THE EFFECT OF 2,4-D ON GENE EXPRESSION IN CULTURED CELLS

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ABSTRACT

The cytotoxic effects of exposure to low concentrations of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) that are typically found in groundwater were investigated, *in vitro*. Most 2,4-D toxicology studies use high concentrations of the herbicide that are above those typically found in groundwater and measure overt biological endpoints. In contrast, this thesis examines the effects of low concentrations of 2,4-D and measures more subtle and sensitive endpoints such as gene expression and the generation of reactive oxygen species. This work derives from recent cDNA microarray analysis conducted in our laboratory that revealed significant alterations in the expression of 238 genes in cells exposed to nanomolar (nM) concentrations of a commercial formulation of 2,4-D. These findings are extended in this thesis to include the *in vitro* cytotoxic effects of low concentrations of both technical and commercial 2,4-D on two cell lines. Cells derived from liver (HepG2) and kidney (HEK293) respectively, were chosen, since liver and kidney are known to metabolize 2,4-D *in vivo*. Cell viability was measured using the Resazurin assay, reactive oxygen species (ROS) were measured with 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA), and real time–polymerase chain reaction (RT-PCR) was used to assess changes in mRNA expression while protein expression was examined by Western blot.

Cell viability studies revealed that low environmental concentrations (0.1 to 100 nM) of 2,4-D induced small, but statistically significant decreases in cell viability. No concentration or time-dependent decreases in cell viability were observed in cells exposed to either forms of low environmental 2,4-D concentrations. HEK293 cells were more susceptible than HepG2 cells to the toxic effects of both forms of 2,4-D, having statistically significant lower viability at all exposure concentrations and durations. Both forms of 2,4-D reduced cell viability in both cell lines, suggesting that cytotoxicity was induced directly by 2,4-D, and not by the 'inert ingredients' in the commercial formulation.

The ROS assays illustrated that 2,4-D induced statistically significant ROS production in HepG2 and HEK293 cell cultures at concentrations greater than 10 µM and 100 nM respectively. This was both a concentration and time-dependent effect in

both cell lines. Although HEK293 cells were more susceptible to 2,4-D, they had 50 to 70% less ROS production than HepG2 cells, at all exposure concentrations and times.

The RT-PCR and Western blot analyses showed that exposure of HepG2 and HEK293 cells to low 2,4-D concentrations induced (< 2 fold) alterations in mRNA and protein levels of FTL, FTH1 and PCNA however these changes did not consistently vary with concentration.

Taken together, cell viability, ROS and gene expression studies show that low environmental 2,4-D concentrations induced subtle *in vitro* cytotoxic effects. However we have no evidence that these subtle changes pose a serious health threat to exposed humans.

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LIST OF ABBREVIATIONS

%	percent
% (v/v)	percent volume per volume
% (w/v)	percent weight per volume
°C	degrees Celsius
µg	microgram
µg/L	microgram per liter
µg/mL	microgram per milliliter
µL	microliter
µM	micromole
2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DB	4-(2,4-dichlorophenoxy) butyric acid
2,4-DP	2-(2,4-dichlorophenoxy) propionic acid
2,4-D CoA	2,4-dichlorophenoxyacetyl-S-acyl-CoA thioester
2,4-D DMA	dimethylamine salt of 2,4-dichlorophenoxyacetic acid
2,4-D-SG	2,4-dichlorophenoxyacetyl-*S*-acyl-glutathione
2',7'-DCF	2',7'-dichlorofluorescein
2',7'-DCFH	2',7'-dichlorofluorescin
2',7'-DCFH-DA	2',7'-dichlorofluorescin diacetate
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4,5-TP	2-(2,4,5-trichlorophenoxy) propionic acid
AHH-1	immortalized human lymphoblastoid cell line
ANOVA	analysis of variance
ALT	alanine aminotransferase

AP	alkaline phosphatase
AST	aspartate aminotransferase
ATCC	American type culture collection
BC	British Columbia
bp	base pair
C6	rat glioma cell line
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaCo2	immortalized human colon cell line
cDNA	complementary deoxyribonucleic acid
CDW	Canadian Federal-Provincial-Territorial Committee on Drinking Water
cm^2	square centimeter
CNS	central nervous system
CO_2	carbon dioxide
COS	monkey cell line
CYP	cytochrome P450
$DT_{½}$	dissipation half-life
DA	dopamine
DEPC	diethyl pyrocarbonate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM	dulbecco's minimum essential medium

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DPBS	dulbecco's phosphate buffered saline solution
DTT	DL-dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylene diamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
EXTOXNET	extension toxicology network
FBS	fetal bovine serum
Fe^{2+}	ferrous ion (reduced form of iron ion)
Fe^{3+}	ferric ion (oxidized form of iron ion)
FEK4	primary human skin fibroblast cell line
FSU	fluorescent signal unit
FTH1	ferritin heavy polypeptide 1
FTL	ferritin light polypeptide
g	gram
GAPDH	glyceraldehyde-6-phosphate dehydrogenase
GD	gestation day
g/L	gram per liter
GSH	glutathione
GSH-Px	glutathione peroxidase
GUP	general use pesticide
GUS	groundwater ubiquity score

Hb	hemoglobin
HBG BC2	immortalized human hepatoma cell line
HeLa	immortalized human cervical cancer cell line
HEK293	immortalized embryonic human renal cell line
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HepG2	immortalized human hepatocellular carcinoma cell line
hr	hour
HSD	honestly significant difference
HSE	heat shock element
HSF1	heat shock factor 1
H_2O_2	hydrogen peroxide
HO-1	heme oxygenase - 1
Hsp	heat shock protein
Hsp72	heat shock protein 72 (inducible form of Hsp70)
IARC	international agency for research on cancer
IMAC	interim maximum acceptable concentration
IRE	iron responsive element
IRP	iron regulatory protein
JC-1	5, 5',6',6'-tetrachloro-1,1',3,3-tetraethybenimidazolycarbocyanine iodide
J/m^2	joules per square meter
KCL	potassium chloride
KH_2PO_4	potassium phosphate monobasic
K_{oc}	adsorption coefficient

K_{ow}	partition coefficient
LAMA-84	immortalized human chronic myeloid leukemic cell line
LDH	lactate dehydrogenase
M	mole per litre
MAC	maximum admissible concentration, or maximum acceptable concentration
MCL	maximum contaminant level
MCPA	4-chloro-2-methyl-phenoxyacetic acid
MCPP	α-(4-chloro-2-methylphenoxy) propionic acid
MDA	malondialdehyde
mg	milligram
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
mg/kg	milligram per kilogram
mg/kg/day	milligram per kilogram per day
mg/L	milligram per liter
min	minute
mL	milliliter
mm	millimeter
mM	millimole
mRNA	messenger ribonucleic acid
MtF	mitochondrial ferritin
MTT	3-(4,5-dimethylthazol-2-yl)-2,5 diphenyltetrazolium bromide
NAC	*N*-acetyl-L-cysteine

