contain 6-thioguanine was changed every 6-7 days. Cloning efficiency dishes for human fibroblasts were stained with crystal violet and clones counted after 13-15 days, while in case of Chinese hamster fibroblasts, the cells were stained after 5-8 days. The cloning efficiency was calculated as following

$$\text{Cloning Efficiency} = \frac{\text{Average \# of colonies from cloning dishes}}{300 (\text{\# of plated cells})}$$

Cells in the selection dishes were stained with crystal violet and 6-thioguanine resistant mutant colonies were counted after 24-26 days in the case of human fibroblasts, while they were stained after 8-12 days in case of Chinese hamster fibroblasts. The induced mutant frequency was expressed as the number of HGPRT mutants per  $10^6$  viable cells, and calculated as shown below.

$$\text{Mutants}/10^6 \text{ viable cells} = \frac{\text{Total \# of colonies in selection dishes}}{\text{Total \# of cells plated in selection dishes}} \times \frac{10^6}{\text{cloning efficiency}}$$

### Vitamin E Treatment

Vitamin E,  $\alpha$ -tocopherol (Sigma, St. Louis, MO), was dissolved in DMSO to give a 0.20 M stock solution. The stock solution was then diluted with complete medium to give a 500.0  $\mu$ M final solution concentration. Vitamin E stock solutions were prepared immediately before use and diluted with the complete medium 1-2



minute before the treatment. Cells were treated as described in the previous section with 500.0  $\mu$ M vitamin E added to medium. Controls were performed in parallel which were not treated with vitamin E. Cells were then assayed for the relative cloning ability and the HGPRT mutant frequency as described above.

### Statistical Analysis

In calculating the relative cloning ability, the numbers expressed in the tables represent the average number of clones in the three tissue culture dishes for each condition. The standard deviation of the three tissue culture dishes is also given for each condition. The effect of nickel on BPDE induced cytotoxicity is considered significant if the difference between treatment conditions is greater than 2 standard deviations. The effect of nickel on BPDE induced mutagenicity is considered significant if the difference between the two experiments is less than one half the reduction in the mutant frequency.

## CHAPTER III

### RESULTS

#### The Effect of BPDE on Cloning Ability and Mutant Frequency in a Human Fibroblasts Cell Line

The purpose of this experiment was to choose the appropriate BPDE concentration that would have a significant effect on the cloning ability and an acceptable mutant frequency. To accomplish this, cells were treated for 2 hours with BPDE. A concentration range between 0.00  $\mu\text{M}$  and 0.45  $\mu\text{M}$  was used. Figure (5) shows the effect of BPDE on cloning ability of normal human fibroblasts. The control was defined as 100%, and the % relative cloning ability for the treated conditions were normalized relative to the control. Figure (5) shows that the effect of BPDE on the ability of human fibroblasts to clone was concentration dependent. Figure (6) shows the effect of BPDE on the HGPRT gene mutant frequency. The number of mutants/ $10^6$  clonable cells was calculated as described in Materials and Methods. Figure (6) shows that the effect of BPDE on the mutant frequency of human fibroblasts was concentration dependent as well. Figures (5) and (6) show that as the % relative cloning ability decreases, the mutant frequency increases. This data is consistent with the data in literature (McCormick, et al., 1982). The concentration of BPDE that we decided to use was 0.3  $\mu\text{M}$  because this concentration was moderately toxic and it gave a good number of mutants.

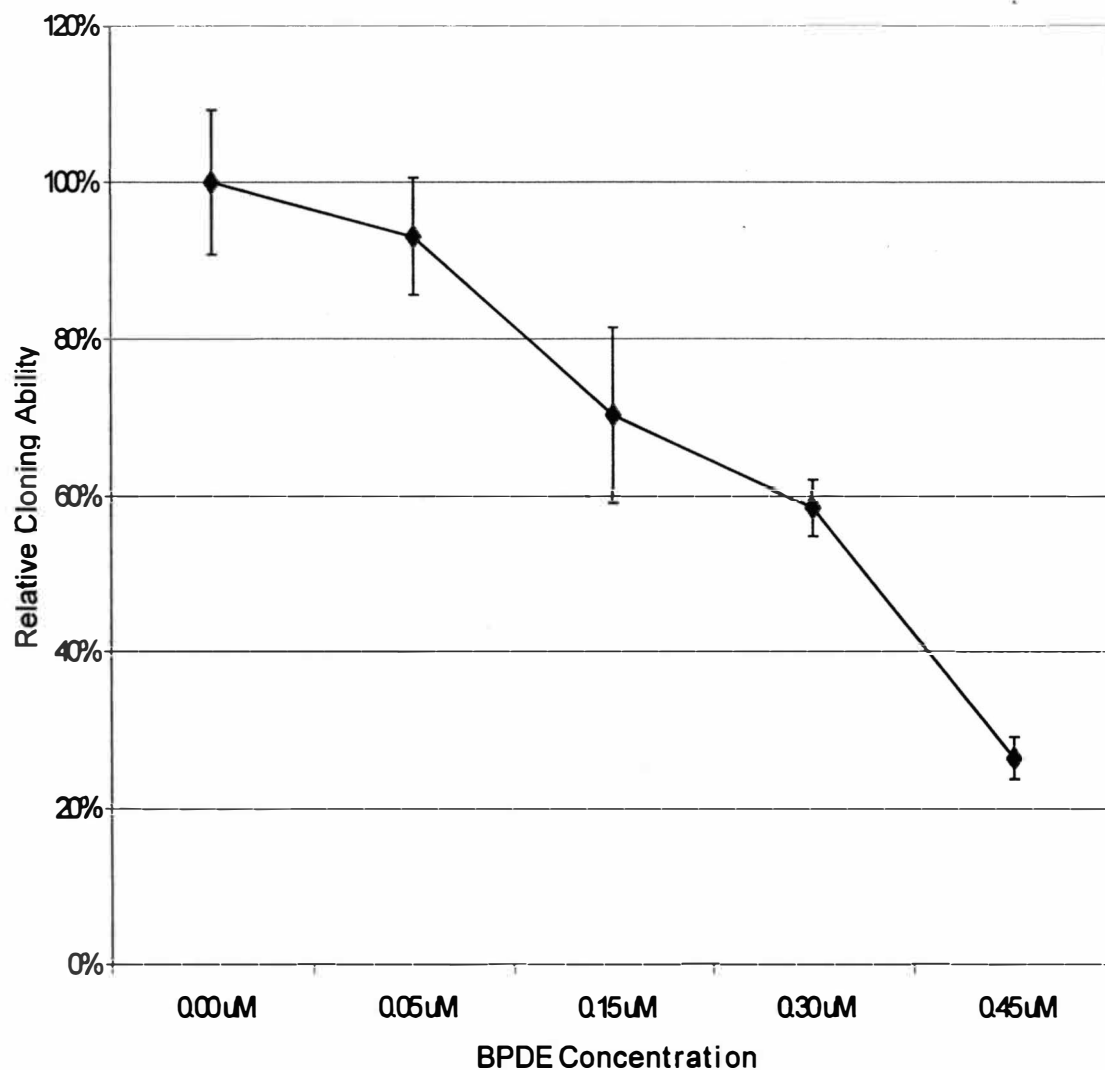


Figure 5. Effect of BPDE on Cloning Ability on Normal Human Fibroblasts. Cells were treated with concentrations of BPDE ranging from 0.00 uM-0.45 uM. The control (no addition) was defined as 100%, and the % of relative cloning ability for the treated conditions was normalized to the control. One experiment was performed and each concentration treatment had 3 replicate dishes. Error bars represent the standard deviation.

