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The Effect of Chromium Compounds on the Mutagenic Rate of Benzo[A]Pyrenediolepoxide in Human Fibroblasts

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THE EFFECT OF CHROMIUM COMPOUNDS ON THE MUTAGENIC RATE OF BENZO[A]PYRENEDIOLEPOXIDE IN HUMAN FIBROBLASTS

by

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Cancer researchers are interested in identifying the environmental causes and synergistic influences of different chemical carcinogens in order to decrease the risk of cancer. There are numerous studies which have investigated the effect of individual carcinogens but relatively few in which a well defined mixture of chemicals have been used. The present study was performed to examine the effect of chromium compounds on benzo[a]pyrene diolepoxide (BPDE) induced cytotoxicity and mutagenicity in human fibroblasts. Cytotoxicity was measured by the ability to form colonies while selection of 6-thioguanine resistant mutants were used to study mutagenesis in human fibroblasts.

Our results indicate that the effect of Cr(VI) on BPDE induced cytotoxicity is more than additive, whereas, the effect of Cr(VI) on BPDE mutagenicity is antagonistic. The effects of two antioxidants, vitamin E and catalase, on BPDE and Cr(VI) induced cytotoxicity and mutagenicity were also examined. Results suggest the involvement of an oxidative stress mechanism in the Cr(VI) induced cytotoxicity as well as the inhibition of mutations at the HGPRT locus by this metal.

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CHAPTER I

INTRODUCTION

Cancer

Cancer is a disease of abnormal gene expression. This altered gene expression occurs through a number of mechanisms including gene mutations, translocations or amplifications and abnormal gene transcription or translation (Ruddon, 1995). Many studies have been done in the last century to investigate the cause, mechanism, and treatment of human cancer. Much has been learned about cancer, yet not nearly enough is understood to completely cure this disease or know the specific mechanisms by which the different types of cancer are induced. The causes of cancer are not clearly defined, but known causes of cancer include both external factors (environmental chemicals, radiation, viruses) and internal factors (immune system defects) as well as aging. Diet is probably one of the most suspected causes of cancer, but the information which is available is often contradictory (ACS, 1993).

Carcinogensis is a multistage process which involves initiation, promotion, progression and metastasis (Waalkes and Ward, 1994). All these stages involve genetic changes which may be a mutation in the genetic information leading to cancer. Initiation is the first stage of carcinogenic process which may involve the exposure of normal cells to chemical, physical or microbial carcinogens (Harris et al., 1991). Promotion is the clonal expansion of the initiated cells. Progression is a stepwise process by which the initiated, promoted cells, evolve into cancer. The last stage of carcinogensis, metastasis, is the spread of cancer cells to distant sites not directly connected to the original cancer (Cooper, 1992). Although these four stages of

carcinogenesis are well recognized, there have been hypotheses that many more stages are involved in the process of cancer cell development (Fearon and Vogelstein, 1990).

It is suggested that about 80% of all cancers may be caused by environmental agents (chemicals, radiation and viruses). Reactive chemical groups which are suspected carcinogens include: free radicals, carbonium ions, epoxides, some metal cations, and ester nitrogens of hydroxamic acids and hydroxylamines. The exact manner by which carcinogens cause cancer is still not well understood, however, it is clearly known that many carcinogens can react with physiologically important molecules such as DNA, RNA and proteins. Not all carcinogens cause cancer in the same manner. Some carcinogens require metabolic activation in order to induce mutagenicity and some do not. Many carcinogens are often organ specific in their effects due to the different ways in which the carcinogens contact or accumulate in certain body tissues.

Cancer researchers are interested in identifying the environmental causes and synergistic influences of different carcinogens in order to decrease the risk of cancer. It is important to investigate how various carcinogens induce DNA damage in order to obtain a better understanding of the relationship between known carcinogens, acquire some information as to their mode of action, and determine the potential carcinogenicity of each carcinogen. Increased understanding of the molecular basis of chemical carcinogens has important implications in the prevention, diagnosis and treatment of human cancer.

Metal Carcinogenesis

Experimental cancer research as well as epidemiological studies have provided evidence that several inorganic metal compounds are involved in carcinogenesis. Metals which are known as potential carcinogens include arsenic, beryllium, cobalt, cadmium,

chromium, nickel, manganese, platinum, and selenium (IARC, 1987). Recent evidence suggest that these metal ions can induce carcinogenicity in multiplistic ways either alone or by enhancing the effect of other DNA damaging agents (Snow, 1994). The role of metal compounds in the development of human cancer may be significant since humans are exposed to these metal ions in considerable amounts through both environmental contamination and occupational exposure.

While carcinogenic metal ions are mostly non-mutagenic in bacteria, different types of cellular damage have been observed in mammalian cells, which may account for their carcinogenic potential. Not all metals induce cytotoxicity and carcinogenicity via a common mechanism (Snow, 1992). Since metals are a diverse group of compounds, each metal ion induces carcinogenicity in a different manner and most induce mutagenicity through more than one mechanism. Table 1 illustrates some of the different mechanisms of mutagenicity induced by carcinogenic metals. The mechanisms and degree by which metals induce carcinogenicity varies considerably not only between different metals but also within different species of the same metal (Hartwig, 1994). These differences may be related to differences in solubility, bioavailability , uptake of metals into cells, oxidation state, absorbability, transport, chemical reactivity and the complexes which are formed within the body (Stohs & Bagchi, 1995; Cupo and Wetterhahn, 1984).

Significant effort has gone into studying the molecular mechanism by which metal ions induce mutagenicity, however, the molecular basis of mutagenesis by metal ions is not well understood at present. Nevertheless, there are some proposed mechanisms by which metals induce mutagenicity. These include: the formation of oxygen and other radical species resulting to oxidative damage, formation of DNA adducts and the interference of metal compounds with DNA repair and/or the DNA replication process (Hartwig, 1995; McBride et al., 1991; Tkeshelashrili et al., 1991).

Table 1

Mechanisms of Mutagenicity Induced by Different Carcinogenic Metals

	Metal Compounds			
Mechanism	Chromium	Nickel	Arsenic	Cadmium
DNA Binding	yes	yes	no	yes
Strand Breaks	yes	yes	no?	yes
Altered DNA Polymerase Fidelity	yes	yes	yes	yes
Excision Repair Inhibition	yes	yes	yes	yes?
Comutagenesis	?	yes	yes	yes
Gene Induction	yes	?	yes	yes
Oxidative Stress	yes	yes?	yes	yes
Enzyme Inhibiton	yes	?	yes	yes
Chromosome Aberration	yes	yes	yes	yes

N.B. A question mark by itself indicates insufficient data and a statement followed by a question mark indicates conflicting results.

Modified from Snow, T. E. (1992). Metal Carcinogenesis: Mechanistic Implications of Carcinogenesis. Pharmac. Ther., 53, 31-65.

One of the mostly widely discussed mechanisms in metal carcinogenesis is oxidative DNA damage. It is believed that metals can induce oxidative stress through generation of free radicals. The most important mechanism of oxygen activation by

transition metals involve Fenton/Haber Weirs chemistry and autoxidation. The reaction involves the conversion of H_2O_2 and superoxide ($O_2^{-\cdot}$), which are two common metabolite products (Imlay and Linn, 1986), into a powerful DNA damaging ·OH radical. The reaction can be written as follows:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + OH^- + {\cdot}OH \quad \text{(Fenton)}$$

$$M^{(n+1)} + O_2^{-\cdot} \rightarrow M^{n+} + O_2$$

The sum of these two reactions is:

$$H_2O_2 + O_2^{-\cdot} \rightarrow O_2 + OH^- + {\cdot}OH \quad \text{(Haber-Weiss)}$$

Two oxidation states of the metal cation (M^{n+} and $M^{(n+1)}$) form a catalytic electron transfer (redox) couple. In the absence of chelators, the above reactions are driven by Cu (I), Fe (II), Co (II), Ti (III) and Cr (VI) ions (Walling, 1975). Some other metal ions become reactive only when they are properly chelated.

Several studies have shown that transition metal compounds which interact with H_2O_2 produce not only free ·OH radical, but also other strong oxidants, such as singlet oxygen $^1O_2$ (Kawanishi et al, 1986) and metal oxo and peroxo species, all of which are capable of damaging DNA and proteins in a site-specific manner (Kwanishi & Yamanoto, 1991). Some researchers have also indicated that the peroxo species and $^1O_2$ play a more important role in metal induced DNA damage than free radicals (Wink et al, 1994).

Another possible pathway by which metals induce mutagenicity is by interfering with repair enzymes and inhibiting DNA repair. It is believed that the inhibition of DNA repair by carcinogenic metals might be associated with structural changes of DNA

or modification of different repair proteins (Hartwig, 1995). Failure to repair genetic damage or improper repair of the damage results in loss of integrity of gene information which in turn can give rise to altered gene expression due to mutation, or cell death due to the inability of cells to replicate. Although, different steps of DNA repair are affected by different metal ions, it is suggested that one common mechanism by which DNA repair is affected might be the competition with essential metals. Figure 1 demonstrates a possible interference of different metal ions with different steps in excision repair (Hartwig, 1994).

Since carcinogenesis is a multistaged process, metal ions may contribute at each of the different stages of carcinogenesis and each metal ion is likely to contribute at more than one step through different mechanisms. Figure 2 describes briefly the possible role of different metal ions at different stages of carcinogenesis (Snow, 1992).

The current study was designed to examine the potential role of chromium compounds in the carcinogenic process. Chromium is a transition metal which was first prepared and characterized by Louis Vanquelini in 1793. Its name reflects the many different colors of its compounds. Chromium exists in different oxidation states of 0, +2, +3, +4, +5 and +6. The most available and environmentally stable oxidation states of chromium are the hexavalent and trivalent chromium compounds (Fishbein, 1981). The most abundant of the two is the trivalent chromium. Unlike the trivalent and hexavalent chromium compounds, most divalent chromium compounds are sensitive to oxidation in air and hence, unstable in most aerobic systems. The tetravalent and pentavalent chromium compounds are also relatively unstable (Dean and Cheeseman, 1987).

Humans are exposed to chromium via both environmental contamination and occupational exposure. One possible source of environmental exposure of chromium is

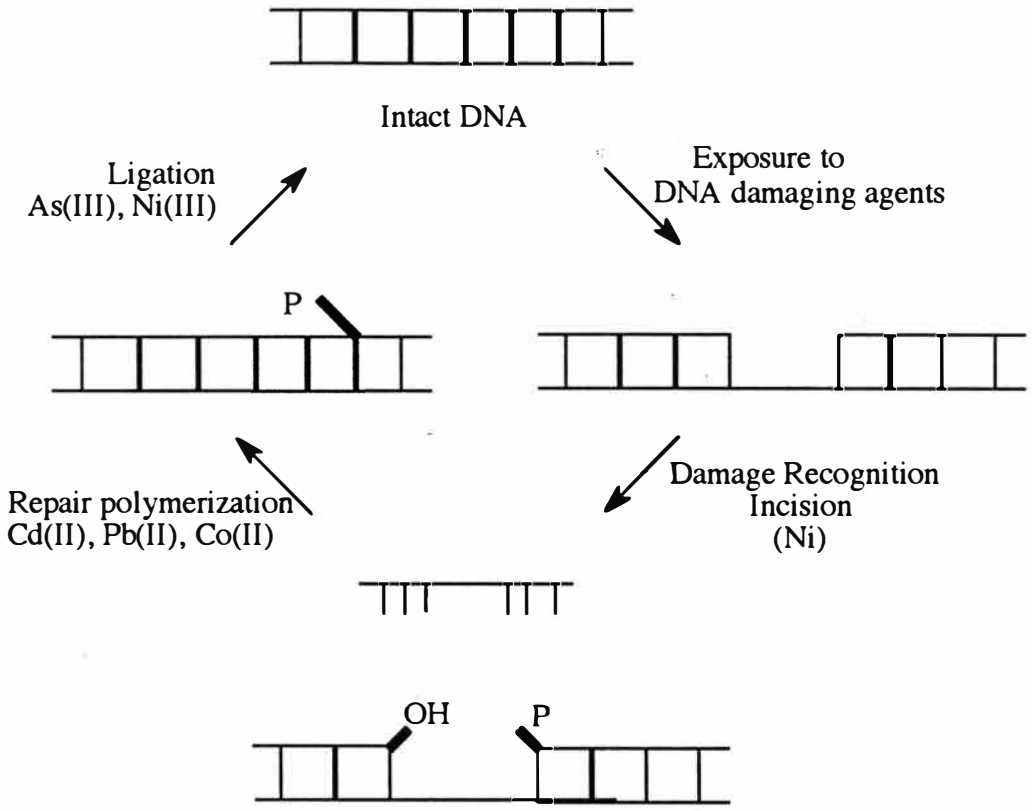

Figure 1. Interference of Metal Ions With Different Steps in Excision Repair.