NaAc	sodium acetate
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic
NCBI	National Center for Biotechnology Information
NH_4Ac	ammonium acetate
NIH	National Institute of Health
nm	nanometer
nM	nanomole
NOAEL	no observed adverse effect level
NOEL	no observed effect level
O_2	oxygen
$O_2^{\cdot -}$	superoxide anion
OFR	oxygen free radical
OH^-	hydroxide ion
$OH^{\cdot}$	hydroxyl radical
PAN	pesticide action network
PBS	phosphate buffered saline solution
PCNA	proliferating cell nuclear antigen
PD	postpartum day
PMRA	pest management regulatory agency
PMSF	phenylmethanesulfonyl fluoride
Polβ	DNA polymerase beta
Ref-1	apurinic/apyrimidinic endonuclease
RLT	RNeasy® lysis buffer (containing guanidine thiocyanate)

RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RPE	RNeasy® wash buffer
RT-PCR	real time – polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TBS	tris phosphate buffered saline solution
TBST	tris phosphate buffered saline solution – tween 20
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TE	tris[hydroxymethyl]aminomethane - ethylene diamine tetraacetic acid
TEMED	N,N,N',N'-tetra-methylethylenediamine
TUNEL	terminal transferase dUTP nick end labeling
Tris	(tris[hydroxymethyl]aminomethane)
Tris-HCL	tris[hydroxymethyl]aminomethane – hydrochloric acid
Tween	polyoxyethylene sorbitol monolaurate
U937	immortalized human histocytic lymphoma cell line
U	units
U/μL	units per microliter
U/mL	units per milliliter
US EPA	United States Environmental Protection Agency

UTR	untranslated region
UV	ultraviolet
UVA	ultraviolet A
UVC	ultraviolet C
WHO	World Health Organization
WSDOT	Washington State Department of Transportation
x g	times gravity
YAC-1	mouse lymphoma cell line

1. INTRODUCTION

In 1944 (PAN Database, 2004), 2,4-dichlorophenoxyacetic acid (2,4-D), a chlorophenoxy herbicide (Li *et al*., 2003) was introduced into the commercial market. The herbicide is extensively used in the control of woody plants (Li *et al*., 2003), broadleaf (Environment Canada, 1991) and aquatic weeds (Environment Canada, 1991). It has been described as the most widely used herbicide in the world (Holland *et al*., 2002; Kwan and Chu, 2004; WSDOT, 2006).

The herbicide typically does not accumulate in the environment (air, water or soil), as it is readily degraded by biological, chemical and physical processes (WHO, 1984). However, alterations in biological, chemical and physical factors may result in the increased persistence of the herbicide in the environment. For instance, anaerobic conditions favours the persistence of 2,4-D in aquatic environments (WHO, 2003), such as groundwater. The persistence of 2,4-D in the environment questions the potential adverse human health effects that may occur following exposure to the chemical for acute or chronic durations of time. This is especially true with respect to its persistence in groundwater; since groundwater may be a significant and possibly the sole source of water for humans residing in rural areas.

Approximately one quarter of Canadians depend on groundwater, of which two-thirds of these are rural habitants (Environment Canada, 2004a). Groundwater is a vital resource (Environment Canada, 2004b) for rural residents (Rudolph *et al*., 1998), for all of their daily water needs, such as drinking (Environment Canada, 2004b). Unfortunately, groundwater contamination with chemicals, such as 2,4-D, has become an increasing problem in Canada (Environment Canada, 2004c). The herbicide, 2,4-D, has been one of the most frequently detected herbicides in groundwater (Grover *et al*., 1997).

Since, chemical contamination of groundwater may pose a human health threat, regulatory agencies such as the Canadian Federal-Provincial-Territorial Committee on Drinking Water (CDW), United States Environmental Protection Agency (US EPA) and

World Health Organization (WHO) have established guideline levels for contaminants in drinking water to protect exposed individuals. Thus, it is important to determine the levels of contaminants, such as 2,4-D, that may be found in groundwater and address the potential adverse human health effects that may be associated with exposure to these contaminants. The herbicide is usually found in groundwater at concentrations (<100 nM (<22 μg/L)) that are below established drinking water guidelines. However, the potential adverse human health effects associated with acute and chronic exposure to low environmental 2,4-D concentrations has not yet been evaluated.

Results from previous *in vivo* and *in vitro* toxicological studies that have tested high doses (1 to 600 mg/kg) or concentrations (1 μM to 45 mM) of the herbicide, suggest that the chemical may induce a plethora of adverse human health effects. These adverse human health effects include; neurotoxicity (Bortolozzi *et al*., 1999, 2004; Garcia, 2001, 2004; Rosso *et al*., 2000), immunotoxicity (de la Rosa, 2003; Lee *et al*., 2001), genotoxicity (Amer and Aly, 2001; Holland *et al*., 2002; Madrigal-Bujaidar *et al*., 2001; González *et al*., 2005; Zeljezic and Garaj-Vrhovac, 2004; Venkov *et al*., 2000), carcinogenesis (Ge *et al*., 2002; Holland *et al*., 2002; Ozaki *et al*., 2001), reproductive toxicity (Amer and Aly, 2001; Madrigal-Bujaidar *et al*., 2001), developmental toxicity (Alpöz *et al*., 2001; Bortolozzi *et al*., 2004; Charles *et al*., 2001; Lee *et al*., 2001) cytotoxicity (Bharadwaj *et al*., 2005; De Moliner *et al*., 2002; Duchnowicz and Koter, 2003; Kaioumova *et al*., 2001a; Palmeira *et al*., 1995; Tuschl and Schwab, 2003, 2004, 2005; Venkov *et al*., 2000) to organ toxicity, including hepatotoxicity (Bharadwaj *et al*., 2005; Charles *et al*., 1996a; Paulino *et al*., 1996; Tuschl and Schwab, 2003, 2004, 2005) and nephrotoxicity (Ozaki *et al*., 2001).