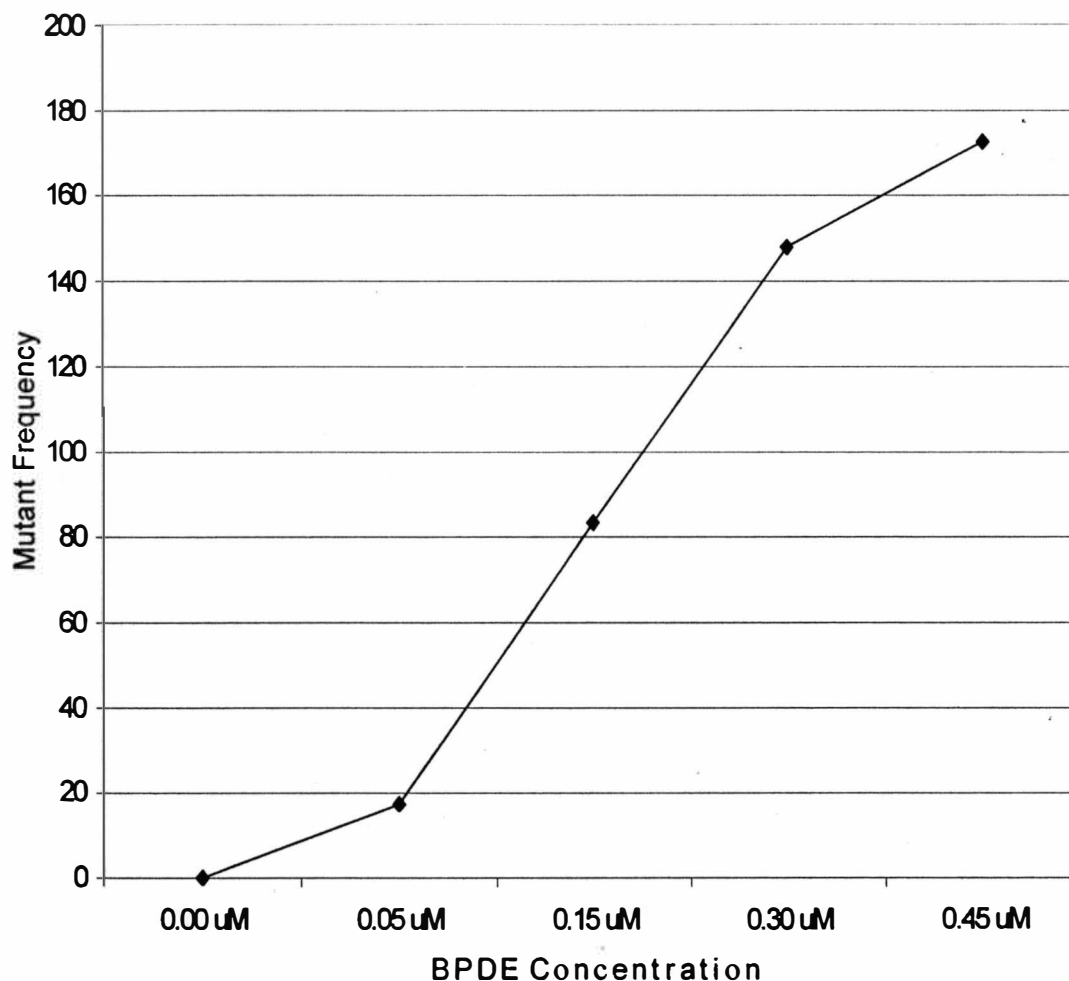


Figure 6. Effect of BPDE on the Mutant Frequency. Cells were treated with different BPDE concentrations ranging from 0.00 uM-0.45 uM. The mutant frequency was measured as the number of mutants in the HGPRT gene per one million viable cells. One experiment was performed and each concentration treatment had 10 dishes. The results of the 10 dishes were pooled to obtain the data shown.

#### The Effect of Nickel Treatment on the BPDE Stimulated Decreases in Cloning Ability and Mutagenicity in Human Fibroblasts

The purpose of this experiment was to determine if the treatment with nickel was capable of protecting human fibroblasts from the mutagenic effect of BPDE, as

was shown with chromium, previously in our lab (Tesfai, et al., 1998). We showed that pretreating human fibroblasts with chromium for 46 hours, followed by the simultaneous treatment with both chromium and BPDE for two hours, had an additive effect on BPDE induced cytotoxicity, but was able to protect normal human fibroblasts from the mutagenic effects of BPDE. Using the same order of treatment that is described in more detail in Figure (7), the effect of nickel on BPDE stimulated decreases in cloning ability and BPDE induced mutagenicity was studied. The nickel subsulfide concentration used in this experiment was 25  $\mu$ M which is based on a cytotoxicity curve carried out by a previous worker in our lab. Table (1) shows that the effect of nickel treatment on BPDE stimulated decreases in cloning ability was additive. Fewer cells were able to clone in the combined treatment than when each carcinogen was used independently. The effect of nickel pretreatment on the BPDE induced mutant frequency, however, was antagonistic. No mutants were observed in the combined treatment. Thus, treatment with nickel subsulfide can protect human fibroblasts from the mutagenic effect of BPDE. These results correlate well with those published in the case of chromium-BPDE co-treatment (Tesfai, et al., 1998), suggesting that this protective effect is not specific for chromium but it might be a general transition metal phenomenon.

#### The Effect of Different Nickel Concentrations on the BPDE Stimulated Decreases in Cloning Ability and BPDE Induced Mutagenicity

The purpose of this experiment was to investigate if the protective effect that

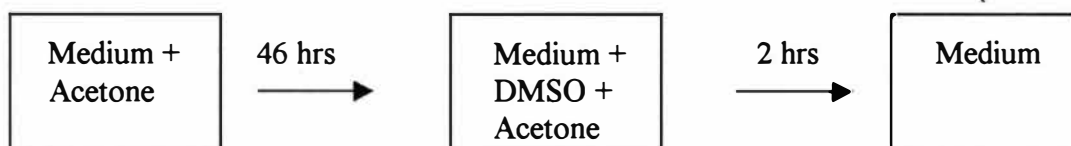
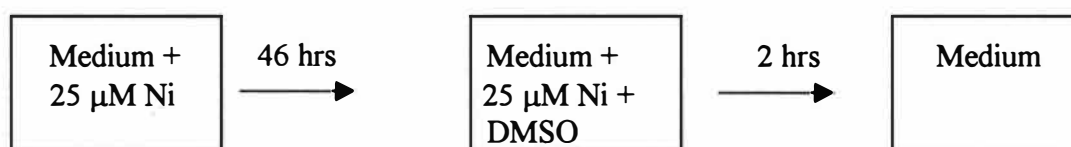
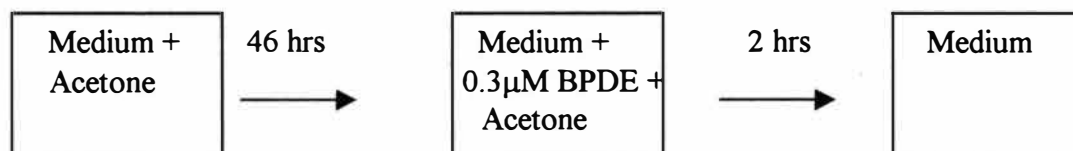
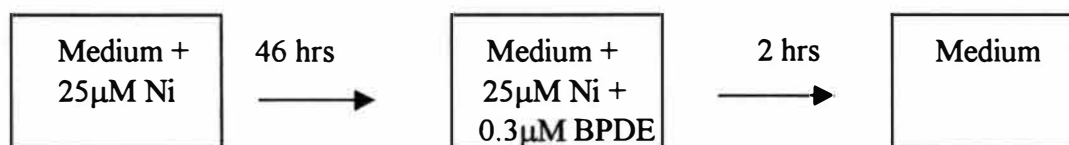
**Control****Nickel****BPDE****Combination**