Modified from Hartwig et al., (1995). Current Aspects in Metal Genotoxicity. Biometals, 8, 3-11.

waste dumps for chromate-producing plants which can cause local air or water pollution (Gemmel, 1973). Water and food are another possible sources of environmental exposure. The daily intake of chromium from food has been estimated to be in the range of 0.03-0.1mg (Langard and Norseth, 1979). Chromium content in municipal drinking water has been estimated to be in the range 1-10μg/l. Therefore, the amount of chromium in food and drinking water has been determined to be of negligible risk to human carcinogenesis (IARC, 1987). Tobaccco has been determined to contain up to 30mg/Kg of chromium but most values are reported below 5mg/kg

(Frank et al., 1977). So far no estimates of the inhaled amount of chromium in tobacco smoke have been found.

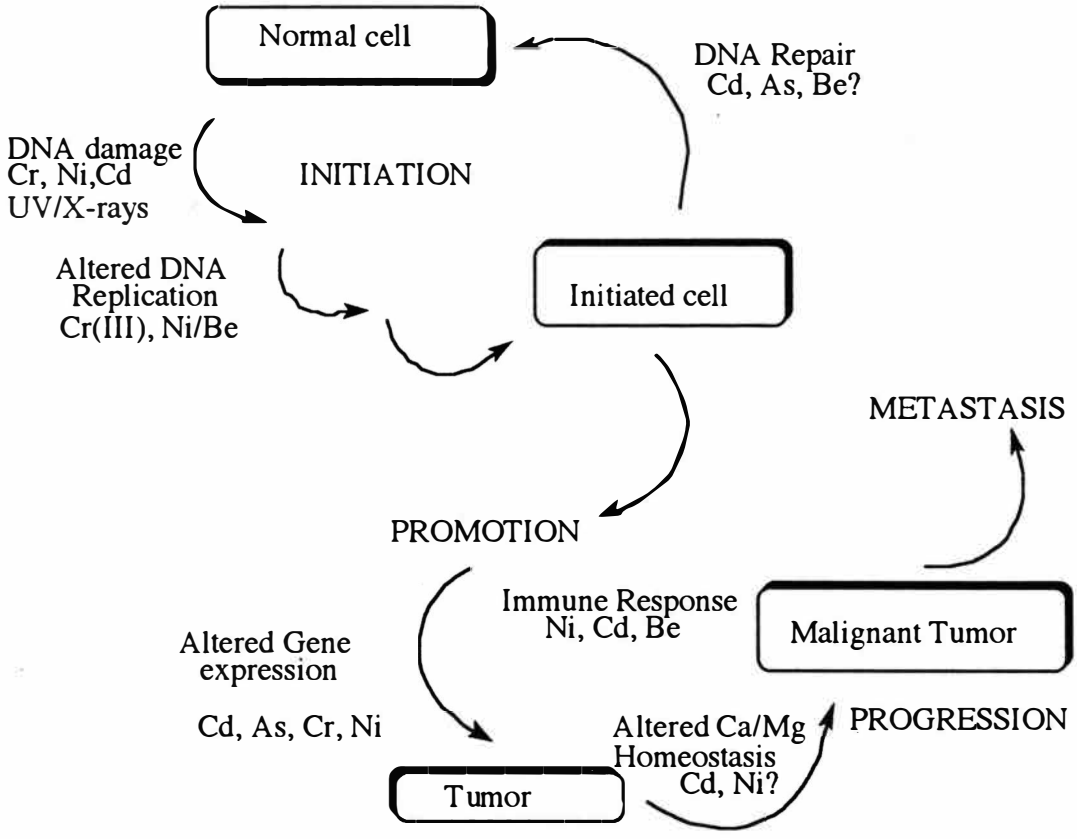

Figure 2. Possible Role of Metal Ions at Different Stages of Carcinogenesis.

Modified from Snow, T. E. (1992). Metal Carcinogenesis: Mechanistic Implications of Carcinogenesis. Pharmac. Ther., 53, 31-65.

The major exposure of humans to chromium is occupational. Occupational exposure to chromium may take place in the metal refining/chromate industry, metallurgic industry and in the refractory brick industry. Considerable amount of chromium exposure is also reported in industries which are secondary users of chromium chemicals (steel, welding and grinding industries and production of

anticorrosive paints). Chromium occurs in the work place primarily as Cr(VI) and Cr(III). Humans are ususally exposed to chromium in the work place through inhalation, ingestion and skin contact.

Chromium is both an environmental toxicant and an essential trace element. The glucose tolerance factor is an example of a chromium containing protein which is required for the optimum activity of insulin (Anderson et al., 1991). Epidemiological studies have shown that chromium is toxic and carcinogen to humans and experimental animals. It has also been shown that chromium exerts genetic toxicity in bacteria and in mammalian cells in *vitro*. Chromium exposure is usually associated with lung cancer (Hayes, 1979). Chromium is also known to be one of the most common sensitizers in allergic eczema (Fregert, 1969). Several studies have shown that Cr(VI) is more toxic than Cr(III). The reason might be because most Cr(III) compounds are not taken up by cells and extracellular reduction of Cr(VI) to Cr(III) is believed to reduce its biological activity (De Flora et al., 1989). In contrast to Cr(III), Cr(VI) in the form of chromate mimics essential phosphate and sulphate anions and enters cells through general anion transport channels (De Flora & Wetterhan, 1989). Once inside the cell Cr(VI) is reduced through relatively unstable Cr(V) and Cr(IV) intermediates to kineticlly stable Cr(III) species (Bjerrum and Bjerrum, 1990). It is suggested that intracellular reduction of Cr(VI) is required for chromium to exert carcinogenicity, however, the ultimate carcinogenic and/or mutagenic species are still unknown (Wetterhann and Hamilton, 1989; Bianchi et al., 1983; De Flora et al., 1989; De Flora & Wetterhahn, 1989).

Several components are involved in the cellular reduction of chromium (VI). These include: ascorbate (Sugyima et al., 1991), glutathione (Kortenkamp et al., 1991), cysteine (Borges & Wetterhahn, 1989) and hydrogen peroxide (Kwanishi et

al., 1986). Figure 3 illustrates the intracellular reduction of hexavalent Chromium through several biological reductants (Sugiyama, 1991).

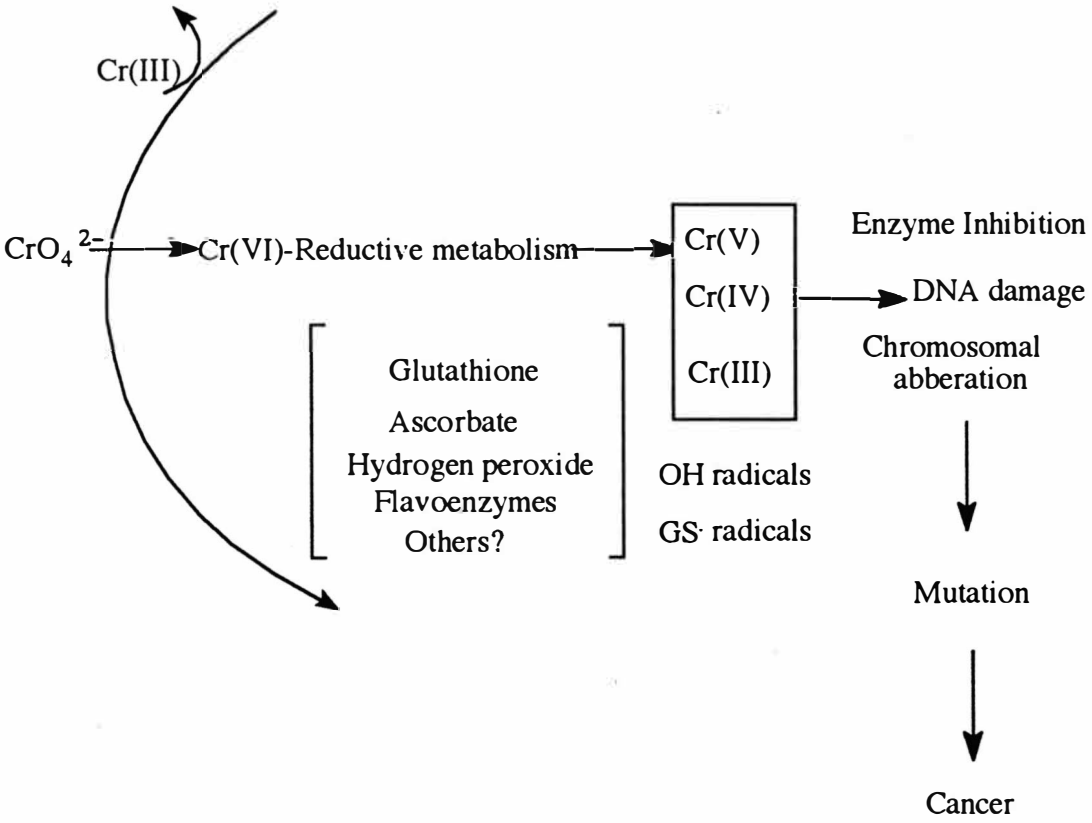

Figure 3. Intracellular Reduction of Cr(VI).

Modified from Sugiyama, M. (1991). Effect of Vitamin on Cr(VI) Induced Damage. Environmental Health Perspectives. 92, 63-70.

Chromium is known to cause different types of genetic damage in cultured mammalian cells including: chromosome aberrations (Bianchi et al., 1983: Sugyima et al., 1991), sister chromatid exchanges (Bianchi et al., 1983: Levis & Bianchi, 1982), and DNA-DNA and DNA-Protein crosslinks and mutations (Borges and Wetterhahn, 1991). Different researchers have shown that chromium (VI) compounds are mutagenic in both prakoryotic and eukaryotic cells in culture (Bianchi et al., 1983: De Flora et al.,

1990). Unlike most metal ions which are mostly non mutagenic, many Cr(VI) compounds have been found to be moderately mutagenic in some mammalian assays (Biedermann and Landolph, 1990: Celotti et al., 1987). The rate of mutation by chromium compounds is maximal at near toxic doses.

Even though it is clearly known that chromium is mutagenic, it is still not well understood why or how. Initially it was assumed that it was the hexavalent chromium compounds themselves that are DNA damaging agents. However, recent reports suggested that while Cr(VI) compounds can cause DNA damage both in *vivo* (Hamilton & Wetterhahn, 1986) and in *vitro* (Sugiyama et al., 1986), they are unreactive toward DNA under physiological conditions (Tsapokos & Wetterhahn, 1983). Therefore, it is now believed that Cr (VI) compounds must undergo further transformation in order to induce mutagenicity (Wetterhahn & Hamilton, 1989). The nature of the crucial intermediate species and the mechanism of transformation, however, is not well defined at present. Although the mechanism of action by which chromium induces mutagenicity is not well known, several mutagenic pathways have been proposed. The two proposed major mechanisms by which chromium induces mutagenicity are: the production of oxidative radicals during intercellular reduction of Cr(VI) (Snow, 1994) and through the formation of a DNA adduct by binding to the phosphate group of DNA. It is believed that once Cr(VI) is inside the cell it is reduced to Cr(III) through different reductive components as shown in Figure 3. During this reduction process, some oxidative radicals such as hydroxyl radicals are formed. These oxidative radicals are believed to be the initiators of the primary events in Cr(VI) mutagenicity (Sugiyama et al., 1993, Aiyar et al., 1989). The reaction can be viewed as follows:

$$Cr(VI) + O_2^{\cdot} \rightarrow Cr(V) + O_2$$

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + \cdot OH + OH^-$$

Until recently it was assumed that Cr(III) was relatively non genotoxic. However, (Ozawa and Hanaki, 1990) demonstrated that Cr(III) can be reduced to Cr(II) by different biological reductants and the resulting Cr(II) might react with hydrogen peroxide to produce DNA damaging hydroxy radicals.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are compounds which represent some of the most potent carcinogens known (Ruddon, 1995) and probably remain the most prevalent of environmental carcinogens. They are also among the most thoroughly studied. Polycyclic aromatic hydrocarbons are of particular interest as carcinogens because they are formed during the incomplete combustion of organic materials (pyrosynthesis) and therefore are widely found in the environment. The precise chemical mechanism of pyrosynthesis are not known, but it is believed that Polycyclic aromatic hydrocarbons are built up from carbon equivalents to acetylene, ethylene and their free radicals (Francis, M. B., 1994). Formation of PAH typically occurs between 600-800°F. Important sources of PAH in the modern human environment include fires, coal combustion and conversion, industries, cigarette smoke, engine exhaust, smoked foods, charcol cooked foods and burnt toast.