In contrast to 2,4-D toxicology, the cell and molecular mechanism(s) of 2,4-D toxicity has not been extensively studied. The results from some existing *in vivo* and *in vitro* studies suggest that the mechanisms of 2,4-D toxicity may include apoptosis (de Moliner *et al*., 2002; Kaioumova *et al*., 2001a, 2001b; Tuschl and Schwab, 2003, 2004; 2005; Zychlinski and Zolnierowicz, 1990), alteration of cell physiology (uncoupling of oxidative phosphorylation, depression of transmembrane potential) (Kaioumova *et al*., 2001b; Palmeira *et al*., 1994; Tuschl and Schwab, 2003), formation of a reactive

metabolite (Li *et al*., 2003) and ROS mediated oxidative stress (Bukowska, 2003; Duchnowicz and Koter, 2003; Palmeira *et al*., 1995; Teixeira *et al*., 2004).

Thus, although the above findings are intriguing and provide evidence of its toxicology and mechanisms of toxicity, the findings were primarily derived from classical toxicological studies that tested high doses and concentrations that do not reflect 2,4-D concentrations that are typically found in the environment. Furthermore, previous studies concentrated on overt biological endpoints, and have not considered subtle cell and molecular changes, such as alterations in gene expression patterns, which are more often sensitive, than currently employed biological endpoints (Aardema *et al*., 2002).

Recently, in our laboratory, it was illustrated that exposure to low nanomolar (nM) environmentally relevant concentrations of a commercial formulation of 2,4-D (containing the dimethylamine salt of 2,4-D; 2,4-D DMA) for 24 hrs resulted in decreased human hepatocellular carcinoma (HepG2) cell viability (Bharadwaj *et al*., 2005). Additionally, cDNA microarray analyses illustrated significant alterations in 238 genes. These genes included those associated with DNA repair and cancer genes (proliferating cell nuclear antigen (PCNA)), immune response genes (interleukin 1 receptor-like 1), cell cycle control genes (CDC-like kinase 1) and stress response genes (ferritin light polypeptide (FTL), ferritin heavy polypeptide 1 (FTH1)). The expression levels of FTL, FTH1 and PCNA genes were consistently up-regulated (approximately 2 fold) over the range of 2,4-D concentrations. The up-regulation of FTL, FTH1 and PCNA genes has been suggested to play a protective role in response to oxidative stress (Balajee *et al*., 1999; Balla *et al*., 1992; Epsztejn *et al*., 1999; Holmes *et al*., 2002; Lin and Girotti, 1997; Orino *et al*., 2001; Regan *et al*., 2002; Savio *et al*., 1998). Therefore, based on previous cDNA microarray analysis and *in vivo* and *in vitro* studies, it is possible that ROS mediated oxidative stress may be one potential mechanism of 2,4-D toxicity.

1.1 Hypotheses

Based on previous *in vivo* and *in vitro* studies, in this thesis it was hypothesized that:

(I) Exposure to environmental 2,4-D concentrations will result in cytotoxicity in exposed human cell cultures.

(II) Reactive oxygen species are produced in human cell cultures exposed to environmental 2,4-D concentrations.

(III) Exposure to environmental 2,4-D concentrations will induce up-regulation of FTL, FTH1 and PCNA gene expression detectable at the level of mRNA and protein accumulation.

1.2 Objectives

The main objectives of this study were to determine:

(I) If environmentally realistic concentrations of 2,4-D will induce cytotoxicity in exposed human cell cultures

To achieve this objective, human HepG2 and embryonic renal (HEK293) cell cultures were exposed to increasing concentrations of a technical grade or commercial formulation of 2,4-D. Technical and commercial 2,4-D form were utilized to determine if cytotoxic effects are enhanced by the presence of 'inert ingredients' in the commercial formulation or a result of the active ingredient; 2,4-D. To determine if there were concentration-dependent cytotoxic effects, cell cultures were exposed to 0.1nM to 1mM. Cells were exposed for 6, 24, 48 and 72 hrs to determine if more pronounced toxic effects would be produced with longer durations of exposure. Cytotoxicity was determined as a measurement of cell viability, using the dye, Resazurin (Sigma-Aldrich Canada Ltd.).

(II) If ROS are produced in human cell cultures exposed to environmental 2,4-D concentrations

To achieve this objective, HepG2 and HEK293 cell cultures were exposed to increasing concentrations of commercial 2,4-D for 2 to 6 hrs. Production of ROS was measured fluorometrically, using 2',7'-dichlorofluorescin diacetate (2',7'- DCFH-DA) (Sigma-Aldrich Canada Ltd.).

(III) If environmentally realistic 2,4-D concentrations induce up-regulation of FTL, FTH1 and PCNA gene expression detectable at the level of mRNA accumulation in exposed human cell cultures

To achieve this objective, HepG2 and HEK293 cell cultures were exposed to increasing concentrations of commercial 2,4-D for 24 hrs. The RNA extracted from cell cultures was used in first-strand cDNA synthesis. Synthesized cDNA was used as the template in real time – polymerase chain reaction (RT-PCR) assays to measure FTL, FTH1 and PCNA mRNA accumulation levels.

(IV) If environmentally realistic concentrations of 2,4-D induce up-regulation of FTL, FTH1 and PCNA gene expression detectable at the level of protein accumulation

To achieve this objective, HepG2 and HEK293 cell cultures were exposed to increasing concentrations of commercial 2,4-D for 24 hrs. Protein extracted from cell cultures was subjected to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and FTL, FTH1 and PCNA protein expression was detected using conventional western blot analysis and quantified densitometrically.

2. LITERATURE REVIEW

The main objective of the research completed in this thesis was to assess the adverse human health effects that may be caused by exposure to low concentrations of 2,4-D that are typically found in groundwater. Therefore, in order to understand the significance and rationale for conducting this current study, a review of 2,4-D (development and use, mechanism of action, environmental fate, relationship with groundwater, 2,4-D toxicology, mechanism of toxicity) is presented in the following paragraphs. A review of ferritin and PCNA gene (structure, function, relationship between gene expression and oxidative stress) is also presented that will allow for an understanding of why, in this thesis; (1) it was hypothesized that, 2,4-D exposure will induce intracellular ROS production, and (2) increases in the expression of ferritin and PCNA mRNA and protein in response to 2,4-D exposure may suggest ROS mediated oxidative stress as a mechanism of 2,4-D toxicity. Finally, a brief review of ROS is presented. The review of ROS is intended for the reader to attain some degree of understanding of ROS, its formation, sources, and association with oxidative stress. The review also includes information on the intricate relationship between iron, ROS and oxidative stress that will enable for an understanding of why the particular up-regulation of intracellular ferritin mRNA or protein expression in the present study may suggest chemical insult via the mechanism of ROS mediated oxidative stress. Thus, taken all together the following literature review allows for a better appreciation, and understanding of why this current study was conducted.