Figure 7. Nickel Subsulfide and BPDE Treatment Protocol. This figure demonstrates the four treatment conditions, and the medium that was added to each condition over the entire 48 hours period of treatment.

Table 1

Effect of Nickel Treatment on the BPDE Stimulated Decreases in Cloning Ability and BPDE Induced Mutagenicity

Treatment	<u>Cloning Ability</u>		<u>Mutants/10<sup>6</sup> Clonable Cells</u>	
	Exp. #1	Exp. #2	Exp. #1	Exp. #2
Control	72.7±1.5(100)	72±3 (100)	0 (0)	0 (0)
Nickel	12.0±2.6 (16.5)	7.3±1.2(10.6)	5.2 (1)	7.5 (1)
BPDE	36.7±6.7(50.4)	38±3.5 (52.8)	92.1(35)	40.7(11)
Both	2.0 ± 1 (2.7)	2.0±1 (2.8)	0 (0)	0 (0)

**Legend.** The cells were treated as described in Figure (7). The number of colonies ± the standard deviation for triplicate dishes is given for the cloning ability. The % relative cloning ability is given in parenthesis in which the control was defined as 100%, and the % relative cloning ability of the treated condition was normalized to the control. The mutagenicity is given as the number of HGPRT mutants per one million viable cells, with the actual number of mutants observed in 10 dishes given in parenthesis.

was observed using 25 uM nickel subsulfide is concentration dependent. To achieve this purpose, the nickel subsulfide concentration was decreased to 5.0 uM and further to 1.0 uM, using the same order of treatment described in Figure (7). Tables (2) and (3) show the effect of these nickel concentrations on BPDE stimulated decreases in the cloning ability and the BPDE mutagenicity. Table (2) shows that decreasing the concentration of nickel subsulfide to 5.0 uM resulted in a decrease in the effect on the

Table 2

Effect of 5.0  $\mu$ M Nickel Subsulfide Treatment on the BPDE Stimulated Decreases in Cloning Ability and BPDE Induced Mutagenicity

Treatment	<u>Cloning Ability</u>		<u>Mutants/<math>10^6</math> Clonable Cells</u>	
	Exp. #1	Exp. #2	Exp. #1	Exp. # 2
Control	45 $\pm$ 2 (100)	118.7 $\pm$ 17.5(100)	0 (0)	0 (0)
Nickel	22.3 $\pm$ 2 (51.8)	55 $\pm$ 8.7 (46.3)	0 (0)	0 (0)
BPDE	17 $\pm$ 2.6 (37.8)	61 $\pm$ 5.2 (51.4)	79.5(14)	73.8(18)
Both	10.7 $\pm$ 0.6(23.7)	28.3 $\pm$ 3.8 (25.3)	35.7(3)	38.2(10)

Legend. The cells were treated as described in Figure (7) except the fact that 5.0  $\mu$ M nickel subsulfide was used in place of 25  $\mu$ M. Data is express as described in Table (1)

cloning ability by nickel alone. The additive effect with BPDE, however, was still observed. Table (3) shows that decreasing nickel concentration further to 1.0  $\mu$ M results in no significant effect on cloning ability either by nickel or in combination with BPDE. Table (2) shows also that decreasing nickel subsulfide concentration to 5.0  $\mu$ M instead of 25  $\mu$ M resulted in a decrease in the protective effect with regard to mutant frequency by almost 50%. Table (3) shows that decreasing nickel concentration further to 1.0  $\mu$ M leads to almost no protective effect. Both Tables (2) and (3) suggest that the effect of nickel treatment on BPDE induced decreases in cloning ability and BPDE



induced mutagenicity is nickel concentration dependent. The results of the mutant frequency experiments are summarized in Figure (8).

Table 3

Effect of 1.0  $\mu$ M Nickel Subsulfide Treatment on the BPDE Stimulated Decreases in Cloning Ability and BPDE Induced Mutagenicity

Treatment	<u>Cloning Ability</u>		<u>Mutants/10<sup>6</sup> Clonable Cells</u>	
	Exp. #1	Exp. #2	Exp. #1	Exp. #2
Control	125 $\pm$ 12.2(100)	119.7 $\pm$ 4 (100)	0 (0)	0 (0)
Nickel	116 $\pm$ 7.9 (92.8)	111 $\pm$ 23.4(92.7)	0 (0)	0 (0)
BPDE	74 $\pm$ 8 (59.2)	69.7 $\pm$ 7.5 (58.2)	89.6(19)	57.7(13)
Both	72.7 $\pm$ 4.6 (58.1)	69.3 $\pm$ 6.5 (57.9)	70 (14)	45 (9)

Legend. The cells were treated as described in Figure (7) except that 1.0  $\mu$ M nickel subsulfide was used in place of 25  $\mu$ M. Data is express as described in Table (1).

#### The Importance of the Pretreatment With Nickel on the BPDE Stimulated Decreases in Cloning Ability and BPDE Induced Mutagenicity

The previous data shown in Tables (1), (2), and (3) suggest that nickel somehow protects cells from the mutagenic effect of BPDE. To investigate the importance of the pretreatment with nickel on this protective effect, an experiment was designed in which the cells were not pretreated with nickel for 46 hours, but were treated with both nickel and BPDE simultaneously for 2 hours. Table (4) shows that

when the nickel pretreatment was eliminated, the effect on BPDE stimulated decrease in cloning ability was similar as in the previous experiments, while the protective effect on BPDE induced mutagenicity was not observed. These results suggest that the pretreatment with nickel is necessary to see the protective effect.