PAH usually require metabollic activation by microsomal enzymes in order to induce DNA damage in a variety of tissues and species. The ultimate carcinogens for many of the polycyclic hydrocarbons are known to be the dihydrodiol epoxides. Conversion of PAH to dihydrodiol epoxide is a crucial pathway in the formation of the ultimate carcinogen. The mutagneic potential of these diol epoxides depends on different interactions of the carcinogen and/or the template, nature of the polymerase

and efficency of DNA repair within the cell. If the changes caused by such carcinogens are not properly repaired, they may give rise to permanent changes in the genome during DNA replication causing mutations (Dipple et al., 1984).

Benzo[a]pyrene is one of the most studied polycyclic aromatic hydrocarbons and is found in cigarette smoke, atmospheric pollution and a variety of foods. It is one of the best understood chemical carcinogens with respect to the structural basis of activity. This compound is metabollically activated by a two step mechanism to a 7,8-dihydrodiol 9, 10-epoxide (BPDE) which is the ultimate carcinogen (Dipple et al., 1984; Roilides et al., 1988; Anderson and Lambert, 1990). Various isomers of BPDE bind covalently to different extents at various positions on DNA bases, particularly to guanine residues forming a guanine adduct which initiates mutagenesis and carcinogenesis. BPDE has been shown to intercalate into DNA and it has been hypothesized that the intercalation is required for covalent binding (Meehan et al., 1982: Geacintov, 1986: Wolfe et al., 1987). The major DNA adduct formed by BPDE is a deoxy-guanine adduct at the N-2 position of guanine. Minor adducts are also formed at N-7 and O-6 position of guanine, N-4 position of cytidine and N-6 position of adenine (Jeffrey et al., 1977). The metabolism of Benzo[a]pyrene and the formation of the major BPDE-guanine adduct is briefly described in Figure 4 (Waalkes and Ward, 1991).

DNA is replicated by enzymes known as DNA polymerases. These enzymes utilize single stranded DNA as templates to catalyze the synthesis of the complementary strand from the appropriate bases resulting in a double stranded DNA. When DNA-polymerase reaches a BPDE-guanine adduct it does not recognize the adduct and may insert a wrong base inducing a point mutation. Several studies have shown that the mutations induced by BPDE are almost exclusively point mutations involving mostly

GC to TA tranversions (Anderson et al., 1992; Anderson and Lambert, 1990; Roilides et al., 1988).

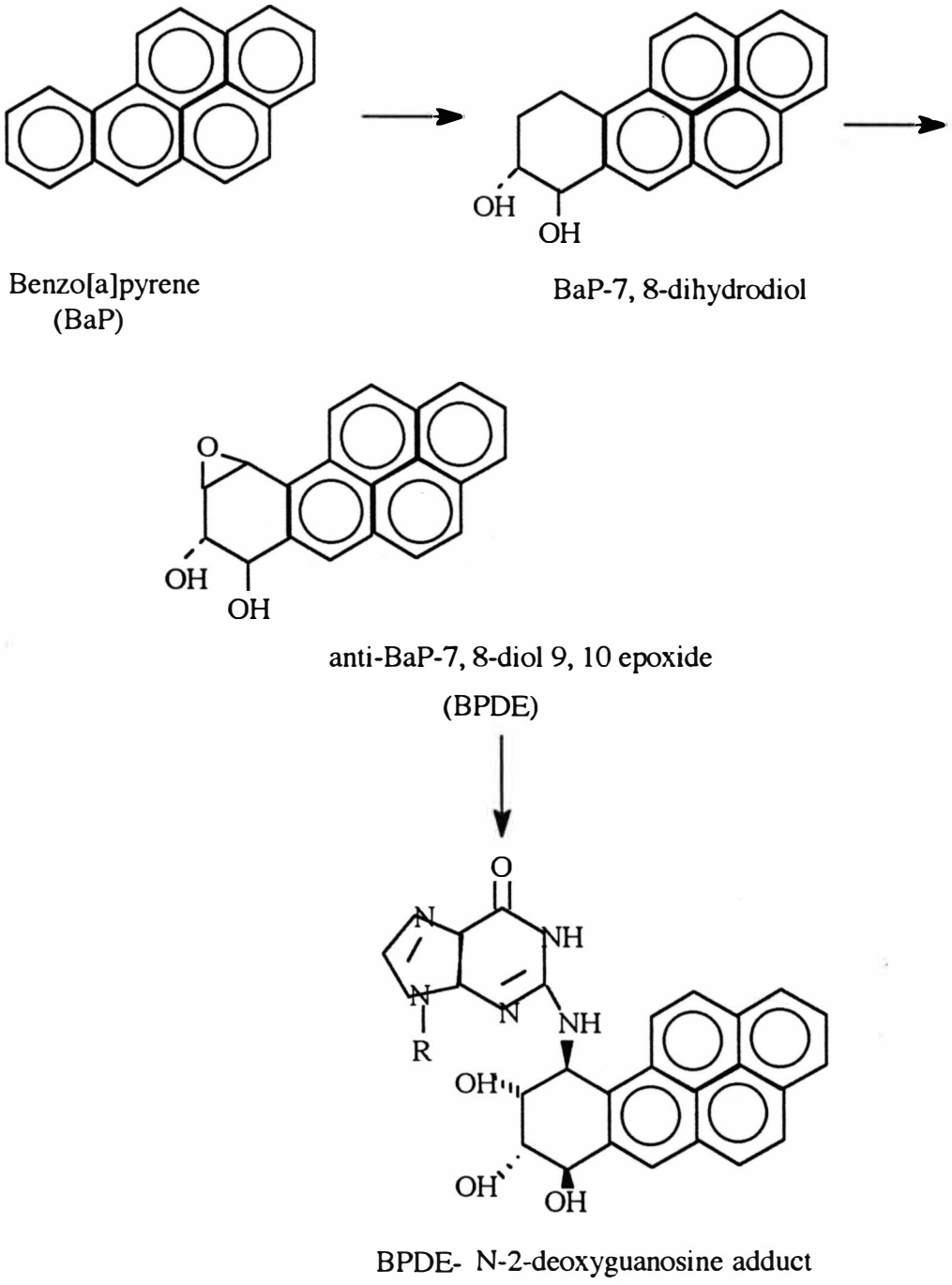

Figure 4. Metabolism and DNA Binding of Benzo[a] Pyrene.

Objectives of the Current Study

Numerous studies have been performed to investigate the carcinogenicity of various individual chemical carcinogens. The studies performed to examine the composite effect of different chemical carcinogens, however, are relatively few. Since humans are exposed to many chemical carcinognes at one time through occupational exposure, air pollution, cigarette smoking etc., the need to study the combined effect of carcinogens is critical. Our objective in the current study was to investigate the interaction between Cr(VI) compounds and benzo[a]pyrene diol-epoxide (BPDE) on the cytotoxicity and mutagenic rate in human fibrobalsts. This study is not the first or only study which has looked at the combined effect of metals and organic compounds. For example, Hartwig and Beyerssman examined the comutagenicity and inhibition of DNA repair by metal ions with different DNA damaging agents in Chinese hamster cells (Hartwig and Beyerssman, 1989). Lavelle and Witmer, studied the mutagenicity of Cr(VI) in combination with sodium azide & ethyl methane sulfonate in the *Salmonella typhrimium* strain (Lavelle and Witmer, 1984). Also, Rivedal and Sanner, looked at the synergistic effect of morphological transformation of Chinese hamster embryo cells by nickel sulfate and benzo[a]pyrene as well as potassium dichromate and benzo[a]pyrene (Rivedal and Sanner, 1980). This study differs from those mentioned above in that normal human fibroblasts were used as a model system instead of rodent or bacterial cells to study the combined effect of chromium and benzo[a]pyrene-diolepoxide (BPDE), on the rate of mutations in the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene. Epidemeological studies have shown that both Chromium and BPDE are established carcinogens humans as well as experimental animals.

We pursued the following specific objectives in the present study,

1. Determine if Cr(VI) has any effect on BPDE cytotoxicity and mutagenicity.
2. If any, is the effect additive, synergistic or antagonistic?
3. Is the effect concentration dependent ?
4. Does the effect depend on the order of addition of Cr(VI) and BPDE?
5. Do antioxidants attenuate the observed effect in any way?

CHAPTER II

MATERIALS AND EXPERIMENTAL METHODS

Cells and Cell Culture

The normal human fibroblast cell line L266-C, derived from human foreskin tissues, was used in this study. Eagle's Minimum Essential Media supplemented with 0.10 μM sodium pyruvate, 0.20% sodium bicarbonate, essential amino acids with 10.0% supplemental calf serum or fetal bovine serum, penicillin (100.0u/ml), streptomaycin (100.0μg/ml) and hydrocortisone (10.0mg/ml) was used as a complete medium for cell growth.

Cell Harvesting and Counting

Cells were washed with phosphate buffered saline (PBS), dissociated from monolayer cultures by treatment of 0.25% trypsin solution for 3-5 minutes, and harvested using 5.0 ml of complete medium. One half ml of the cell suspension was diluted with 19.5 ml of isotone solution and the cells were counted using a Coulter cell counter. The average of three readings was used to calculate the cell concentration, cells were plated at the appropriate density, and then incubated in a humidified incubator maintained at 37^0C and 5.0% CO_2.

Test Chemicals

$CrCl_3$. $(H_2O)_6$ and $K_2Cr_2O_7$ were purchased from Sigma Chemical Company (St. Louis, Mo). Compounds were dissolved in MilliQ purified water to give 1.0 mM stock solutions. These stock solutions were then sterile filtered through a 0.22 μM

Millipore filter and diluted with the complete medium to the desired final concentration shortly before use. BPDE was purchased from ChemSyn Science Laboratories (Lenexa, KS). BPDE was dissolved in DMSO just prior to use to give a 1.0 mM solution and then diluted with the complete medium to the desired final concentration. The final concentration of DMSO used was consistently less than 0.20%.

$K_2Cr_2O_7$ and $CrCl_3.(H_2O)_6$ Toxicity Curves

Three hundred cells per dish were plated onto 100mm dishes. Three dishes per chromium concentration were used. Cells were allowed to attach to the dishes for twenty four hours, and then were treated with the chromium compounds for 48 hours by replacing the medium with a medium containing the desired final concentration of each compound. The concentration range used for $CrCl_3$ was 10.0μM-10.0mM and 0.010μM-1μM for $K_2Cr_2O_7$. After 48 hours, medium was removed from cells, the cells were washed with PBS and supplemented with a freshly prepared complete medium. Medium was changed every 5-6 days and cells were allowed to grow for 14 days. After 14 days, dishes were stained with crystal violet and visible colonies were counted. The average colony number of three dishes was used to calculate the relative % survial for each concentration of the chromium used. The control (no treatment) was defined as 100.0% cloning. Results are reported as % relative survival ± standard deviation.

Chromium and BPDE Treatment and Analysis

On day one, three hundred cells per dish in 10.0 ml complete medium were plated on 100mm culture dishes for the cytotoxic assay, and 500,000-1000,000 cells per dish in 25.0 ml of medium were plated on 150mm culture dishes for the experimental set. Cells were then placed in the humidified incubator overnight to attach

to the dishes. There were three replicates per treatment group for the cytotoxic assay and one dish each for the experimental set. Four treatment conditions were used as follows: (1) Control, no treatment; (2) Potassium dichromate treated; (3) BPDE treated; and (4) Combination, Potassium dichromate and BPDE treated.