2.1 2,4-Dichlorophenoxyacetic acid

2.1.1 2,4-D: development and use

The herbicide, 2,4-D, a chlorophenoxy compound (Li *et al*., 2002; PAN Pesticide Database, 2004) was introduced as the first phenoxy herbicide, or hormone weed killer (Ware, 1978). Additional members of the chlorophenoxy family of herbicides include; 2-(2,4-dichlorophenoxy) propionic acid (dichloroprop, 2,4-DP), 4-(2,4-

dichlorophenoxy) butyric acid (2,4-DB), 2-(2,4,5-trichlorophenoxy) propionic acid (fenoprop, silvex, 2,4,5-TP), α-(4-chloro-2-methylphenoxy) propionic acid (mecoprop, MCPP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 4-chloro-2-methyl-phenoxyacetic acid (MCPA) (WHO, 1984). The herbicide, 2,4-D was synthesized in 1942 and introduced into the commercial market over 60 years ago (WSDOT, 2006). It is currently used in the control of: (1) broadleaf weeds (Environment Canada, 1991; Stevens and Sumner, 1991; US EPA, 2005; Li *et al*., 2003) that are found in cereal crops (Kaioumova *et al*., 2001; WHO, 1984) (i.e wheat and corn (US EPA, 2005), pastures, lawns (Kaioumova *et al*., 2001; WHO, 1984) and recreational areas (i.e parks and golf courses (Kaioumova *et al*., 2001; Kwan and Chu, 2004; WHO, 1984)), (2) aquatic weeds (WHO, 1991; Zeljezic and Garaj-Vrhovac, 2004) and (3) woody plants (Li *et al*., 2003; US EPA, 2005) that are found along roadsides, railways and utilities right of way (US EPA, 2005). The herbicide is the third most widely used herbicide in the USA and Canada (WSDOT, 2006). For instance, as much as 20 million kilograms of 2,4-D are annually applied to the Canadian prairies (Grover *et al*., 1997). This suggests that there may be a high risk of human exposure to the chemical from the environment.

The application rate of 2,4-D is dependent upon a combination of (1) the physical and chemical formulation of the herbicide and (2) the target. For example, amine salts of 2,4-D are applied at a rate of 0.2 to 2.0 kg active ingredient per hectare to control broad-leaved weeds in cereal crops, pastures, lawns, golf courses and parks (WHO, 1989). Ester formulations of 2,4-D are applied at a higher rate of up to 6.0 kg active ingredient per hectare to suppress weeds, brush and deciduous trees along rights-of-way, conifer plantations and conifer reafforestation areas (WHO, 1989). Granular forms of 2,4-D may be applied at rates ranging from 1 to 122 kg active ingredient per hectare to aquatic weeds (WHO, 1989).

The herbicide is commercially available to the public and is considered a general use pesticide (GUP) (Beyond pesticides, 2004; EXTOXNET, 1996). Thus, a license is not required to use or purchase this herbicide (Beyond pesticides, 2004). It is commercially prepared as esters and amine salts (EXTOXNET, 1996; PMRA, 2005; WHO, 1984). The herbicide is available to consumers (i.e. farmers and homeowners) for purchase under commercial trade names such as agent white, bladex-B, red devil dry

weed killer, weedar (EXTOXNET, 1996; US EPA, 2005), weedone (EXTOXNET, 1996; Stevens and Summer, 1991), savage, planotox, aqua-kleen, barrage, malerbane, lawn-keep, salvo, weedtrine-II and plantguard (EXTOXNET, 1996). Commercial formulations of 2,4-D are found as emulsions, aqueous solutions and dry compounds (EXTOXNET, 1996).

2.1.2 Mechanism of action of 2,4-D as a herbicide

The parent compound of 2,4-D is an acid (Charles *et al*., 1996; PMRA, 2005). The parent 2,4-D acid has an empirical formula of $C_8H_6Cl_2O_3$ and a molecular weight of 221.04 (PMRA, 2005; Stevens and Sumner, 1991). Physically, the acid is a white powder with a slight phenolic odor (Stevens and Sumner, 1991). It is the parent acid that is the active ingredient in 2,4-D commercial formulations (PMRA, 2005). An active ingredient in herbicide products is one that induces the desired effect (i.e. plant regulation, defoliation) (US EPA, 2006) on the plant. The parent acid binds to target sites on plants, while the amine or ester portion of the chemical allows for absorption of the herbicide into plants (PMRA, 2005). The commercial forms of 2,4-D differ in their ability to facilitate absorption into the plant (PMRA, 2005). For instance, the more hydrophobic form (i.e. ester form) of 2,4-D allows for easier penetration of the herbicide into the waxy cuticle of the leaves of plants, while the more hydrophilic form (i.e. amine form) allows for easier uptake of the herbicide from the roots of plants (PMRA, 2005).

The herbicide functions as a systemic herbicide (EXTOXNET, 1996), and is thus translocated throughout the plant (Ware, 1978). It is a chlorinated form (WHO, 1984) or structural analogue of the natural plant hormone, auxin indole-3-yl-acetic acid (Venkov *et al*., 2000). Auxin indole-3-yl-acetic acid plays an integral role in the division, differentiation and elongation of plant cells (Venkov *et al*., 2000). Thus, since it is a synthetic 'auxin-like' (Reuber, 1983) herbicide, the primary mode of action of 2,4-D is to alter plant metabolism (Li *et al*., 2003). The alteration of plant metabolism results in over stimulation of plant growth and ultimately plant death (Chu *et al*., 2004; Holland et al., 2002).

2.1.3 Environmental fate of 2,4-D in water

The herbicide is considered a biodegradable compound (Industry Task Force II on 2,4-D Research Data, 1999) as it is readily degraded by biological, chemical and physical processes (WHO, 1984). Thus, 2,4-D is believed to be non-persistent or non-accumulative in the environment (atmosphere, soil and aquatic (i.e. surface and groundwater)) (WHO, 1984). However, the chemical has been found to have a half-life of approximately 800 to 1900 days in experimental groundwater environments (reviewed by Cox. (1999)); suggesting that 2,4-D may be quite persistent in groundwater.