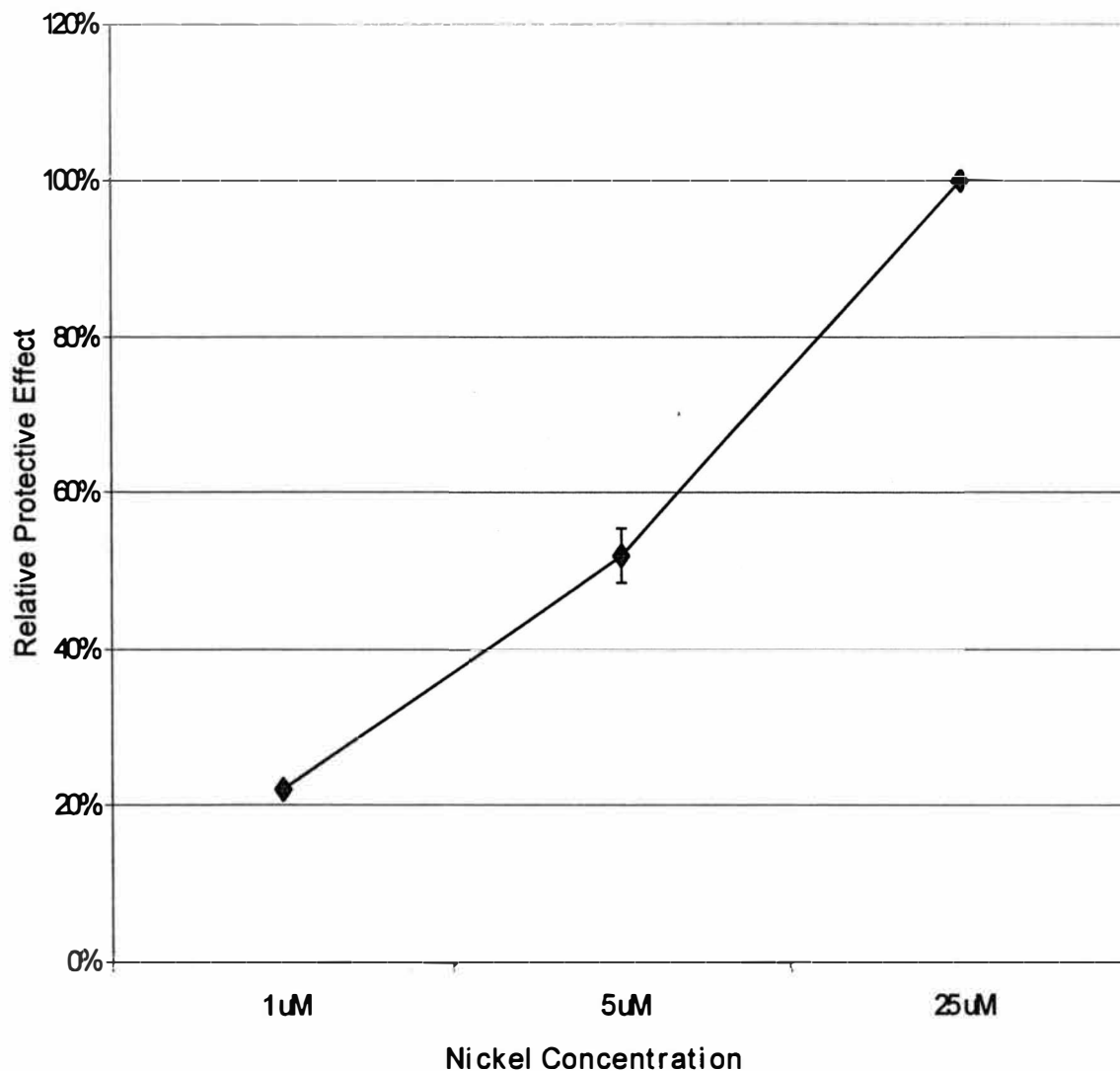


Figure 8. Summary of the Effect of Nickel Concentration on the Protective Effect of BPDE Induced Mutagenicity.

Table 4

The Importance of the Pretreatment With Nickel on the BPDE Stimulated  
Decreases in Cloning Ability and BPDE Induced Mutagenicity

Treatment	<u>Cloning Ability</u>		<u>Mutants/10<sup>6</sup> Clonable Cells</u>	
	Exp. #1	Exp. #2	Exp. #1	Exp. # 2
Control	82.7±9.6 (100)	74.7±2.1(100)	0 (0)	0 (0)
Nickel	13.7±4.2 (16.7)	10.7±3.8(14.3)	3.3 (1)	6.5 (1)
BPDE	43.2±17.7(52.6)	27±3.6(36.1)	43.1(10)	40.6(13)
Both	2±2.0 (2.4)	5.3±2.0(7.1)	119 (15)	64 (9)

**Legend.** The cells were treated as described in Figure (7), except that there was no pretreatment with nickel for 46 hours. The data is presented as described in Table (1).

The Effect of Nickel on BPDE Stimulated Decreases in Cloning Ability  
and BPDE Induced Mutagenicity in Chinese Hamster Fibroblasts

This study is not the first study to examine the effect of multiple carcinogen treatment. Several co-treatment experiments have been done using nickel compounds and B(a)P (Sunderman, et al., 1971; Sunner and Rivedal, 1980; Sunner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989). In those experiments, the effect of nickel on B(a)P induced mutagenicity was shown to be additive or synergistic, which is different from the data seen in our study. Our data, however, correlates well with the previously published data from our lab in which chromium was used instead

of nickel (Tesfai, et al., 1998). There are three major differences between our study and those of previous studies (Sunderman, et al., 1971; Sunner and Rivedal, 1980; Sunner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989). First, we used BPDE, the most potent metabolite form of B(a)P, while the previous studies used B(a)P. Second, a normal human fibroblast cell line is used in this study, while in the previous studies, rodent cell lines were used. Finally, the order of treatment used in our study was not done in the previously reported studies.

To investigate if using BPDE instead of B[a]P was responsible for the differences in observations, we planned an experiment in which B[a]P was used instead of BPDE. Prior to doing the experiment, however, we needed to find a B[a]P concentration which would produce about 50% reduction in cloning ability. Thus a dose-response experiment was done to correlate B[a]P concentration with reduction in cloning ability. Figure (9) shows the cytotoxic effect of B[a]P on human fibroblasts in which cells were treated with B[a]P concentrations ranging between 1.0  $\mu$ M and 500  $\mu$ M for two hours. This data shows that human fibroblasts cannot metabolize B[a]P since B[a]P did not decrease the cloning ability even at elevated concentrations that are orders of magnitude higher than the BPDE concentration that we used. As a result of this, the experiment using B[a]P instead of BPDE could not be performed.