Twenty four hours after plating the cells, complete media containing the desired final concentration of potassium dichromate and/or BPDE were prepared. Culture dishes were taken out from the incubator, medium aspirated off the dishes and replaced with the medium containing the desired final concentration of the carcinogens. In order to eliminate any effects by DMSO an equal amount of DMSO was added to each dish for each condition when cells were not treated with BPDE. The time of treatment used throughout the experiments was 48 hours with potassium dichromate and two hours with BPDE. Concentrations used for Potassium dichromate were 0.20μM and 0.30μM. The concentration of BPDE used througout was 0.30μM. Four different orders of treatment were used to examine the effect of varying the order for the combined treatments. After the required time of treatment, the medium was removed, cells were washed with PBS and supplemented with fresh medium. Medium was changed every 5-6 days for both experimental dishes and cytotoxicity assay dishes. Cytotoxicity assay dishes were stained with crystal violet and counted after 14-17 days. The colony forming ability was calculated as the average number of colonies formed as a percentage of the number of colonies formed in the control. Control (no treatment), was defined as 100.0% cloning.

$$\% \text{ Relative Survival} = \frac{\text{Average \# of colonies from treated dish}}{\text{Average \# of colonies from control dish}} \times 100\%$$

Cells in the experimental dishes were grown for 10 days to allow expression of mutants. The cells were then trypsinized and 200,000 cells per dish were replated in

100mm culture dishes for each condition in a medium containing 30.0μM 6-thioguanine (Sigma Chemcial Company) to select for HGPRT mutants. Ten dishes were used for each condition. In parallel experiments, 300 cells per dish in 100 mm culture dishes were replated per each condition in triplicates in normal medium for the determination of cloning efficency. Medium for both the selection and the cloning efficiency dishes was changed every 4-5 days. After incubation for 15-20 days, the cell colonies formed from both selection and cloning efficency dishes were stained with crystal violet and counted. The observed number of 6-thioguanine resistant mutant colonies was corrected for the colony-forming ability of cells in each treatment group. The induced mutation frequency was expressed as the number of induced mutants per 10^6 viable cells.

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

Selection of mutants was based on selecting cells resistant to the guanine analogue 6-thioguanine. Resistance to 6-thioguanine is usually achieved in a single step selection and is caused by the reduction in activity of hypoxanthine guanine phosphoribosyltransferase (HGPRT) due to a mutation in the HGPRT locus on the X chromosome (Bradley et al., 1981; Meehan, 1979,). A mutation at this locus prevents cells from utilizing the toxic guanine anolugue. Therefore, cells are selected by their ability to grow in a medium containing 6-thioguanine.

Vitamin E and Catalase Treatment and Analysis

Vitamin E, α-tocopherol (Sigma, St. Louis, MO) was dissolved in 100.0% ethanol to give a 0.20M stock solution. The 0.20M stock solution was then diluted with a complete medium to give a 500.0μM solution. Vitamin E was prepared immediately

before use. Cells were treated as described in the previous section with 500.0μM vitamin E added to medium containing chromium and/or BPDE. Controls were performed in parallel which were not treated with vitamin E. Cells were then assayed for cytotoxicity and mutation at the HGPRT gene as described above.

Catalase (from Bovine Liver) was purchased from Sigma chemical company (St. Louis, Mo) with a specific activity of 2800.0 units/mg. Complete medium containing a final concentration of 130.0u/ml of catalase was made and sterile filtered through 0.22μM millipore filter prior to use. Cells were treated with catalase, potassium dichromate and BPDE in the same manner as described for Vitamin E and were assayed for cytotoxicity and mutation rate at the HGPRT gene.

CHAPTER III

RESULTS AND DISCUSSION

Effects of Cr(III) and Cr(VI) on the Cloning Ability of Human Fibroblasts

The purpose of these experiments was to choose a chromium concentration which could be used for the study of the combined mutagenic effects of chromium compounds and BPDE. It has been shown that most metals induce mutagenicity at toxic doses. Our goal therefore, was to determine a concentration of chromium which produced a significant level of cytotoxicity so as to get optimum mutation rates.

The experiments were done as described in Materials and Methods and cytotoxicity was determined by the ability of individual cells to form colonies. Figures 5 and 6 show the cytotoxicity of Cr(III) and Cr(VI) respectively. Control (no treatment) was defined as 100.0% cloning and results are expressed as the average relative percent survival ± average deviation of two independent experiments.

Both Cr(III) and Cr(VI) caused dose-dependent cytotoxicity in human fibroblasts. Cr(III) induced dose-dependent cytotoxicity over a wide concentration range of 10.0μM-10.0mM in contrast to Cr(VI) which caused dose-dependent cytotoxicity over a more narrow range, of 0.010-1.0μM. Chromium (VI) induced cytotoxicity at approximately 1000 fold lower concentration than Cr(III). These results confirmed a lower toxicity of Cr(III) compounds in human fibroblasts which is consistent with previously published data (Whiting et al., 1979: Biederman & Landolph, 1990 : Kasprazk 1992). We decided to use concentrations of 0.20μM and 0.30μM potassium dichromate which were moderately toxic to human fibroblasts for our studies.

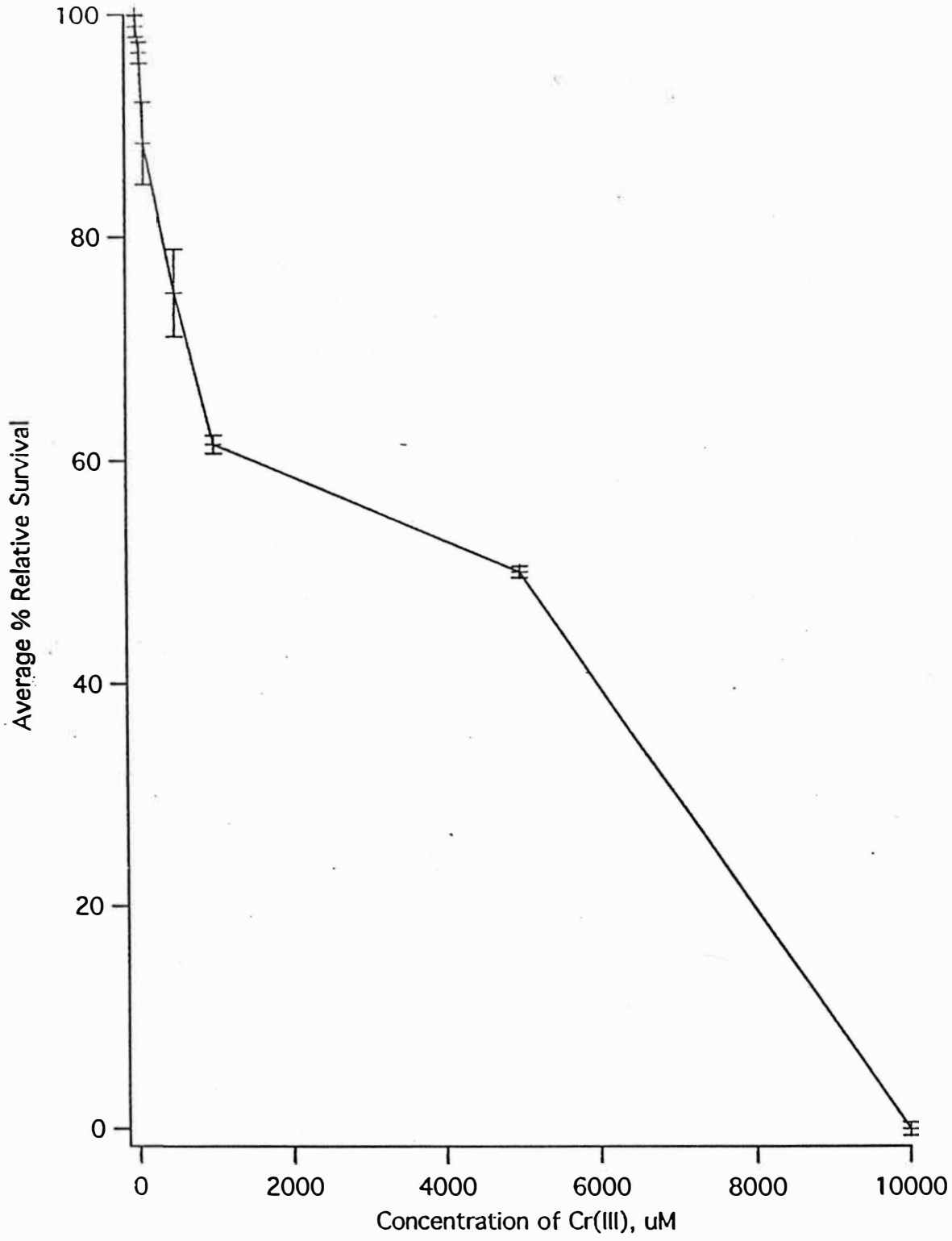

Figure 5. Cytotoxic Effect of Cr(III) in Human Fibroblasts.

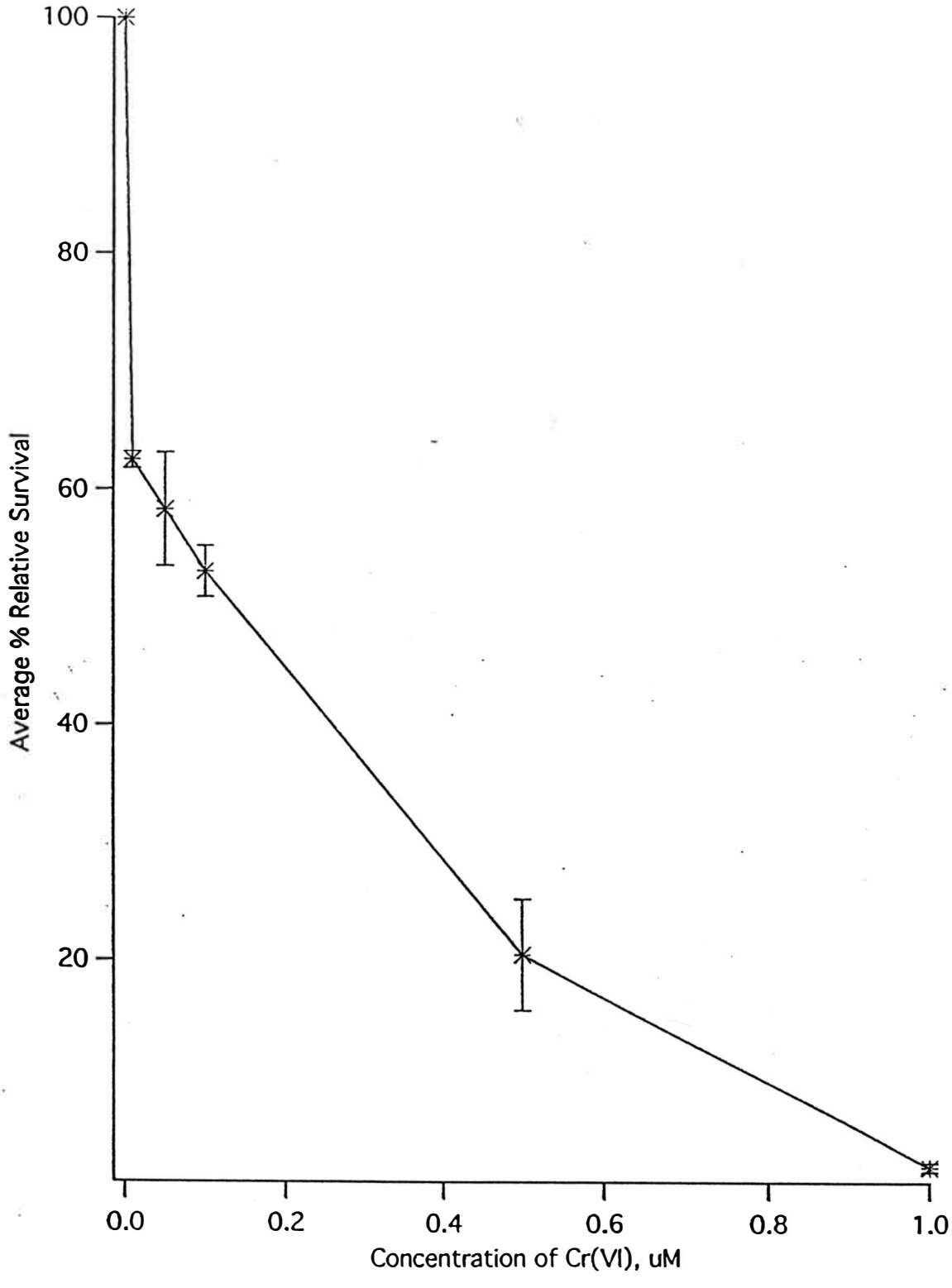

Figure 6. Cytotoxic Effect of Cr(VI) in Human Fibroblasts.

Combined Effects of Cr(VI) and BPDE on the Cloning Ability and Mutation Rate in Human Fibroblasts

These experiments were designed to examine the effect of Cr(VI) on BPDE induced cytotoxicity and mutagencity. Cytotoxicity was measured using the ability of cells to form colonies and mutations at the HGPRT gene was used to monitor genetic damage. In all experiments throughout the study, cells were incubated with potassium dichromate for 48 hours and with BPDE for 2 hours but the order of treatment of the carcinogens was varied from one set of experiments to another. Cells were first pretreated with 0.30μM potassium dichromate followed by 0.30μM BPDE. The treatment condition used in these experiments is briefly described in Figure 7. The response obtained for cytotoxicity was slightly greater than that predicted by simple addition of the effects of each carcinogen separately (Table 2), suggesting that Cr(VI) has a more than additive effect on the cytotoxicity of human fibroblasts induced by BPDE.