The rate of 2,4-D degradation in water is dependent upon several factors (i.e. (water type, temperature, nutrient levels, sunlight and oxygen content) (reviewed by Environment Canada, 1991). For instance 2,4-D is more persistent in groundwater, than surface water, possibly due to increased photolysis of 2,4-D residues in surface waters (i.e. lakes, ponds, streams (Cox, 1999)). Extreme low or high water temperatures (Rushings *et al*., 2007) and low nutrient concentrations (Government of Ontario, 2003) also favor 2,4-D persistence in water. These conditions inhibit the growth of microorganisms, and microorganisms are responsible for the biodegradation of 2,4-D in water (WHO, 2003). Oxygen content also plays a crucial determinant factor in the rate of 2,4-D degradation in water. Under aerobic conditions, the half-life of 2,4-D in water can be equal to or greater than 7 days (reviewed by Environment Canada, 1991). In contrast, under anaerobic conditions, the half-life of 2,4-D in water may be greater than 80 to 120 days (reviewed by Environment Canada, 1991). Thus, depending upon biological, chemical and physical factors (mentioned above), 2,4-D may be fairly persistent in aquatic environments, such as groundwater. This is a grave health concern for humans who may be acutely or chronically exposed to the chemical via consumption of groundwater.

2.1.4 2,4-D and groundwater

Groundwater, described as one of nature's hidden treasures (Environment Canada, 2004b) is the world's largest reservoir of fresh water (Environment Agency, 2007). It is located below the earth's water table (Environment Agency, 2007;

Environment Canada, 2004; U.S. Geological Survey, 2005) and is contained in bodies of rocks below the earth's surface, known as aquifers (Environment Agency, 2007). This body of water can be accessed; (1) anthropogenically (i.e. by digging wells (U.S. Geological Survey, 2005) and dugouts (Grover *et al*., 1997)) or (2) naturally (i.e. springs, swamps, lakes and rivers (U.S. Geological Survey, 2005).

Groundwater serves as an essential and vital source of water (Environment Canada, 2004b) to some individuals (Rudolph *et al*., 1998) for all of their daily water needs (i.e. drinking, washing, cooking, farming (Environment Canada, 2004b)). Rural Canadians account for the largest percentage of Canadians that depend on groundwater. Approximately 9 million Canadians depend on groundwater, and 6 million of these are rural habitants (Environment Canada, 2004a). The natural filtering of groundwater through aquifers typically renders it free of disease-causing microorganisms (Environment Canada, 2004d). However, human use of contaminants (industrial chemicals, pesticides) in close proximity to groundwater sources, such as wells, may pollute (via run-offs, spray-drifts, spills, leaks) these sources (Environment Canada, 2004c, 2004e). Pollution of groundwater renders it unsuitable for use and increases the risk of occurrence of adverse health effects in humans that consume the contaminated water. Unfortunately, due to the increased used of toxic chemicals in industries and agriculture; groundwater contamination has become an increasing problem in Canada (Environment Canada, 2004c). Even more worrisome, is the fact that, the overall extent of the problem of groundwater contamination is currently unknown and some contamination even goes unnoticed until populations fall ill following consumption of the contaminated water (Environment Canada, 2004c). Contamination of groundwater can occur from either point or non-point sources (Environment Canada, 2004c, 2004e). Accidental spills, leaks from septic systems, livestock wastes and effluents from mining processes serve as point sources of groundwater (Environment Canada, 2004c, 2004e). In contrast, non-point, or distributive sources of groundwater include run-offs of fertilizers and pesticides (i.e 2.4-D) from agricultural land, and contaminants from the rain, snow and dry atmosphere (Environment Canada, 2004c, 2004e).

Previously mentioned, 2,4-D has been one of the most frequently detected herbicide in groundwater (Grover *et al*., 1997). The ability of 2,4-D to contaminate

groundwater is most likely associated with its leaching from soil environments (reviewed by Cox *et al*., 1999). The herbicide has an increased potential to leach into groundwater from soils that are coarse-grained, sandy with low organic content, and basic (US EPA, 2005). However, in general, little run-off from the soil into groundwater environments occurs with 2,4-D acid or its amine salts (US EPA, 2005). The US EPA has classified 2,4-D as a marginal leacher (reviewed by Environment Canada, 1991) from soil. The leaching potential of chemicals from soil is a result of its adsorption and persistence properties (Gan, 2002). The leaching rating of pesticides are assigned based on the groundwater ubiquity score (GUS), which is calculated using the adsorption coefficient (K_{oc}) and dissipation half-life ($DT_{½}$) of the pesticide in soil (Gan, 2002). A non-leacher is a pesticide that has a GUS <1.8 and a marginal leacher is a pesticide that has a GUS between 1.8 and 2.8 (Gan, 2002).

Groundwater is a significant source of water for rural habitants (Environment Canada, 2004b). Therefore, it is important to acknowledge the concentrations of contaminants, such as pesticides, found in groundwater and address the adverse human health effects that may occur from exposure to these contaminants. In groundwater, 2,4-D is usually detected at concentrations that are below established drinking water guidelines. For instance, in a groundwater monitoring study (total of 21 rural Saskatchewan farm dugouts sampled between the fall of 1987 and the spring of 1989), 2,4-D residues were detected in 75% of the total dugouts that were sampled (Grover *et al*., 1997). Concentrations of 2,4-D were below (median and maximum 2,4-D residue concentrations were determined as 0.07 and 2.67 µg/L, respectively (Grover *et al*., 1997)) established guideline values for the herbicide in drinking water (see below for established guideline values for 2,4-D in drinking water). However, there have been some studies that have found 2,4-D in well water samples at concentrations that were alarmingly approaching and exceeding established drinking water guideline values for the widely used herbicide. For example, in an investigation of pesticide contamination in 359 rural wells in Ontario over a duration of 5 years (1979 and 1984), the authors found that 71% of the wells were contaminated with pesticides and 2,4-D was involved in 30% of those well contaminations at concentrations ranging from 0.1 to 60 µg/L (Frank *et al*., 1987). In a survey conducted in 6 Canadian provinces from 1971 to 1986,

2,4-D residue was found at a concentration of approximately 29 µg/L in 52 out of 805 samples of raw and treated drinking water (reviewed by Environment Canada, 1991; WHO, 2003). A concentration that was approaching the international WHO drinking water guideline for 2,4-D (see below).