To investigate if our observed protective effect was species specific, an experiment was designed in which Chinese hamster fibroblasts were used instead of human fibroblasts, and the same order of treatment described in Figure (7) was used. The nickel subsulfide concentration used was 25  $\mu$ M, and the BPDE concentration

was 1.2  $\mu\text{M}$ . Those concentrations were chosen based on cloning ability dose-response curves for both nickel and BPDE on Chinese hamster fibroblasts. Table (5)

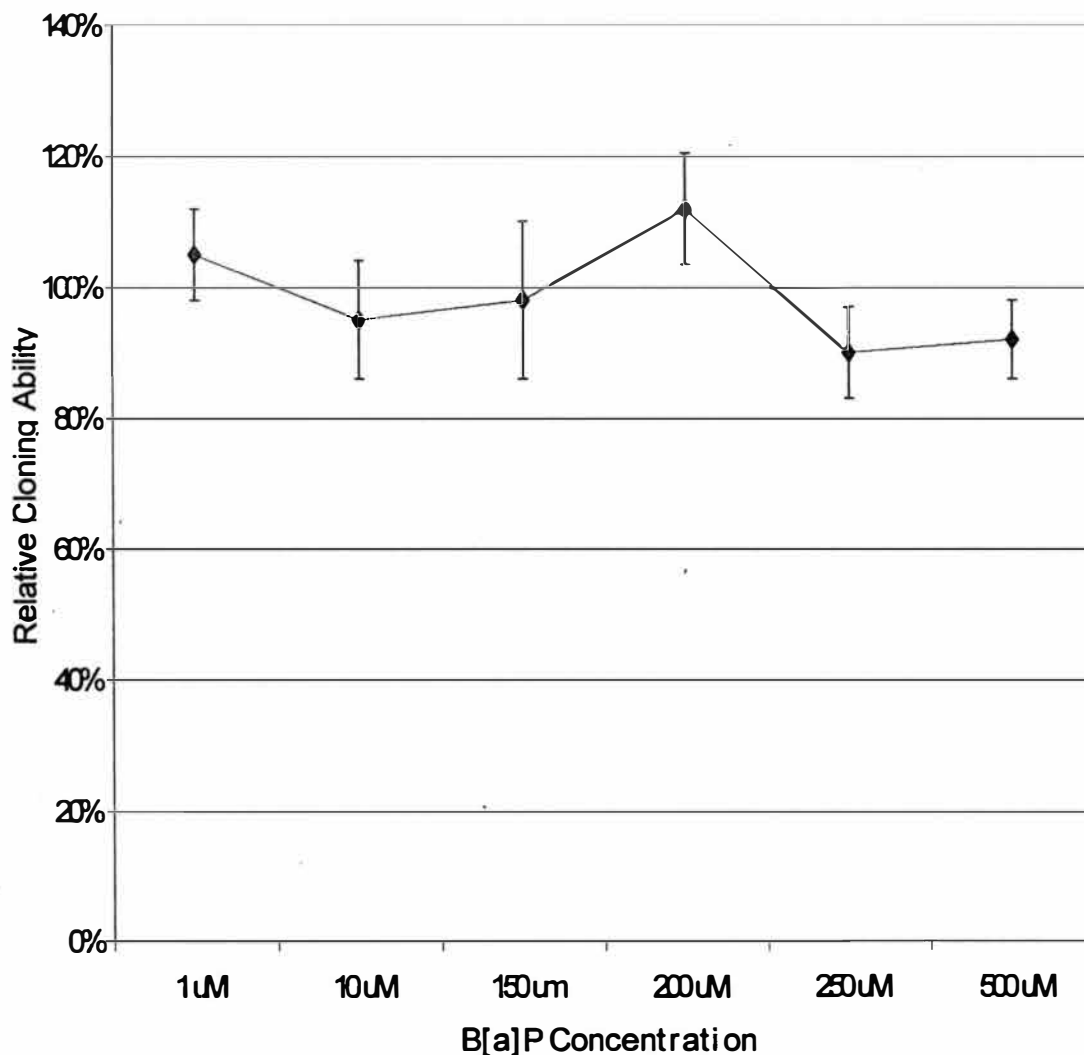


Figure 9. B[a]P Induced Reduction in Cloning Ability. Cells were treated with B[a]P ranging from 0.00  $\mu\text{M}$ -500  $\mu\text{M}$ , in which the control (no addition) was defined as 100%, and the % of relative cloning ability for the treated condition was normalized to the control. One experiment was performed and each concentration treatment had 3 replicate dishes. Error bars represent the standard deviation for the three dishes.

shows that the effect of nickel on the BPDE stimulated decrease in cloning ability was at least additive. These results were similar to the results obtained using human fibroblasts. The effect of nickel on BPDE induced mutagenicity, however, was slightly synergistic, which is different than the effect on human fibroblasts. Our results correlate well with the previously published data with B[a]P and nickel co-treatment (Sunderman, et al., 1971; Sunner and Rivedal, 1980; Sunner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989). These results validate our data and suggest that the protective effect is species specific, since it was seen in human fibroblasts but not in Chinese hamster fibroblasts. It should be noted in that Chinese hamster fibroblasts were shown to be different from human fibroblasts in that mutants were obtained in the controls. This may reflect a decrease in DNA fidelity in rodent cell lines.

#### The Effect of Vitamin E on the Nickel Protective Effect on BPDE Induced Mutagenicity

Since one of the most commonly hypothesized mechanisms of nickel carcinogenicity is via oxidative stress, an experiment was designed to investigate if the protective effect involves a reactive oxygen species. To achieve this purpose, cells were treated with vitamin E, a free radical scavenger, using the same order of treatment described in Figure (7). Incubation with vitamin E was done through the entire 48 hour incubation period. All conditions previously tested were also examined in the presence and absence of vitamin-E. Table (6) shows that the treatment with

Table 5

Effect of Nickel Treatment on the BPDE Stimulated Decreases in Cloning Ability and BPDE Induced Mutagenicity in Chinese Hamster Fibroblasts

<u>Relative Cloning Ability</u>			
Treatment	Exp. #1	Exp. #2	Exp. #3
Control	103.3±34.6(100)	112±13 (100)	84.3±9.7(100)
Nickel	15.3±2.3 (15.3)	42.3±2.5(37.8)	14.3±1.2(17)
BPDE	96±5.3 (92.9)	78±6.1 (70)	46.7±14 (55)
Both	3±1 (3)	4±3.1 (4.6)	2.7±1.2 (3.1)

<u>Mutants/10<sup>6</sup> Clonable Cells</u>			
Treatment	Exp. #1	Exp. #2	Exp. #3
Control	50.3 (13)	117 (45)	84 (71)
Nickel	100 (44)	100 (39)	64 (76)
BPDE	1144.5(293)	252 (107)	851.6 (454)
Both	1553.7(463)	607.2(205)	870.0 (601)

Legend. Cells were treated as described in Figure (7) except 1.2 uM BPDE was used instead of 0.3 uM. The data is presented as described in Table (1).

vitamin E resulted in a slight decrease in the effect of nickel on the BPDE stimulated decrease in cloning ability, suggesting an involvement of an oxidative stress mechanism in nickel cytotoxicity. Table (6) also shows that the treatment with vitamin E almost completely reversed the protective effect with regard to the mutant frequency. This experiment suggests that the protective effect is going through an oxidative stress dependent mechanism.