The effect of pretreatment with 0.30μM Cr(VI) on BPDE induced mutations at the HGPRT locus in human fibroblasts is shown in Table 3. The mutation frequency induced by BPDE was in agreement with previously published results (Rivedal & Samer, 1981: Roilides et al., 1988: Ochi et al., 1982: Anderson et al., 1992). The mutagenic responses to potassium dichromate in the human fibroblast cell lines were not as great as those observed with BPDE. Contrary to the cytotoxic effects, however, pretreatment with potassium dichromate greatly decreased the mutation rate at the HGPRT gene caused by BPDE. Cr(VI) caused about 89.0% decreased in the BPDE-induced mutations. Similar protection effects by Cr(VI) have been reported with the combined treatment of Potassium dichromate and Ethyl-Methanesulfonate (Lavelle & Witmer, 1984) in *Salmonella typhimurium* as well as with the combined treatment of Cr(VI) and Ni (Katsfifis et al., 1996) in human lymphocytes. These results,however,

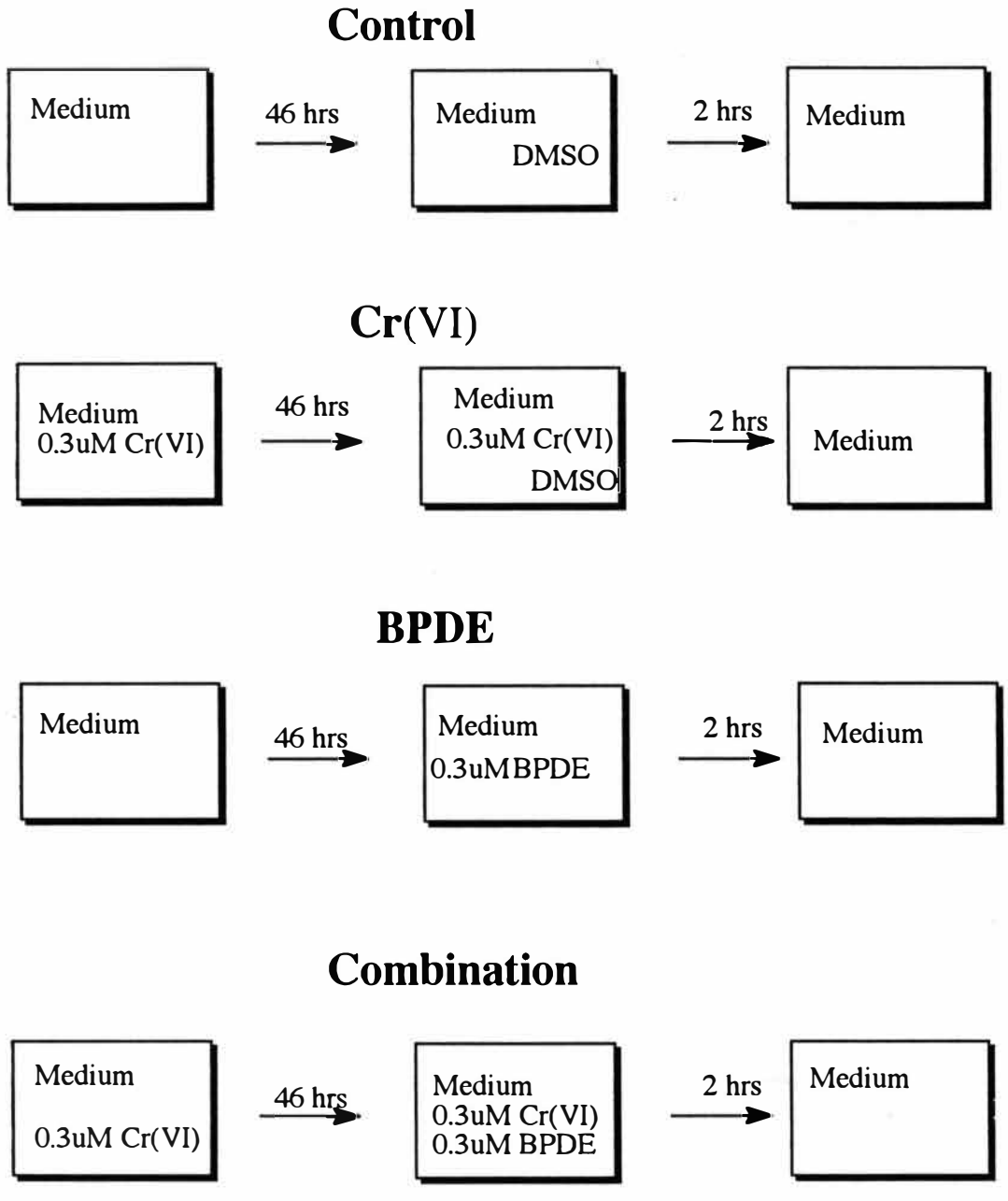

Figure 7. Chromium Pretreatment Protocol.

were not in agreement with other studies that have shown that potassium dichromate and nickel sulfate enhanced the transformation frequency of hamstro embryo cells in the presence of benzo[a]pyrene (Rivedeal and Sanner).

Table 2

The Effect of Pretreatment With 0.30μM Cr(VI) on BPDE Induced Cytotoxicity in Human Fibroblasts

	Average % Relative Survival (± Standard Deviation)		
Treatment	Experiment 1	Experiment 2	Experiment 3
Control	100.0	100.0	100.0
Cr(VI)	37.7 (5.2)	42.0 (4.3)	45.1 (5.0)
BPDE	40.0 (6.5)	48.6 (5.6)	45.8 (5.0)
Combination	4.6 (1.4)	5.7 (2.1)	4.4 (1.8)

Table 3

The Effect of Pretreatment With 0.30μM Cr(VI) on the BPDE Induced Mutation Rate at the HGPRT Gene in Human Fibroblasts

	Mutants /10^6 Viable Cells		
Treatment	Experiment 1	Experiment 2	Experiment 3
Control	0.0	0.0	0.0
Cr(VI)	18.9	14.5	8.9
BPDE	164.6	68.3	150.8
Combination	18.2	7.9	16.6

The potassium dichromate was reduced to 0.20μM and cells were treated in the same manner as described in Figure 7 in order to determine if the effect of Cr(VI) on the BPDE mutational rate was concentration dependent. The cytotoxicity and mutagenicity results obtained from such treatments are shown in Tables 4 and 5 respectively. As shown in Table 4, 0.20μM Cr(VI) induced less cytotoxicity compared to 0.30μM Cr(VI) in human fibroblasts. The effect of pretreatment with 0.20μM Cr(VI) on BPDE induced cytotoxicity was more than additive but there was no significant difference between the cytotoxicity of the combined treatment when 0.20μM Cr(VI) was used instead of 0.30μM Cr(VI) suggesting that the effect of pretreatment with Cr(VI) was not dependent on the concentration of Cr(VI) within the concentration range tested.

The mutagenicity induced by potassium dichromate alone was very small, but suggested a dose related response. It has been shown that Cr(VI) compounds in bacterial and mammalian cell culture are only weakly mutagenic, if at all. Klein and coworkers showed that chromate induces no more than a 5 to 10 fold increase in mutation frequency compared to background (Klein et al., 1994). It should also be noted that chromate induces persistent toxicity and at the higher doses the plating efficency of the treated cells is reduced even one week after treatment. This residual effect may contribute to a selective loss of mutant cells and to the frequently observed decreased mutant yield at higher doses (Snow, 1992). In contrast to the cytotoxicity effect, 0.20 μM potassium dichromate inhibited the mutagenicity induced by BPDE at HGPRT locus, but the resulting antagonistic effect was less pronounced than that observed with 0.30μM Cr(VI). The number of mutants was reduced 74.0% by 0.20μM versus 89.0% by 0.30μM Cr(VI) indicating that the effect of Cr(VI) on BPDE induced mutations is concentration dependent.

Table 4

The Effect of Pretreatrement With 0.20μM Cr(VI) on BPDE Induced Cytotoxicity in Human Fibroblasts

	Average % Relative Survival (± Standard Deviation)	
Treatment	Experiment 1	Experiment 2
Control	100.0	100.0
Cr(VI)	70.6 (8.2)	70.2 (1.8)
BPDE	41.7 (6.3)	40.5 (1.2)
Combination	4.9 (1.0)	11.0 (1.1)

Table 5

The Effect of Pretreatment With 0.20μM Cr(VI) on the BPDE Induced Mutation Rate at the HGPRT Gene in Human Fibroblasts

	Mutants/ 10^6 Viable Cells	
Treatment	Experiment 1	Experiment 2
Control	0.0	0.0
Cr(VI)	1.9	2.2
BPDE	157.9	160.4
Combination	41.3	43.5

We next studied the effect of simultaneous treatment of Cr(VI) and BPDE on BPDE induced cytotoxicity and mutation rate using 0.30μM Cr(VI) and 0.30μM BPDE. The sequence of treatment used for each condition is descriebed briefly in Figure 8. The cytotoxicity and mutagenicity results obtained from such treatments are shown in Tables 6 and 7 respectively. The effect of simultaneous treatment of Cr(VI)

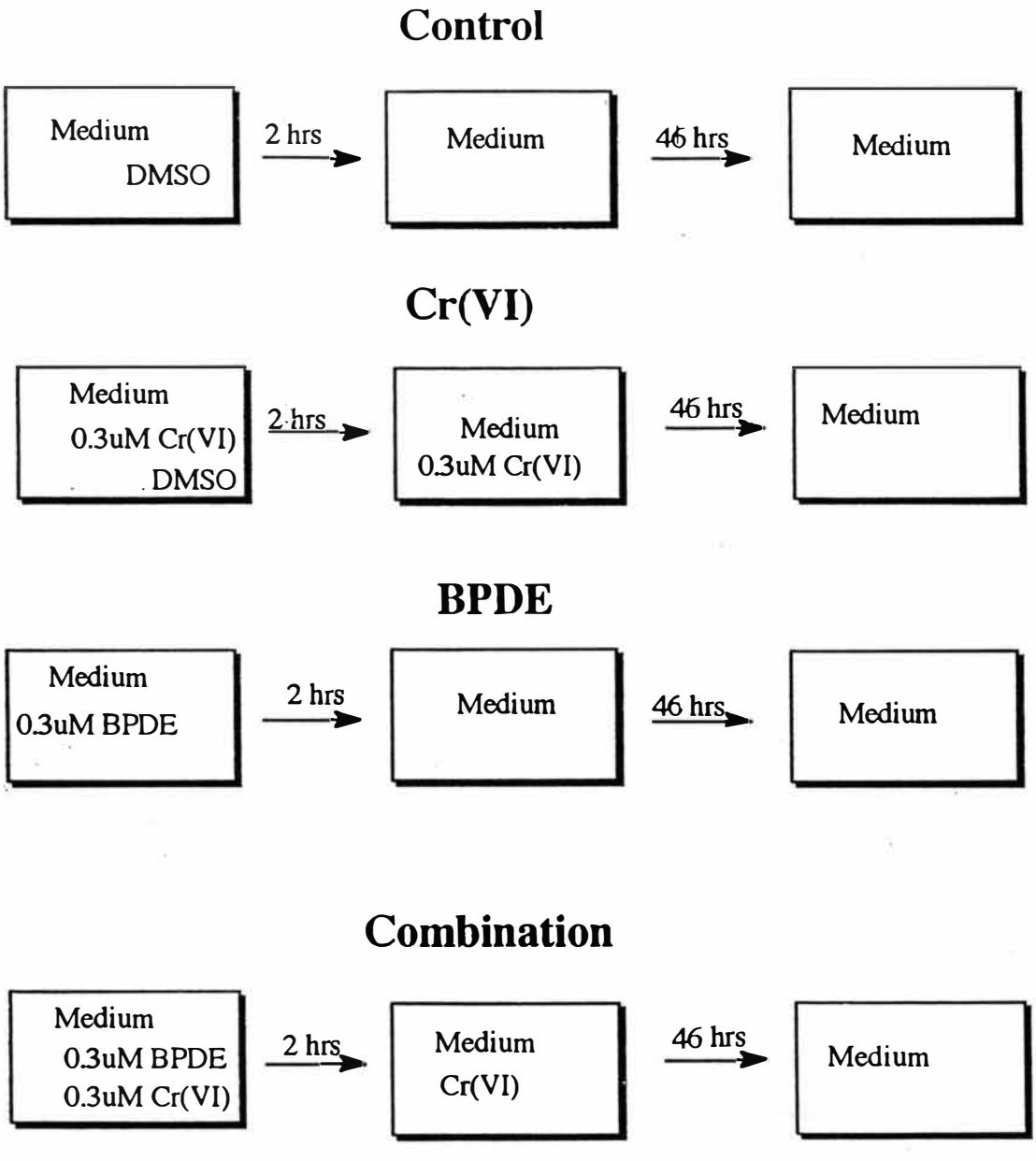

Figure 8. Chromium and BPDE Simultaneous Treatment.