The herbicide has been suggested to induce a plethora of adverse human health effects (see sections 2.1.6.1 to 2.1.6.9), thus regulatory guideline levels for 2,4-D in drinking water have been established by both national and international agencies to help protect the health of humans who may be exposed to the chemical via drinking water. There are vast variations between individual regulatory guidelines established for 2,4-D in drinking water. Regulatory guidelines for water contaminants, such as 2,4-D are established by agencies based on the best available scientific research conducted on the potential adverse human health effects that may be associated with consumption of high concentrations of the chemical over a chronic period of time (Health Canada, 2006). Currently, the highest guideline level for 2,4-D in drinking water has been established by Canada. The Canadian CDW has established an interim maximum acceptable concentration (IMAC) for 2,4-D in drinking water of 100 µg/L (Environment Canada, 1991). Other agencies have established guideline levels that are well below that of Canada. For example, the US EPA has established a maximum contaminant level (MCL) for 2,4-D in drinking water of 70 µg/L (US EPA, 2005). In contrast, WHO has established an international drinking water guideline of 30 µg/L (WHO, 2003), while the European council of agriculture ministers has established a maximum admissible concentration (MAC) for all pesticides in drinking water of 0.1 µg/L (Harrison *et al*., 2000).

2.1.5 Exposure to and fate of 2,4-D in humans

Humans may be exposed to 2,4-D via several routes. These include the respiratory, dermal, eyes and oral routes (PAN Pesticide Database, 2004). The oral route is the most significant route of exposure to 2,4-D from groundwater. Although water represents a significant source, it is not the sole source of 2,4-D exposure. Soil (contaminated during herbicidal application (WHO, 2003)) and air (contaminated through spray drifts and volatilization of the herbicide (reviewed by Cox *et al*., 1999))

are also sources of 2,4-D exposure to humans. Consumption of foods (i.e. alfalfa, apple, apricot, cranberry, flax, maple, pear, peach and plum (PMRA, 2005)) contaminated with 2,4-D also exposes humans to the herbicide. Other sources of 2,4-D exposure may include occupational and bystander exposure. Personnel occupationally responsible for handling and applying 2,4-D may unintentionally be exposed to high concentrations of the herbicide (PMRA, 2005; WHO, 1984) via all routes. For instance the herbicide has been found at concentration of up to 453.6 μg/L in the urine of backpack sprayers of 2,4-D (Garry *et al*., 2001). Homeowners who apply 2,4-D to their lawns may be also be unintentionally exposed to the chemical (PMRA, 2005) via all routes of exposure. Finally, bystanders, such as persons (adults, children) who enter an area (residential lawns, parks, golf-course) where 2,4-D has been applied may also be exposed to the herbicide through similar routes (PMRA, 2005).

In humans, once orally ingested, as would occur with consumption of groundwater, the esters and amine forms of 2,4-D rapidly undergo hydrolysis to yield the parent 2,4-D acid (BC Ministry of Forests, 2003; Garabrant and Philbert, 2002). The parent 2,4-D acid has been described to be well absorbed from the gastrointestinal tract following oral ingestion (Sauerhoff *et al*., 1977). However, at human physiological pH (pH 7.4), the parent acid exists predominantly in the ionized form (Garabrant and Philbert, 2002). Thus, ionization of the 2,4-D acid molecule at human physiological pH limits the diffusion across cell membranes (Garabrant and Philbert, 2002), suggesting that in humans, the probability of 2,4-D to enter, accumulate and induce adverse effects in cells and/or tissues is low.

Excretion of 2,4-D occurs mainly in the urine via first-order rate kinetics, with an average half-life of approximately 17 hrs (Sauerhoff *et al*., 1977). The majority of absorbed 2,4-D (82.3%) is excreted in the urine mainly unchanged. A small portion (12.8%) is excreted as an unidentified 2,4-D conjugate (Sauerhoff *et al*., 1977). Thus, due to its pharmacokinetic properties, accumulation of 2,4-D in human organs and tissues is considered highly unlikely (Garabrant and Philbert, 2002). However, several studies have illustrated that 2,4-D may accumulate at high concentrations in humans through multiple exposure sources (i.e via consumption of groundwater, occupational and bystander). For instance, in a study conducted in rural Saskatchewan, during spring

application (June-July 1996) of the herbicide, 2,4-D was detected at a maximum concentration of 230 µg/L in the plasma of rural residents (Semchuk *et al*., 2004). In these cases, the residents may have been unintentionally exposed to 2,4-D via multiple routes (i.e. oral, dermal, respiratory, eyes) and sources (groundwater consumption for drinking, washing, and cooking, bystander, air and food). The accumulation of high concentrations of 2,4-D in humans have also been observed in persons working with the herbicide (Arbuckle *et al*., 1999; Garry *et al*., 2001; Holland *et al*., 2002). Results from a study performed in Ontario also reported high 2,4-D concentrations of up to 106.9 µg/L in the semen of farmers (Arbuckle *et al*., 1999) occupationally exposed to the herbicide. High concentrations of 2,4-D have also been detected at maximum levels of 240 µg/L (Holland *et al*., 2002) and 453.6 µg/L (Garry *et al*., 2001) in the urine of backpack sprayers who were occupationally exposed to the herbicide. The results of these studies are worrisome, since the entrance and accumulation of toxicants (i.e. 2,4-D) into cells may induce toxicity in cells and tissues (Gregus and Klaassen, 2001). A review of studies that have examined the adverse human health effects of 2,4-D will now be presented.

2.1.6 Toxicological evidence of the adverse human health effects induced by 2,4-D exposure

The toxicology of 2,4-D has been well studied and its current literature is overwhelming. In the following review of 2,4-D toxicology, several studies that have used *in vivo* and *in vitro* experimental models to assess the adverse human health effects (i.e. cytotoxicity, organ toxicity, genotoxicity, carcinogenesis, neurotoxicity, immunotoxicity, developmental toxicity and reproductive toxicity) that may occur following 2,4-D exposure will be discussed. The purpose of this comprehensive review, is to illustrate that although 2,4-D toxicology has been well studied, the majority of previous studies failed to assess the adverse human health effects of low concentrations (<100 nM) of the herbicide; concentrations that are typically found in the environment and hence to which humans may more likely be exposed to from the environment over acute, subacute, subchronic and chronic durations of time.

However, before commencing the review, the following paragraphs consist of a dissection of some information that will allow for better interpretation of the studies reviewed. Firstly, in most *in vivo* studies reviewed, doses of 2,4-D have reported in mg/kg units. Additionally, some *in vitro* studies that were reviewed expressed 2,4-D concentrations in µg/L units. Therefore, in order to attain an understanding of the difference between the low 2,4-D concentrations that was used in this present study and the high concentrations that were used in those particular *in vivo* and *in vitro* studies, below is a presentation of the conversion of the 2,4-D concentration range used in the current study to the equivalent dose (mg/kg) or concentrations (µg/L) units. The conversion from molar concentration to dose assumed that adult male rats were exposed to a single dose of commercial 2,4-D (containing 2,4-D DMA) from their drinking water. The average weight of an adult male rat is 250-550 g and drinks an average of 10-12 mL of water per 100 g per day (Grant, 2000) Thus, the conversion assumed that the average weight of a rat was equal to 250 g and drank an average of 25 mL of water per day. Therefore, 0.1 nM to 1 mM of commercial 2,4-D in the present study is equivalent to 2.2×10^{-6} to 22 mg/kg of 2,4-D acid. The concentration range of 0.1 nM to 1 mM of commercial 2,4-D in the present study is equivalent to 0.022 to 2.2×10^{5} µg/L of 2,4-D acid.