Table 6  
Effect of Vitamin E on Nickel Protective Effect

Treatment	<u>Relative Cloning Ability</u>	
	Exp. #1	Exp. #2
Control	79.4±4.1(100)	90±8.8 (100)
Nickel	34.7±3.4(43.5)	44±2.9 (48.9)
BPDE	53±3.3 (66.5)	58±3.6 (64.5)
Both	15.5±1.7(19.5)	19.7±1.7(21.8)
Control <sup>+</sup>	78±4.6 (97.8)	90.3±9.5(100)
Nickel <sup>+</sup>	31.7±3.4 (39.7)	42.3±3.9(47)
BPDE <sup>+</sup>	52.7±4.6 (66)	60.6±4.5(67)
Both <sup>+</sup>	24.3±4.1 (30.5)	28.3±1.7(31.4)



Table 6-Continued

Treatment	<u>Mutants/10<sup>6</sup> Clonable Cells</u>	
	Exp. #1	Exp. #2
Control	0 (0)	0 (0)
Nickel	0 (0)	6.4 (2)
BPDE	47.5(13)	33.3(12)
Both	21 (5)	17.8(5)
Control <sup>+</sup>	0 (0)	0 (0)
Nickel <sup>+</sup>	0 (0)	3.12(1)
BPDE <sup>+</sup>	53.3 (14)	44 (16)
Both <sup>+</sup>	42.9 (12)	31.3(10)

Legend. The cells were treated as described in Figure (7) except that 500 uM vitamin E was added to the medium for the entire 48 hours in half the conditions. A control experiment without the vitamin E treatment (no superscript) was performed in parallel with the vitamin-E addition experiment (superscript +). The data is presented as described in Table (1) except that the % relative cloning ability of the treated condition was normalized to the control with no vitamin E addition.

## CHAPTER VI

### DISCUSSION

This study is an extension of previous work done in our lab. We previously have shown that pretreating human fibroblasts with potassium dichromate for 46 hours, followed by simultaneous treatment with both potassium dichromate and BPDE, has a synergistic effect on BPDE induced cytotoxicity as determined by the dramatic decrease in cloning efficiency when cells treated with both carcinogens are compared to either chromium or BPDE treated cells. In addition, an antagonistic has been observed on BPDE induced mutagenicity as shown by a dramatic decrease in the number of BPDE induced HGPRT mutants in the combined treatment compared to cells treated with BPDE alone (Tesfai, et al., 1998). The work described here shows that nickel subsulfide is capable of having a similar effect on BPDE induced cytotoxicity and mutagenicity suggesting a general metal dependent effect rather than a chromium specific one (Table 1).

Our results also show that the nickel synergistic cytotoxic effect and antagonistic mutagenic effect are dependent on the concentration of nickel subsulfide. Decreasing the concentration of nickel subsulfide from 25  $\mu$ M to 5.0  $\mu$ M resulted in a smaller nickel additive effect on BPDE induced cytotoxicity (Table 2), and decreasing it further to 1.0  $\mu$ M resulted in the lose of the nickel additive effect on BPDE induced cytotoxicity, (Table 3). The antagonistic effect of nickel on BPDE

induced mutagenicity decreased from a total loss of mutants in the case of 25  $\mu\text{M}$  to a 50% reduction in mutants for 5.0  $\mu\text{M}$  (Table 2), and only a 20% reduction when the nickel subsulfide concentration was further reduced to 1.0  $\mu\text{M}$  (Table 3). In actual environmental situations, we are exposed to low concentrations of carcinogens for a long period of time. Thus, our lab is currently testing the possibility of treating human fibroblasts with lower nickel and chromium concentrations for long periods of time to examine if the protective effect can be seen under such conditions.

The pretreatment with nickel was necessary to see the protective effect, since treating human fibroblasts simultaneously with nickel subsulfide and BPDE for two hours without the 46 hours pretreatment, resulted in no antagonistic effect on BPDE induced mutagenicity, even though it still had an additive effect on BPDE induced cytotoxicity (Table 4). This differential effect on cytotoxicity and mutagenicity may be explained by an experiment in which we looked at the uptake of nickel by human fibroblasts at various periods of time. It was found that nickel subsulfide can be phagocytosized very quickly and there was not much difference between the uptake of nickel at two hours and at six hours. This result is also supported by data in the literature in which it was shown that the majority uptake of nickel subsulfide occurs within the first two hours (Costa and Mollenhauer, et al., 1980). It is possible that the protective effect is due to a synergistic response to both BPDE and nickel subsulfide. If the effect of BPDE is short lived and ends when the BPDE is removed, the signal may be lost before the signal from nickel subsulfide is initiated. The kinetic characteristics of the protective effect can be tested by treating cells with different

nickel preincubation periods varied from 2 hours to 46 hours in order to see how fast the protective effect can be stimulated. The kinetic profile of nickel and chromium might be different due to differences in their physical state. These differences are supported by an unpublished data from our lab in which chromium-BPDE simultaneous treatment for two hours showed a 50% protective effect, while in the case of the nickel-BPDE simultaneous treatment for two hours in this study, no protective effect was observed.

The effect of vitamin E on nickel induced BPDE cytotoxicity and mutagenicity was studied in order to investigate if the protective effect involve reactive oxygen species. Vitamin E is an antioxidant that has been observed to restrict the chain of reactions induced by free radicals (Farrio, et al., 1994).  $\alpha$ -Tocopherol, the most active form of vitamin E, is considered one of the chief cell components which maintains the structural and functional membrane integrity by acting as a free radical scavenger preventing the peroxidation of membrane, fatty acids and stabilizing membrane system (Conti, et al., 1990). Addition of vitamin E, during the incubation with the carcinogens, was able to reverse the protective effect (Table 6). These results are similar to what has been shown in the previous chromium-BPDE study (Tesfai, et al., 1998). This suggests that the protective effect of nickel is via an oxidative stress dependent mechanism. This oxidative stress dependent mechanism is consistent with the data in the literature, which show that nickel stimulates the generation of variety of reactive oxygen species that play an important role in the action of the metal carcinogenicity (Kawanashi, et al., 1989; Cottele, 1992; Costa, 1991 a; Costa, et al.,

1994 a; and Costa, 1994 b). Since this study and the previous chromium-BPDE co-treatment work (Tesfai, et al., 1998) have shown that the protective effect is going through a similar oxidative stress mechanism, our lab is currently testing the possibility of treating human fibroblasts with both nickel and chromium followed by the simultaneous treatment with nickel, chromium and BPDE for two hours. This experiment will test the possibility that the protective effect might be synergistic with the addition of a mixture of metals. This would be even closer to the real environmental situation in which we are exposed to several carcinogens at any one time.

There are several possible explanations for this protective effect. First, it may be due to a simple intracellular interaction between the generated reactive oxygen species and BPDE, leading to nongenotoxic form of BPDE. This hypothesis might be tested by work that we are doing currently in which BPDE is replaced with ultraviolet light (UV). There is no reaction between UV and the generated reactive oxygen species so one would not expect the protective effect with UV if this hypothesis is correct.

A second hypothesis is based on the possibility that either DNA repair is stimulated, or cell cycle progression is delayed. The possibility of delaying the progression of the cell cycle by nickel is supported in the literature, since nickel was been shown to stop the cell cycle at the G<sub>1</sub> phase (Christie, et al., 1995). This inhibition would give the cell more time to correct the mutations caused by BPDE before the cell goes into S-phase where the DNA will replicate and the mutation will

get incorporated into the genome. The possibility of delaying the progression of the cell cycle can be tested by looking at the expression of some cell cycle regulatory proteins in the four treatment conditions. If these proteins are stimulated, the next step would be to look at the signal transduction pathways that are involved in the regulation of these proteins.