Table 6

The Effect of Simultaneous Treatment With 0.30μM Cr(VI) and 0.30μM BPDE on BPDE Induced Cytotoxicity in Human Fibroblasts

	Average % Relative Survival (± Standard Deviation)	
Treatment	Experiment 1	Experiment 2
Control	100.0	100.0
Cr(VI)	49.3 (8)	35.6 (5.1)
BPDE	43.8 (5.1)	39.1 (4.4)
Combination	3.0 (0.6)	2.5 (0.3)

Table 7

The Effect of Simultaneous Treatment With 0.30μM Cr(VI) and 0.30μM BPDE on the BPDE Induced Mutation Rate at the HGPRT Gene in Human Fibroblasts

	Mutants/ 10^6 Viable Cells	
Treatment	Experiment 1	Experiment 2
Control	0.0	0.0
Cr(VI)	16.2	10.8
BPDE	195.6	195.2
Combination	45.4	51.7

and BPDE on BPDE induced cytotoxicity is more than additive and similar to that observed when Cr(VI) was added 46 hours before BPDE. The effect of simultaneous treatment on the mutation rate at the HGPRT gene was again antagonistic however, the inhibition of BPDE induced mutations by Cr(VI) was less pronounced compared to when Cr(VI) was given first (75.0% inhibition versus 89.0% inhibition). These results suggest that the mutation rate is dependent on the order of treatment. These results also

might explain epidemiological studies reporting conflicting results for cytogenic effects in lymphocytes of workers exposed to chromium compounds and other DNA damaging agents in welding and electroplating industries. Some epidemiological studies report that populations exposed to such mixtures show increased incidence rates of cancer (Nordberg and Anderson, 1981) while some report that there is no significant effect (Katsfis, 1996). These different results might depend on the order of exposure to the different carcinogens.

We then treated cells in the same manner as described above, but lowered the concentration of potassium dichromate to 0.20μM. Results for cytotoxicity and mutation frequency are shown in Tables 8 and 9 respectively. Potassium dichromate at 0.20μM induced an increase in BPDE induced cytotoxicity similar to that observed in the previous experiments using this concentration of Cr(VI). The effect of simultaneous treatment of 0.20μM Cr(VI) and BPDE on the BPDE induced mutation rate ,however, was not significant. These results along with those of the previous experiments indicate that both the concentration of Cr(VI) and order of treatment play an important role in determining the effect of Cr(VI) on the BPDE induced mutation rate at the HGPRT gene in human fibroblasts. The effect of chromium on BPDE induced cytotoxicity, however, is not affected by either order of addition or concentration of metal within the range of concentration tested here.

Cells were then treated as described in Figure 9 to see if BPDE induced cytotoxicity and mutation at the HGPRT locus in human fibroblasts were affected by posttreatment with Cr(VI). Tables 10 and 11 depicit the results obtained from the cytotoxicity and the mutation assays respectively. Once again, cytotoxicity results show that posttreatment with Cr(VI) has an effect on BPDE induced cytotoxicity and the effect was more than additive as previously observed in the other experiments.

Table 8

The Effect of Simultaneous Treatment With 0.20μM Cr(VI) and 0.30μM BPDE on BPDE Induced Cytotoxicity in Human Fibroblasts

	Average % Relative Survival (± Standard Deviation)	
Treatment	Experiment 1	Experiment 2
Control	100.0	100.0
Cr(VI)	76.5 (1.9)	70.4 (3.3)
BPDE	45.2 (2.5)	42.8 (3.4)
Combinaiton	2.3 (4.3)	4.3 (0.5)

Table 9

The Effect of Simultaneous Treatment With 0.20μM Cr(VI) and 0.30μM BPDE on the BPDE Induced Mutation Rate at the HGPRT Gene in Human Fibroblasts

	Mutants/ 10^6 Viable Cells	
Treatment	Experiment 1	Experiment 2
Control	0.0	0.0
Cr(VI)	1.9	1.9
BPDE	149.3	152.6
Combination	143.4	147.4

Posttreatment with Cr(VI) however, was found to be largely ineffective in affecting the number of mutations induced by BPDE at the HGPRT locus. Similar results were observed by Lee and coworkers with respect to the pre and post treatment with sodium arsenite on the genotoxicity of methyl methanesulfonate in Chinese hamster ovary cells (Lee et al., 1986). Pretreatment with sodium arsenite reduced the

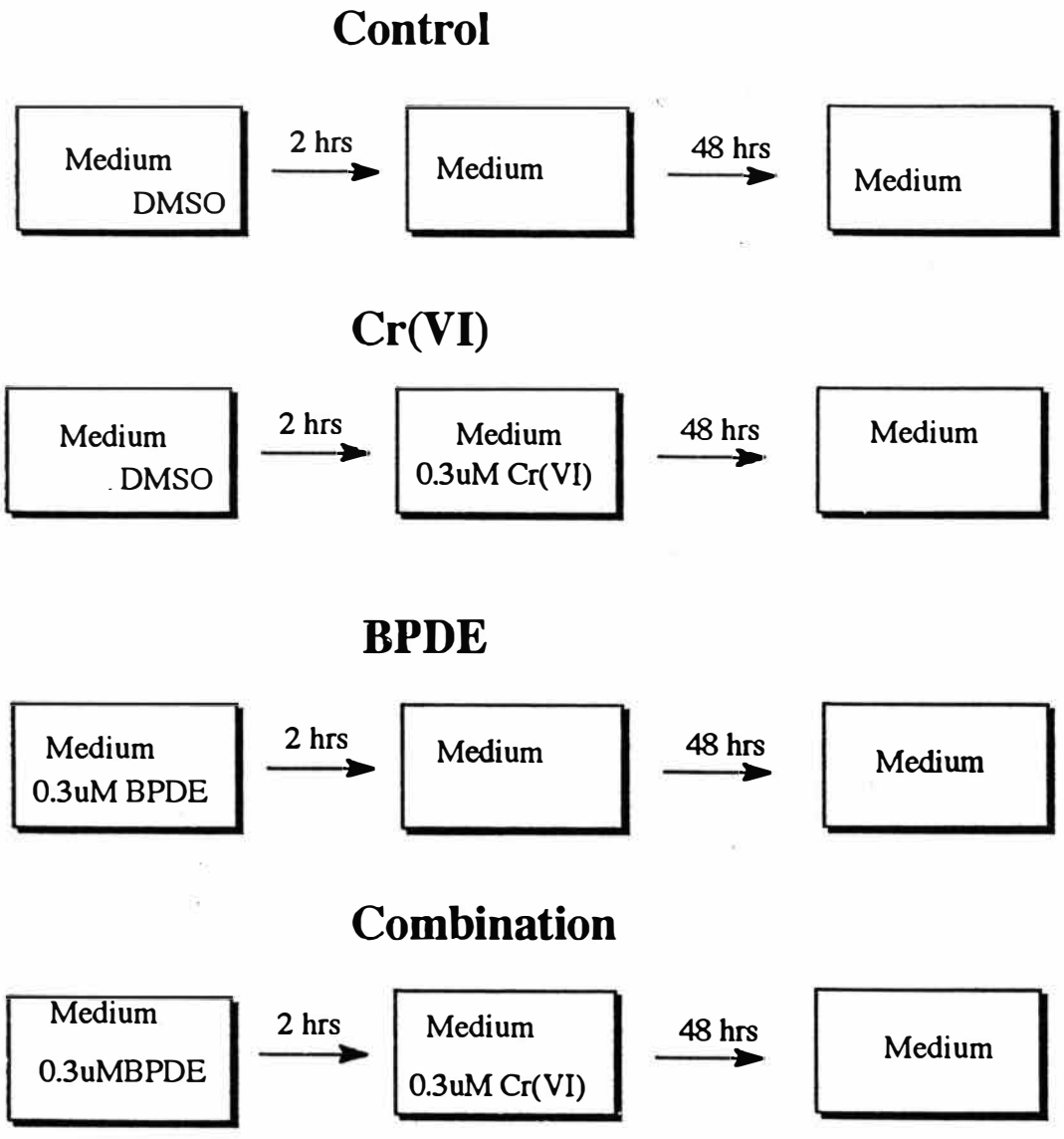

Figure 9. Chromium Posttreatment Protocol.

Table 10

The Effect of Posttreatment With 0.30μM Cr(VI) on BPDE Induced Cytotoxicity in Human Fibroblasts

	Average % Relative Survival (± Standard Deviation)		
Treatment	Experiment 1	Experiment 2	Experiment 3
Control	100.0	100.0	100.0
Cr(VI)	45.3 (5.8)	42.0 (3.5)	41.7 (2.8)
BPDE	47.3 (4.8)	40.9 (2.3)	42.6 (4.5)
Combination	12.4 (2.1)	12.3 (0.4)	12.8 (0.6)

Table 11

The Effect of Posttreatment With 0.30μM Cr(VI) on the BPDE Induced Mutation Rate at the HGPRT Gene in Human Fibroblasts

	Mutants/10^6 Viable Cells		
Treatment	Experiment 1	Experiment 2	Experiment 3
Control	0.0	0.0	0.0
Cr(VI)	0.0	9.4	11.3
BPDE	197.2	196.0	196.2
Combination	204.3	200.0	194.8

methyl-methanesulfonate induced mutations at the HGPRT gene, while posttreatment with sodium arsenite drastically increased the methyl-methanesulfonate induced mutations at the HGPRT gene suggesting that the responses were dependent on the order of treatment. In one of the experiments (Table 11) it can be seen that chromium did not induce any mutations, but this is not so surprising since the number of colonies obtained from Cr(VI) treated dishes were very few throughout the experiments.

The effect of a recovery period of 24 hours between treatments of the two compounds was also tested. The protocol used is shown in Figure 10 and the results are shown in Tables 12 and 13. It can be seen that the posttreatment with Cr(VI) following a twenty four hour recovery period had a more than additive effect on the cytotoxicity induced by BPDE similar to that seen in all previous treatments. There was, however, little effect on the mutation rate of BPDE at the HGPRT gene in human fibroblasts. The 24 hour recovery period did not seem to have any significant effect on the mutation rate and the results were similar to those obtained when posttreatment with Cr(VI) immediately followed treatment with BPDE.

In summary, our results from these experiments performed with different concentration of Cr(VI) and different orders of treatment suggest that the effect of Cr(VI) on the BPDE induced cytotoxicity in human fibroblasts is more than additive. The cytotoxicity effect did not appear to be concentration dependent in the range tested (Tables 2 & 4, Tables 6 & 8). There was not any significant difference in the relative survival of cells in the combined treatment when 0.20μM Cr(VI) was used instead of 0.30μM Cr(VI) . The effect did not change significantly with the order of treatment except some slight increase when Cr(VI) was added immediately after addition of BPDE (Table 10). These results indicate that the cytotoxic effect was not dependent on the order of treatment.

Results from mutational assay indicate that Cr(VI) seems to protect human fibroblasts from BPDE induced mutations. Results are summarized in Tables 14 and 15 and are expressed as the average ratio of mutants induced by the combined tratement to the mutants induced by BPDE treatment ± average deviation. The protective effect appears to be dependent on the concentration of Cr(VI) since 0.30μM Cr(VI) showed a larger protective effect against BPDE induced mutations when compared to 0.20μM Cr(VI).