Secondly, different forms (i.e. acid, amine or ester forms) of 2,4-D are used in 2,4-D toxicological studies. However, from the review of toxicity data, the toxic effects induced by this herbicide are through the actions of the parent 2,4-D acid, regardless of the 2,4-D form used in experimental studies. It was reported that, in humans, the esters and amine forms of 2,4-D undergo hydrolysis to yield the parent 2,4-D acid (BC Ministry of Forests, 2003). Additionally, in experimental studies performed in animals, similar toxicity has been produced by 2,4-D acid, 2,4-D DMA and 2,4-D ester (Charles *et al*., 2001). It has been suggested that those observations confirm previous suggestions of the rapid metabolic conversion of 2,4-D salts to 2,4-D acid *in vivo* (Charles *et al*., 2001). Similarity between 2,4-D acid and commercial formulations containing 2,4-D DMA have also been observed (González *et al*., 2005). The authors observed that the two compounds exerted similar genotoxic effects. Therefore, based on its metabolism, any adverse human health effects associated with 2,4-D acid or commercial forms is

most likely due to the parent 2,4-D acid. In the current review, studies that have employed technical 2,4-D acid or commercial forms containing 2,4-D DMA were discussed, since a commercial form of 2,4-D containing 2,4-D DMA was used in the present study.

2.1.6.1 Cytotoxic effects of 2,4-D

Cytotoxic effects are defined as the adverse effects that result from the disruption of structures and processes that are essential for cell survival, proliferation and function by exposure to chemical agents (Tuschl and Schwab, 2004). Several investigators have assessed the cytotoxic effects of 2,4-D. A review of the *in vivo*, followed by the *in vitro* studies is presented below.

The cytotoxic effects of 2,4-D have been assessed using an *in vivo* model (rats) (Kaioumova *et al*., 2001a). Rats were orally gavaged with single high doses of 2.8, 22.8, 228 mg/kg of 2,4-D DMA. Thymus, spleen and blood were obtained 4, 8, 12 or 24 hrs after 2,4-D treatment. The DNA laddering pattern (produced by DNA gel electrophoresis) demonstrated that exposure to 2,4-D induced dose- and time-dependent cell death in the thymus, spleen and blood. The results suggest that 2,4-D may induce cytotoxic effects in various organs in humans. However, the study examined 2,4-D doses (2.8 to 228 mg/kg) that reflects concentrations of the herbicide that are more than 100 fold higher than that typically found in the environment. Thus, the results of the study failed to elucidate the adverse human health effects of low 2,4-D concentrations that are routinely found in the environment. A review of the *in vitro* 2,4-D cytotoxicity studies that have been conducted will now commence.

Various *in vitro* models (HepG2, human cerebellar granule, human lymphoblastoid, human histocytic lymphoma and chronic myeloid leukemic, human erythrocytes, rat hepatocytes, mouse lymphoma cells) have been utilized to assess the cytotoxic effects of 2,4-D. Similar to this thesis, the cytotoxic effects of low 2,4-D concentrations were assessed using HepG2 cells (Bharadwaj *et al*., 2005). The researchers were the first to examine the cytotoxic effects of low concentrations (0.1 to 100 nM) of the herbicide (commercial 2,4-D (containing 2,4-D DMA)). Cell viability was determined using the neutral red uptake assay. Results illustrated that acute

exposure (24 hrs) to low 2,4-D concentrations (0.1 to 100 nM) induces cytotoxicity, reflected by non concentration-dependent cell death (10 to 15% decreases in cell viability). The results suggest that low 2,4-D concentrations may induce cytotoxic effects in humans.

In contrast to the above study and this current study, the cytotoxicity of high concentrations (>100 nM) of 2,4-D has been evaluated by several other *in vitro* studies (De Moliner *et al*., 2002; Duchnowicz and Koter, 2003; Palmeira *et al*., 1995; Tuschl and Schwab, 2003, 2004, 2005; Venkov *et al*., 2000). Similar to this present research, Tuschl and Schwab used HepG2 cells to investigate 2,4-D cytotoxicity (Tuschl and Schwab, 2003, 2004, 2005). The authors used flow cytometry and the annexin-V assay to determine cell death. Cells were exposed for 24 and 48 hrs to 2,4-D acid concentrations (4, 8 and 16 mM) that were more than 1000 fold higher than that typically found in the environment (Tuschl and Schwab, 2003). Exposure resulted in a concentration-dependent prolongation of the GI phase of the cell cycle and an increase in cell death (Tuschl and Schwab, 2003). Effects were exacerbated following exposure to 16 mM. At 16 mM, more than 50% of cells were dead and a total disruption of the cell cycle was observed after 48 hrs of exposure (Tuschl and Schwab, 2003). Similar results were obtained in later studies, where HepG2 cells were exposed to 4, 8 and 16 mM of 2,4-D acid for 24 hrs (Tuschl and Schwab, 2004; 2005).

The cytotoxicity of high 2,4-D concentrations (>100 nM) that are 1000 fold or higher than that found in the environment, has also been evaluated using other *in vitro* cell models (De Moliner *et al*., 2002; Duchnowicz and Koter, 2003; Palmeira *et al*., 1995; Tuschl and Schwab, 2004, 2005; Venkov *et al*., 2000). For instance, results from flow cytometry and the 3-(4,5-dimethylthazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assays illustrated that exposure to 1 and 2 mM of 2,4-D acid for 12, 20 or 24 hrs induced non concentration- and time-dependent decreases in human cerebellar granule cell viability. In cytotoxicity studies performed by Tuschl and Schwab, comparative effects were observed in human lymphocytes isolated from whole blood (Tuschl and Schwab, 2005), human lymphoblastoid (AHH-1) and mouse lymphoma (YAC-1) cells (Tuschl and Schwab, 2004, 2005).