The possibility of stimulation of DNA repair is not documented in literature. On the contrary, it has been shown that nickel can inhibit DNA repair and this is a hypothesized mechanism of its carcinogenicity (Hartwig, et al. 1998; Lynn, et al. 1997; Kasrpezak, et al., 1997; Hartwig, et al. 1992; Hartwig, and, Beyersmann, 1989). (see introduction) . There are, however, two major differences between our study and those studies that have reported inhibition of DNA repair. The first difference is that the previous study used soluble nickel compounds, while we used insoluble nickel subsulfide. This might be important since it has been shown that vitamin E has different effects on insoluble nickel sulfide and soluble nickel chloride. Vitamin E treatment was able to significantly inhibit the formation of some but not all the chromosomal aberrations induced by NiS particles, but it did not affect the chromosome damage induced by NiCl<sub>2</sub>. This suggests some possible differences in the carcinogenic mechanism of the soluble and insoluble nickel compounds (Costa, et. al., 1991 a). The second difference is that the previous studies used much higher nickel concentrations. The protective effect shown in our study maybe the result of stimulation of DNA repair, while higher concentrations of nickel might inhibit DNA repair. The idea of nickel having opposite effects depending on the concentration is

supported by work done in Chrestie's lab, in which nickel has been shown to activate DNA polymerase  $\alpha$  at concentrations below 250  $\mu\text{M}$  and inhibit the polymerase at concentrations above 250  $\mu\text{M}$  (Chrestie, et al., 1994). Despite the fact that stimulation of DNA repair by nickel has not been reported in literature, there is some evidence that nickel can alter gene expression and protein synthesis. Nickel has been shown to have an effect on transcriptional regulation (Costa, et al., 1998; Costa, et al., 1997 b; Costa, et al., 1995 a and Costa, 1995 b) and on translational regulation (Davis, et al., 1975, Holts and Nordlind, 1988, Sorge, et al., 1993, and Cole, et al, 1997). In addition, other carcinogens have been shown to stimulate DNA repair. For example, asbestos was shown to stimulate AP-endonuclease, gene expression, protein levels, and enzyme activity (Mossman, et al., 1998). The possibility of the stimulation of the DNA repair machinery can be tested by looking at the expression of some DNA repair proteins involve in the nucleotide excision repair mechanism. Also, replacing BPDE with other carcinogens that get repaired by different mechanisms might provide a clue about whether the protective effect is specific for nucleotide excision repair or a general phenomena.

The data reported here is contradictory to data previously obtained by other laboratories in which additive or synergistic effects of nickel-B[a]P co-treatment were reported (Sanner, and Rivedal, 1980; Sanner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989). The major differences between our results and the previous B[a]P and nickel co-treatment studies which show synergistic results, include the fact that they used rodent cell lines while we used human fibroblasts, the previous studies used

B[a]P while we used the ultimate metabolite, BPDE, and we used a different order of treatment. To test if using BPDE instead of B[a]P might make a difference, a preliminary experiment was performed in which human fibroblasts were treated with B[a]P instead of BPDE. It was found that human fibroblasts showed no cytotoxic effect to high concentrations of B[a]P, suggesting that they do not metabolize B[a]P efficiently, even at a concentration of 500  $\mu$ M (Figure 9). Thus, we could not test B[a]P in our system.

To test the possibility that using human fibroblasts instead of rodent cells might be the reason for our contradictory results, Chinese hamster fibroblasts (V79-4) were treated with BPDE using the same order of treatment as used with the human cells. It was observed that the effect of nickel on BPDE induced cytotoxicity was synergistic which is similar to the previous reported data in the literature (Sanner, and Rivedal, 1980; Sanner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989) and to our human fibroblast data (Table 5). The effect of nickel on BPDE induced mutagenicity the Chinese hamster cells, however, was synergistic, which correlates well with the previous reports (Sanner, and Rivedal, 1980; Sanner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989), but is completely opposite to the results obtained using human fibroblasts (Table 5). This suggests that the synergistic cytotoxic effect is not species specific while the mutagenic effect is species specific.

None of the previous studies have pretreated with nickel and then followed it by a simultaneous treatment with both nickel and B[a]P (Sanner, and Rivedal, 1980; Sanner and Rivedal, 1981; Uziel, et al., 1986; and Christie, 1989). Some of these



studies have done the simultaneous treatment with nickel and B[a]P (Sanner, and Rivedal, 1980; Sanner and Rivedal, 1981; Uziel, et al., 1986) and their data correlate well with our two hour incubation results (Table 4). Some other studies have done sequential order of treatment, as well (Sanner and Rivedal, 1981; and Christie, 1989). Since none of these studies have used our order of treatment, it maybe that the different results are due to treatment differences. However, since the nickel antagonistic effect on BPDE induced mutagenicity was clearly not seen in Chinese hamster fibroblasts using the same order of treatment which produced the protective effect in human fibroblasts, this suggests that the difference between our study and the previous reports is not due to differences in the order of treatment, but is due to a species differences. This hypothesis is supported by Chrestie's work which showed a synergistic effect of nickel with other DNA damaging agents in a rodent cell line (Christie, 1989), but showed an antagonistic effect using the same DNA damaging agents in human lymphocytes (Christie, 1996).

**Appendix A**  
**Experimental Raw Data**

The data shown in Tables 1-6 and Figure (5) express the average number of clones from the cytotoxicity plates  $\pm$  the standard deviation of the three plates, and the % relative cloning ability is given in parenthesis. Tables 7-13 below contain the actual number of clones obtained in each dish with the average and relative cloning ability.

The data shown in Tables 1-6 and Figure (6) express the mutant frequency and the actual number of mutants is given in parenthesis. The cloning efficiency values used in calculating the mutant frequency, however, were not given. Tables 14-20 below contain the actual number of clones obtained in each dish with the average and the cloning efficiency.

Table 7

Actual Numbers Used in Calculating the Cloning Ability in Table 1

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A**	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	70	73	67	72.0	100	73	71	74	72.7	100
Nickel	9	7	7	7.3	10.5	11	15	10	12	16.5
BPDE	42	36	36	38	52.8	40	41	29	36.7	50.4
Both	3	2	1	2	2.8	1	3	2	2	2.7

Legend. P\*: Plate

C.A.\*\*: Cloning Ability

Table 8

Actual Numbers Used in Calculating the Cloning Ability in Table 2

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A.**	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	43	45	47	45	100	138	114	104	118.7	100
Nickel	29	25	27	23.3	51.8	61	45	59	55	46.3
BPDE	20	15	16	17	37.8	58	58	67	61	51.4
Both	11	10	11	10.7	23.7	24	31	30	28.3	25.3

Legend. P\*: Plate

C.A.\*\*: Cloning Ability

Table 9

Actual Numbers Used in Calculating the Cloning Ability in Table 3

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A.**	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	133	131	111	125	100	124	119	116	119.7	100
Nickel	107	119	122	116	92.8	99	96	138	111	92.7
BPDE	82	74	66	74	59.2	70	62	77	69.7	58.2
Both	70	78	70	72.7	58.1	63	69	76	69.3	57.9

Legend. P\*: Plate

C.A.\*\*: Cloning Ability

Table 10

Actual Numbers Used in Calculating the Cloning Ability in Table 4

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A.**	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	74	81	93	82.7	100	74	73	77	74.7	100
Nickel	17	9	15	13.7	16.7	9	15	8	10.7	14.3
BPDE	31	56	C***	43.2	52.6	30	28	23	27	36.1
Both	2	4	0	2	2.4	7	3	6	5.3	7