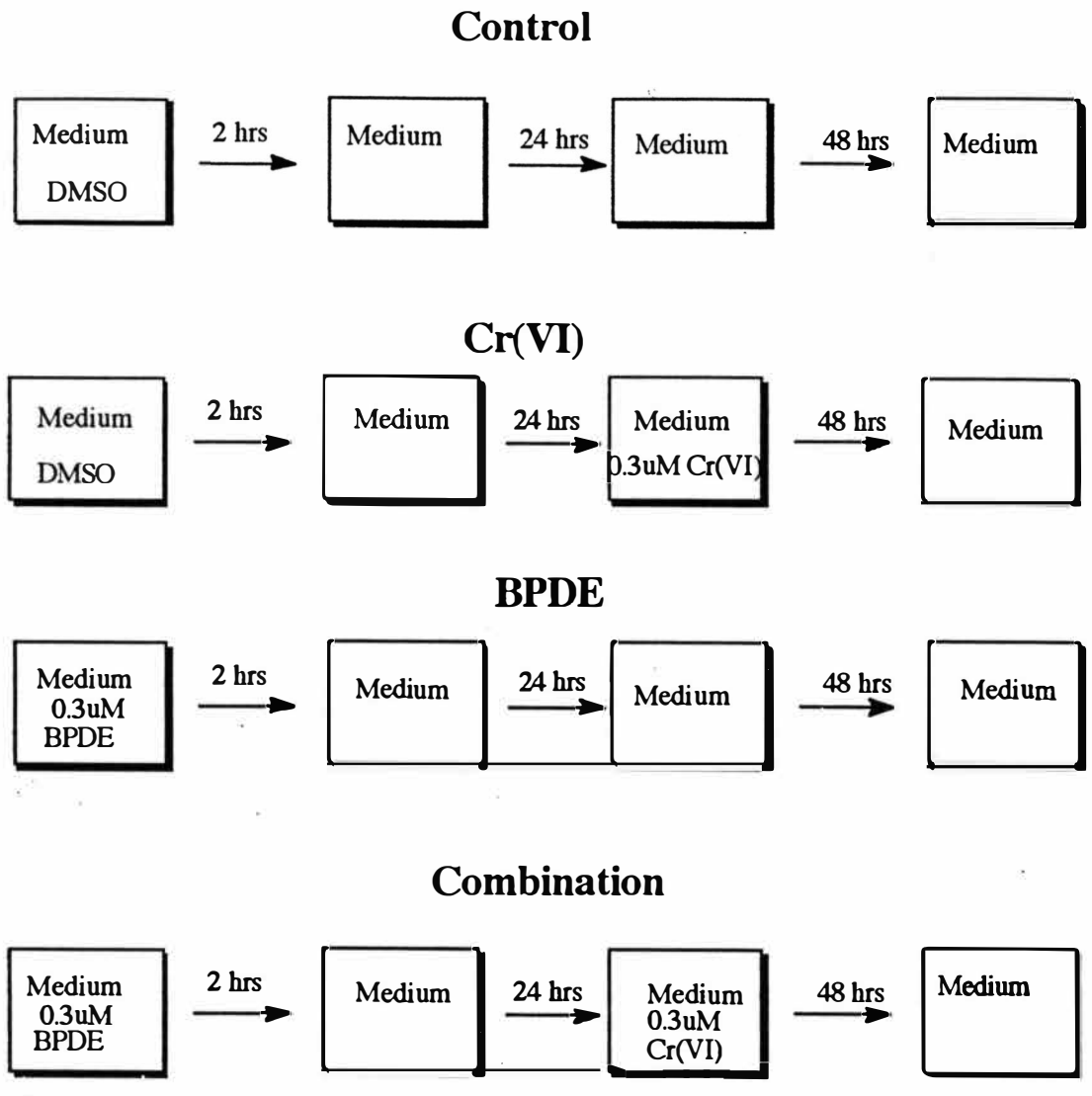

Figure 10. Delayed Chromium Posttreatment Protocol.

Table 12

The Effect of Posttreatment With 0.30μM Cr(VI) 24 Hours After BPDE on the BPDE Induced Cytotoxicity In Human Fibroblasts

	Average % Relative Survival (± Standard Deviation)	
Treatment	Experiment 1	Experiment 2
Control	100.0	100.0
Cr(VI)	44.3 (1.4)	49.6 (2.3)
BPDE	43.0 (1.8)	46.1 (1.8)
Combination	3.3 (0.5)	3.8 (0.3)

Table 13

The Effect of Posttreatment With 0.30μM Cr(VI) 24 Hours After BPDE on the BPDE Induced Mutation Rate at the HGPRT Gene in Human Fibroblasts

	Mutants/ 10^6 Viable Cells	
Treatment	Experiment 1	Experiment 2
Control	0.0	0.0
Cr(VI)	12.6	10.0
BPDE	184.2	141.3
Combination	171.4	135.9

The effect was also dependent on the order of treatment used. Our results indicate that for Cr(VI) to inhibit BPDE induced mutation at the HGPRT gene, either preincubation with Cr(VI) or at least simultaneous exposure with BPDE using 0.30μM Cr(VI) is necessary. Posttreatment with Cr(VI) seems to have no significant effect on BPDE induced mutations at the HGPRT gene in human fibroblasts (Table 15). The

present results indicate that pretreatment of Cr(VI) protects cells from induction of mutations by BPDE. On the other hand, posttreatment with Cr(VI) has little or no effect on the mutation rate induced by BPDE .

Table 14

The Effect of Cr(VI) Concentration on BPDE Induced Mutation at the HGPRT Gene in Human Fibroblasts

Treatment Condition	Concentration of Cr(VI) (μM)	Mutants by Combination / Mutants by BPDE
1	0.2	0.266 ± 0.006
1	0.3	0.118 ± 0.009
2	0.2	0.963 ± 0.004
2	0.3	0.248 ± 0.004

Table 15

The Effect of the Order of Treatment of Cr(VI) on BPDE Induced Mutation at the HGPRT Gene in Human Fibroblasts

Treatment Condition	Concentration of Cr(VI) (μM)	Mutants by Combination / Mutants by BPDE
1	0.3	0.118 ± 0.009
2	0.3	0.248 ± 0.023
3	0.3	1.040 ± 0.021
4	0.3	0.946± 0.036

Condition 1: Pretreatment with Cr(VI).
Condition 2: Simultaneous treatment with Cr(VI) and BPDE.
Condition 3: Posttreatment with Cr(VI).
Condition 4: Delayed Posttreatment with Cr(VI).

Effects of Vitamin E and Catalase on the Cr(VI) Effect in Human Fibroblasts

The results above show that the Cr(VI) effect on toxicity was more than additive with that of BPDE, but the metal suppressed the mutagenicity induced by BPDE at the HGPRT gene. We next began to seek an explanation for the effects which were observed. Since different metal ions have been known to induce oxidative stress, one possible explanation for the decrease in HGPRT mutants with Cr(VI) treatment could be that the induced oxidative stress stimulates DNA repair enzymes, which in turn, inhibits the induction of mutations by BPDE. Experiments were thus performed with antoxidants to determine if the hypothesis could be correct.

The effects of two antioxidants, vitamin E (α-tocopherol) and catalase, on the cytotoxicity and mutations induced by Cr(VI) and BPDE were examined. Vitamin E and catalase are antioxidants that have been observed to restrict the chain of reactions induced by free radicals in different cell types in culture (Elattar and Sin., 1992). Alpha-tocopherol, the most active form of vitamin E, is considered one of the chief cell components that maintain the structural and functional membrane integrity by acting as free radical scavengers preventing the peroxidation of membrane fatty acids and stabilizing membrane systems (Conti, 1990). Catalase is an enzyme which plays an essential role in protecting cell integrity and is mainly known for its detoxification of hydrogen peroxide into oxygen and water (Sies, 1985).

Cells were treated with the antoxidants at the same time when they were treated with Cr(VI) and BPDE and cytotoxicity and mutation assays were performed as described in Materials and Methods. The order of treatment used for vitamin E is briefly described in Figure 11. As can be seen from Table 16, 500.0μM vitamin E did not have any effect on the survival of normal human fibroblasts. The data does show, however, that vitamin E significantly inhibits the cytotoxicity of human fibroblasts induced by

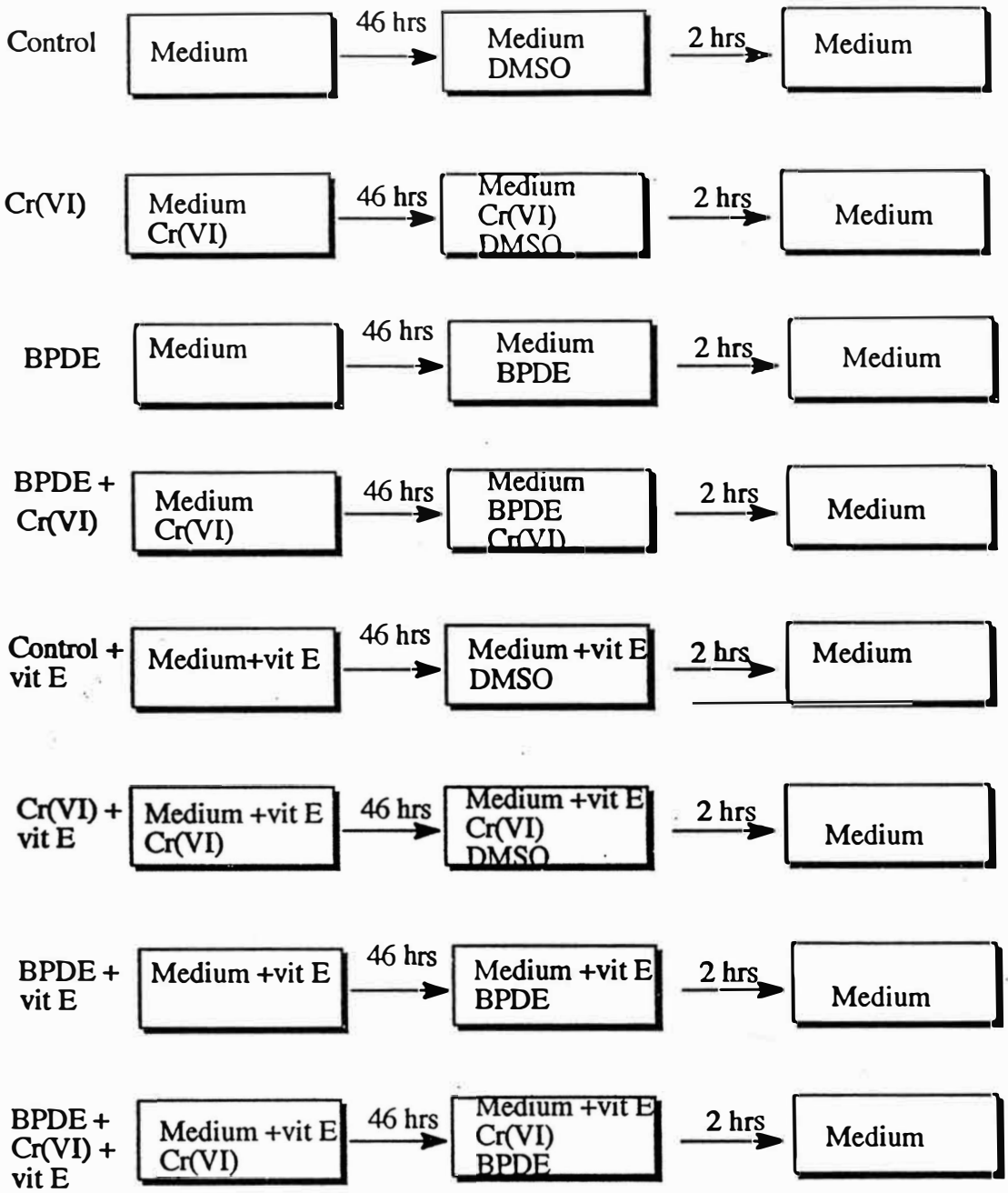

Figure 11. Vitamin E Treatment Protocol.

The concentrations used were: 0.30μM Cr(VI), 0.30μM BPDE and 500.0μM Vit E.

Table 16

The Effect of 500.0μM vitamin E on the Cytotoxicity Induced by 0.30μM Cr(VI) and 0.30 μM BPDE in Human Fibroblasts

	Average % Relative Survival ± (Standard Deviation)	
Treatment	Experiment 1	Experiment 2
Control	100.0	100.0
Cr(VI)	53.3 (3.2)	46.6 (1.8)
BPDE	52.3 (1.6)	47.6 (1.6)
Cr(VI) + BPDE	9.9 (0.5)	7.4 (1.3)
Control + vitamin E	104.4 (3.2)	105.7 (2.9)
Cr(VI) + vitamin E	74.3 (3.2)	74.7 (2.0)
BPDE + vitamin E	51.6 (2.3)	47.9 (1.3)
Cr(VI) + BPDE + vit E	42.0 (2.4)	38.9 (2.8)

Cr(VI) as compared to controls suggesting that an oxidative component might be involved in the cytotoxicity of Cr(VI). Vitamin E did not have any significant effect on BPDE induced cytotoxicity which might suggest that BPDE induced cytotoxicity may not involve an oxidative component. The cytotoxicity induced by the combined treatment of Cr(VI) and BPDE was also significantly reduced by vitamin E, which might be due to the reduction of oxidative species and similar to the protective effect vitamin E had on Cr(VI) cytotoxicity . Similar protection effect against chromium cytotoxicity, in the presence of vitamin E were also reported by Sugiyama (Sugiayama, 1991).