Legend. P\*: Plate    C.A.\*\*: Cloning Ability    C\*\*\*: Contaminated Plate

Table 11

Actual Numbers Used in Calculating the Cloning Ability in Table 5

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A.**	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	70	93	138	103.3	100	120	97	119	112	100
Nickel	18	14	14	15.3	15.3	40	42	45	42.3	37.8
BPDE	98	90	100	96	92.9	84	80	72	78	70
Both	2	3	4	3	3	8	4	2	4	4.6

Table 11-Continued

Treatment	Exp. #3				
	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	82	76	95	84.3	100
Nickel	15	13	15	14.3	17
BPDE	61	46	33	46.7	55
Both	2	4	2	2.7	3.1

Legend P\*: Plate

C.A.\*\*: Cloning Ability

Table 12

Actual Numbers Used in Calculating the Cloning Ability in Table 6

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A.**	P*#1	P*#2	P*#3	Avg.	C.A.**
Control	75	79	85	79.4	100	102	87	81	90	100
Nickel	30	36	38	34.7	43.5	41	43	48	44	48.9
BPDE	49	57	53	53	66.5	55	56	63	58	64.5
Both	13	17	16	15.5	19.5	18	22	19	19.7	21.8
Control <sup>+</sup>	72	83	79	78	97.8	102	81	83	90.3	100

Table 12-Continued

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.A.**	P*#1	P*#2	P*#3	Avg.	C.A.**
Nickel <sup>+</sup>	27	35	33	31.7	39.7	37	46	44	42.3	47
BPDE <sup>+</sup>	48	59	51	52.7	66	58	57	67	60.6	67
Both <sup>+</sup>	19	25	29	24.3	30.5	29	30	26	28.3	31.4

Legend. P\*: Plate

C.A.\*\*: Cloning Ability

Table 13

Actual Numbers Used in Calculating the Cloning Ability in Figure (5)

[BPDE]	Exp. #1				
	P*#1	P*#2	P*#3	Avg.	C.A.**
0.00 uM	90	75	80	82	100
0.05 uM	70	81	79	76.3	93.1
0.15 uM	65	55	57	57.6	70.3
0.30 uM	46	49	49	48	58.5
0.45 uM	22	22	21	21.7	26.4

Legend. P\*: Plate

C.A.\*\*: Cloning Ability

Table 14

Actual Numbers Used in Calculating the Cloning Efficiency in Table 1

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.E.**	P*#1	P*#2	P*#3	Avg.	C.E.**
Control	52	55	75	62.7	0.21	70	C***	C***	70	0.23
Nickel	42	35	29	35.3	0.12	45	C***	C***	45	0.15
BPDE	64	52	55	57	0.19	20	C***	C***	20	0.07
Both	35	24	31	30	0.10	19	31	C***	26	0.09

Legend. P\*: Plate      C.E.\*\*: Cloning Efficiency      C\*\*\*: Contaminated Plate

Table 15

Actual Numbers Used in Calculating the Cloning Efficiency in Table 2

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.E.**	P*#1	P*#2	P*#3	Avg.	C.E.**
Control	38	40	40	39.3	0.13	58	74	70	67.3	0.22
Nickel	38	40	62	46.7	0.16	77	60	55	64	0.21
BPDE	21	25	33	26.3	0.09	35	37	38	36.7	0.12
Both	11	12	15	12.7	0.04	38	37	43	39.3	0.13

Legend. P\*: Plate      C.E.\*\*: Cloning Efficiency



Table 16

Actual Numbers Used in Calculating the Cloning Efficiency in Table 3

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.E.**	P*#1	P*#2	P*#3	Avg.	C.E.**
Control	50	51	60	53.7	0.18	50	50	46	48.7	0.16
Nickel	72	57	60	63	0.21	40	47	39	42	0.14
BPDE	37	28	30	31.7	0.11	39	40	39	39.3	0.13
Both	25	27	39	30.3	0.10	31	24	35	30	0.10

Legend. P\*: Plate

C.E.\*\*: Cloning Efficiency

Table 17

Actual Numbers Used in Calculating the Cloning Efficiency in Table 4

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.E.**	P*#1	P*#2	P*#3	Avg.	C.E.**
Control	49	44	47	46.7	0.16	50	43	75	56	0.19
Nickel	36	54	48	46	0.15	23	C*	C*	23	0.08
BPDE	28	45	31	34.7	0.12	53	51	47	50.7	0.17
Both	21	19	17	19	0.06	25	22	C*	23.5	0.08

Legend. P\*: Plate

C.E.\*\*: Cloning Efficiency

Table 18

Actual Numbers Used in Calculating the Cloning Efficiency in Table 5

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.E.**	P*#1	P*#2	P*#3	Avg.	C.E.**
Control	33	40	43	38.7	0.13	49	59	64	57.3	0.19
Nickel	70	70	58	66	0.22	43	34	50	42.3	0.14
BPDE	36	39	40	38.3	0.128	51	78	62	63.7	0.21
Both	44	51	42	44.7	0.149	64	54	34	50.7	0.17

Treatment	Exp. #3					
	P*#1	P*#2	P*#3	Avg.	C.E.**	
Control	108	133	133	124.7	0.42	
Nickel	170	200	157	175.7	0.59	
BPDE	95	95	96	95.3	0.32	
Both	105	108	100	104.3	0.35	

Legend. P\*: Plate

C.E.\*\*: Cloning Efficiency

Table 19

Actual Numbers Used in Calculating the Cloning Efficiency in Table 6

Treatment	Exp. #1					Exp. #2				
	P*#1	P*#2	P*#3	Avg.	C.E.**	P*#1	P*#2	P*#3	Avg.	C.E.**
Control	45	40	46	43.7	0.15	55	42	35	44	0.16
Nickel	36	32	44	37.3	0.12	45	56	45	47	0.16
BPDE	34	34	55	41	0.137	51	56	55	54	0.18
Both	40	42	25	35.7	0.12	40	45	39	41.3	0.14
Control <sup>+</sup>	52	34	38	41.3	0.14	68	56	59	58.7	0.20
Nickel <sup>+</sup>	34	31	40	35	0.12	54	50	40	48	0.16
BPDE <sup>+</sup>	47	40	30	39	0.13	58	49	55	54	0.18
Both <sup>+</sup>	43	35	48	42	0.14	48	47	53	49.3	0.16

Legend. P\*: Plate

C.E.\*: Cloning Efficiency

Table 20

Actual Numbers Used in Calculating the Cloning Efficiency in Figure (6)

[BPDE]	Exp. #1					
	P*#1	P*#2	P*#3	Avg.	C.E.**	#of Mutants
0.00 uM	77	81	70	76	0.25	0
0.05 uM	70	81	79	76.3	0.29	10
0.15 uM	53	56	58	55.7	0.19	31

Table 20-Continued

Exp. #1						
[BPDE]	P*#1	P*#2	P*#3	Avg.	C.E.**	#of Mutants
0.30 uM	23	30	35	29.3	0.01	29
0.45 uM	50	65	62	60.8	26.4	68

Legend. P\*: Plate

C.E.\*\*: Cloning Efficiency

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