Table 17 shows the effect of vitamin E on Cr(VI) and BPDE induced mutations at the HGPRT locus. Vitamin E alone showed no ability to induce 6-thioguanine

resistant mutations in human fibroblasts. In addition, vitamin E did not have any significant effect on the mutation rate induced by BPDE at the HGPRT gene supporting the hypothesis that oxidative stress might not be involved in the mutagenicity of BPDE. The mutation frequency at the HGPRT gene induced by Cr(VI) was slightly less in the presence of vitamin E which might suggest the involvement of oxidative stress in Cr(VI) mutagenicity, however, since the mutation frequency at the HGPRT gene induced by Cr(VI) is very small the relative difference may not be significant. When human fibroblasts were treated with both Cr(VI) and BPDE in the presence of vitamin E, the mutation at the HGPRT gene was comparable to the mutation rate of BPDE alone. The increase in mutations in the presence of vitamin E was significant and consistent. These results show that vitamin E can completely block the effect of chromium on the mutational rate of BPDE suggesting an oxidative stress mechanism. Vitamin E might be playing a role by decreasing the oxidative species induced by Cr(VI) and thus decreasing the stimulation of DNA repair enzymes.

We next examined the effect of 130units/ml catalase on the cytotoxicity as well as on the mutations at the HGPRT gene induced by Cr(VI) and BPDE in human fibroblasts in order to see if different antioxidants have the same response to the cytotoxicity and mutation rate at the HGPRT gene induced by chromium and BPDE. The order of treatment used is briefly illustrated in Figure 12 and results are shown in Tables 18 and 19. Catalase, unlike vitamin E, showed an effect on the survival rate of human fibroblasts by slightly decreasing the cloning ability of cells relative to control. At present, we do not have any explanation for the cause of cytotoxicity of catalase to human fibroblasts. Catalase, like vitamin E, did not have any detectable effect on the BPDE induced cytotoxicity in human fibroblasts supporting the previous conclusion that oxidative mechanism is not involved in the BPDE induced cytotoxicity. The

Table 17

The Effect of 500.0μM Vitamin E on the Mutation Rate at the HGPRT Gene Induced by 0.30μM Cr(VI) and 0.30μM BPDE in Human Fibroblasts

	Mutants / 10^6 Viable Cells	
Treatment	Experiment 1	Experiment 2
Control	0.00	0.00
Cr(VI)	11.50	8.41
BPDE	132.47	123.08
Cr(VI) + BPDE	23.04	29.67
Control + vitamin E	0.00	0.00
Cr(VI) + vitamin E	6.30	0.00
BPDE + vitamin E	140.77	127.99
Cr(VI) + BPDE + vit E	133.77	127.78

Cytotoxicity induced by the combination of Cr(VI) and BPDE was also reduced in the presence of catalase but to a lesser extent than vitamin E. This reduced protection of cytotoxicity might be due to the slight cytotoxic effect that catalase caused in human fibroblasts. This data indicates the involvement of an oxidative component in chromium cytotoxicity.

Table 19 shows the effect of catalase on BPDE and Cr(VI) induced mutations at the HGPRT gene in human fibroblasts. Catalase, like vitamin E, did not show any detectable mutagenicity in human fibroblasts. The mutations induced by Cr(VI) were slightly reduced in the presence of catalase but it may not be significant since the mutation frequency induced by Cr(VI) itself was very small. Similar to the effects of

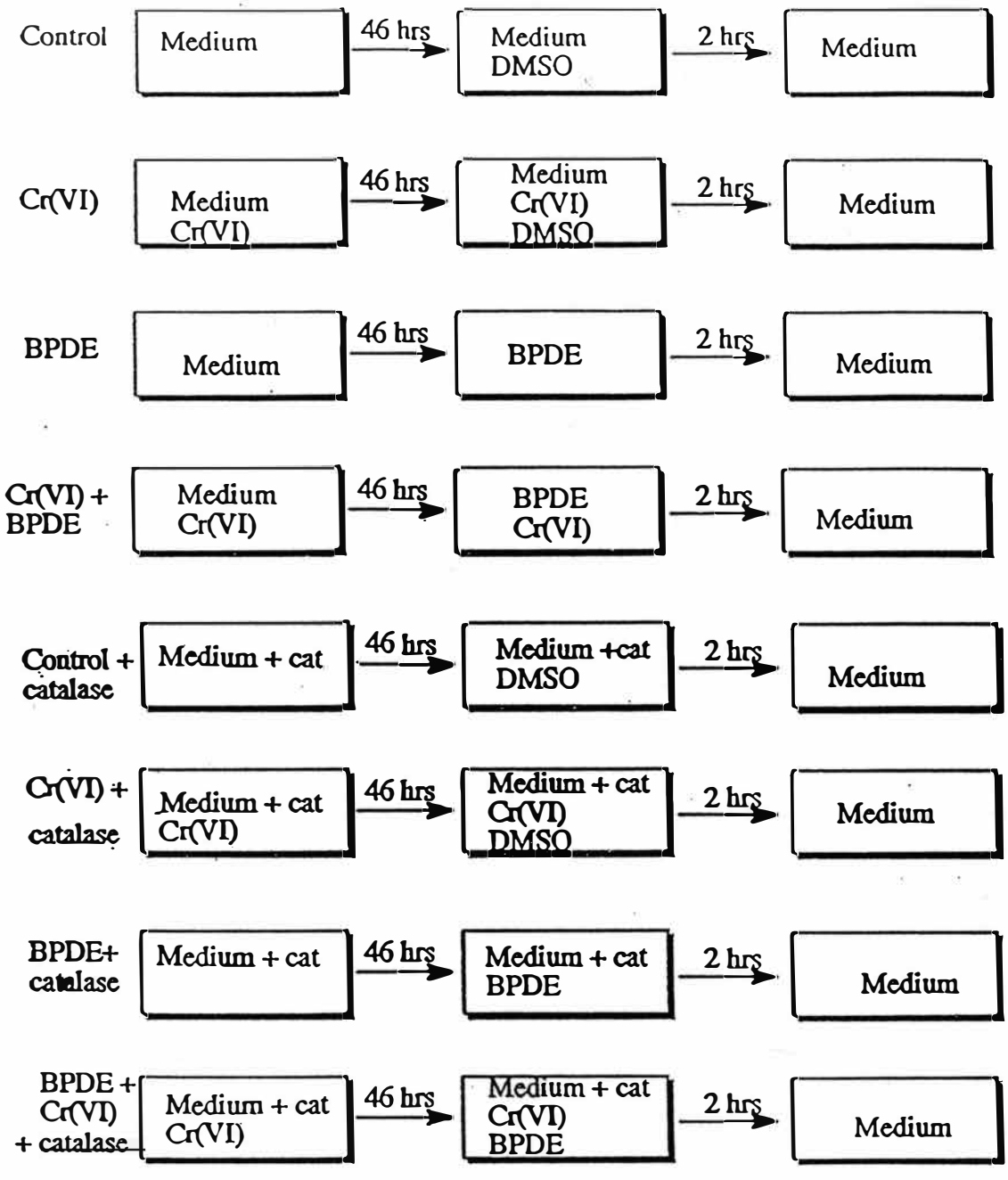

Figure 12. Catalase Treatment Protocol.

The concentrations used were: 0.30μM Cr(VI), 0.3μM BPDE and 130u/ml catalase.

vitamin E, catalase did not have any marked effect on the mutation rate at the HGPRT gene induced by BPDE which again suggests that oxidative effects might not be involved in the mutations induced by BPDE. Catalase increased the mutation frequency of human fibrobalsts induced by the combined treatment of Cr(VI) and BPDE in a similar manner to vitamin E, but complete reversal was not obtained. Again this reduction might be partly due to the slight toxicity of catalase.

Table 18

The Effect of 130.0u/ml Catalase on the Cytotoxicity Induced by 0.30μM Cr(VI) and 0.30μM BPDE in Human Fibroblasts

	% Average Relative Survival ± (Standard Deviation)	
Treatment	Experiment 1	Experiment 2
Control	100.0	100.0
Cr(VI)	39.5 (1.2)	45.0 (3.1)
BPDE	42.4 (1.5)	46.0 (3.7)
Cr(VI) + BPDE	1.2 (0.2)	5.0 (1.2)
Control + catalase	88.8 (3.8)	91.2 (2.8)
Cr(VI) + catalase	58.8 (3.6)	63.6 (2.7)
BPDE + catalase	43.3 (3.3)	44.3 (2.1)
Cr(VI) + BPDE + catalase	11.7 (1.1)	21.8 (4.3)

Table 19

The Effect of 130.0u/ml Catalase on the Mutation Rate at the HGPRT Gene Induced by 0.30μM Cr(VI) and 0.30μM BPDE in Human Fibroblasts

	Mutants/10^6 Viable Cells	
Treatment	Experiment 1	Experiment 2
Control	0.00	0.00
Cr(VI)	9.89	12.04
BPDE	136.15	129.87
Cr(VI) + BPDE	16.37	14.83
Control + catalase	0.00	0.00
Cr(VI) + catalase	4.86	5.67
BPDE + catalase	131.28	126.74
Cr(VI) + BPDE + catalase	92.85	93.96

CHAPTER IV

CONCLUSIONS

The present study was designed to examine the effect of Cr(VI) on the BPDE induced cytotoxicity and BPDE induced mutations at the HGPRT gene in human fibroblasts. In the studies presented here, we have shown that the effect of Cr(VI) on BPDE induced cytotoxicity is more than additive, whereas the effect of Cr(VI) on BPDE mutagenicity at the HGPRT gene is antagonistic. Our results have also indicated that the effect of Cr(VI) on BPDE cytotoxicity does not appear to depend on the concentration of Cr(VI) in the range tested (0.20μM-0.30μM). The response obtained from BPDE induced cytotoxicity was also independent on the order of treatment of Cr(VI) and/or BPDE. Unlike BPDE induced cytotoxicity, the BPDE induced mutations at the HGPRT gene is dependent on the concentration of Cr(VI) as well as the order of treatment. In the present study, it has been shown that pretreatment and simultaneous treatment with Cr(VI) using 0.30μM BPDE has an antagonistic effect on BPDE induced mutation at the HGPRT gene, while posttreatment with Cr(VI) does not have any significant effect on the mutation rate induced by BPDE at the HGPRT gene.

In an attempt to better understand the observed effects, the effects of two antioxidants, vitamin E and catalase on both Cr(VI) and BPDE induced cytotoxicity and mutations at the HGPRT locus were examined. The presence of the antioxidants, vitamin E and catalase increased the observed cloning ability induced by the combined treatment of BPDE and Cr(VI). Furthermore, vitamin E and catalase reversed the protective effect of chromium pretreatment on BPDE mutations. Vitamin E reversed the

effect to a higher degree than catalase. Results indicate that the observed effects are at least partly due to oxidative stress.

One possible reason for the different effects observed by Cr(VI) on BPDE induced cytotoxicity and mutations at the HGPRT gene could be that the toxicity occurs due to damage at the protein level and not at the DNA levels. Recently, Persson and his coworkers have shown that the DNA binding of AP-1 (activator protein-1) transcription factor, an important protein which is involved in transcription, can be inhibited by the formation of a BPDE-adduct (Persson et al., 1996). Induction of DNA repair enzymes by chromium would not protect cells from BPDE cytotoxicity, if it were due to effects of proteins. Another possibility is that DNA repair may be different at different genes. The repair enzymes which repair the damage at the HGPRT gene might not repair the damage leading to cytotoxicity as efficiently. In order to have a better understanding of the observed effects, however, more specific studies addressing the mechanism of the observed effects need to be done.

Appendix A

Statistical Analysis

Statistical Analysis

Cytotoxicity assays for each condition in every experiment were done in triplicate, and the cloning ability of the cells was expressed as the average percent relative survival ± the standard deviation. A difference between conditions greater than 2 standard deviations was considered to be significant.

Ten dishes per condition were used for the mutation rate assays and the total number of colonies obtained from ten dishes was used to calculate the number of mutants. Mutation rate was expressed as number of mutants per one million viable cells. Significant differences were determined by first calculating the ratio of the mutation rate for the conditions in question versus the BPDE mutation rate. A significant difference between the two conditions was defined as a difference of more than three times the variation between these ratios of the two or three experiments conducted.

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