In vitro studies were also performed using high concentrations (>100 nM) of 2,4-D to assess cytotoxicity in human histocytic lymphoma (U937) and chronic myeloid leukemic (LAMA-84) cells (Venkov *et al*., 2000). Results from MTT assays demonstrated that exposure to 0.16 mM of 2,4-D acid for 48 hrs induced a 40% decrease in cell viability in U937 and LAMA-84 cells. Complete cell death was observed in cells exposed to 1.6 mM of 2,4-D for 48 hrs.

The cytotoxic effects of high concentrations of 2,4-D have also been investigated using human erythrocytes cultures (Duchnowicz and Koter, 2003) and primary rat hepatocytes (Palmeira *et al*., 1995). Spin probe analysis revealed that exposure to 1 mM of 2,4-D acid for 1 hr induced hemolysis of human erythrocytes (Duchnowicz and Koter, 2003). Results from trypan blue exclusion assay demonstrated that 0 to 200 min exposure to 2,4-D acid concentrations (1, 5 and 10 mM) induced concentration- and time-dependent decreases in the viability of rat hepatocytes (Palmeira *et al*., 1995).

Thus, taken all together, the above review illustrates that; although the cytotoxicity of 2,4-D has been investigated by several researchers who used various models (*in vivo* (rats) and *in vitro* (HepG2, human cerebellar granule, human lymphoblastoid, human histocytic lymphoma and chronic myeloid leukemic, human erythrocytes, rat hepatocytes and mouse lymphoma cells)), assays (annexin-V, flow cytometry, MTT, neutral red uptake, spin probe, trypan blue exclusion and TUNEL assays), durations of exposure (12 to 72 hrs) and forms (technical 2,4-D acid or commercial 2,4-D (containing 2,4-D DMA)), most studies used overt concentrations (>100 nM) and did not examine the cytotoxic effects of low 2,4-D concentrations (<100 nM). This is an alarming finding that warrants attention, since humans are typically exposed to low concentrations of 2,4-D in the environment. The research conducted in this thesis assesses the cytotoxicity of low 2,4-D concentrations.

2.1.6.2 Hepatotoxic effects of 2,4-D

The hepatotoxic effects of 2,4-D have been assessed by several investigators. However, many researchers failed to examine the hepatotoxic effects that may be associated with exposure to low environmental concentrations of 2,4-D. A review of some *in vivo*, followed by the *in vitro* studies is presented below.

In an *in vivo* study, rats were exposed (via drinking water) to doses (600 mg/kg for 1 day) or concentrations (200,000 µg/L for 30 and 180 days) of commercial 2,4-D (containing 2,4-D DMA) (Paulino *et al*., 1996) that are more than 1000 fold higher than would be typically encountered by humans in the environment. Spectrophotometric analyses demonstrated that acute exposure to such a high dose of 2,4-D induced disruption of hepatic function, indicated by elevated levels of several hepatic enzymes markers (i.e. aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (AP)) that are routinely used for determination of hepatic dysfunction. Interestingly, subchronic and chronic exposure to 2,4-D induced lesser degrees of the above effects. This may have been an adaptive response to longer exposure to the herbicide. Subchronic exposure induced an increase in AST levels, while chronic exposure induced increases in AST, AP and LDH levels. A greater degree of hepatic dysfunction may have been observed following chronic, compared to subchronic exposure due to saturation of repair and/or detoxification mechanisms following chronic, compared to sub-chronic exposure to the herbicide. Sub-chronic exposure refers to exposure to a chemical for no longer than 3 months (Eaton and Klaassen, 2001). Chronic exposure refers to exposure to a chemical for more than 3 months (Eaton and Klaassen, 2001).

The adverse hepatic effects of high concentrations of 2,4-D has also been documented by Charles and colleagues. In a subchronic experimental study, rats were exposed to doses of 2,4-D (1, 15, 100 and 300 mg/kg/day of 2,4-D acid or 2,4-D DMA from their feed for 3 months (Charles *et al*., 2001)) that were equal to one half or less than that used by Paulino and co-workers. Histopathology revealed that the highest dose, a dose that humans are not likely to be exposed to from the environment, of 2,4-D induced centrilobular hepatocellular hypertrophy. Thus, the above *in vivo* studies have examined and illustrated the adverse hepatic effects of high 2,4-D doses (1 to 600 mg/kg) or concentrations (200,000 µg/L). However, humans are not likely to be exposed to such high doses or concentrations of 2,4-D from the environment. A review of the *in vitro* studies will now be presented.

In vitro studies have assessed the hepatotoxic effects of 2,4-D concentrations (2 to 16 mM) that are more than 1000 fold or higher than that encountered by humans in

the environment. The *in vitro* cytotoxic effects that were reported by Tuschl and Schwab (see section 2.1.6.1) were observed in HepG2 cells (Tuschl and Schwab, 2003; 2004; 2005), cells that are derived from human liver (ATCC, 2004). Therefore, the results also suggest that high 2,4-D concentrations (≥2 mM) may induce hepatotoxic effects (i.e. cell death) in humans. The results from the cytotoxicity study (see section 2.1.6.1) that was conducted by Palmeira et al. (1995) and illustrated that 2,4-D induced death of rat hepatocytes, also suggest the same; that is, high 2,4-D concentrations (≥ 1mM) may induce hepatotoxic effects (i.e. cell death) in humans. In addition to the studies performed by Tuschl and Schwab, the results from the *in vitro* study conducted by Bharadwaj and colleagues also suggest 2,4-D may induce hepatotoxic effects, since the study was also conducted using HepG2 cells (Bharadwaj *et al*., 2005). In contrast to the studies conducted by Tuschl and Schwab, and similar to this thesis, the authors tested low environmental 2,4-D concentrations that humans are more likely to be exposed to from the environment. The study illustrated that low 2,4-D concentrations induced cell death and gene alteration. Genes whose expression levels were altered included those involved in stress response (FTL, FTH1, PCNA), for example. An intriguing finding that, for the very first time showed that low 2,4-D concentrations may induce adverse human health effects.

Taken altogether, the majority of previous *in vivo* and *in vitro* studies failed to assess the hepatotoxic effects of low environmental concentrations of 2,4-D. Studies examined 2,4-D doses (1 to 600 mg/kg) and concentrations (2 to 16 mM) that reflects 2,4-D concentrations that are more than 100 fold higher than that found in the environment, and hence to which humans are not typically exposed to. Thus, the results of such studies may not accurately predict the adverse human hepatic effects of exposure low environmental 2,4-D concentrations over acute or chronic durations of time.

2.1.6.3 Nephrotoxic effects of 2,4-D

The nephrotoxic effects of 2,4-D have been evaluated by some researchers, using *in vivo* models. In the first study reviewed, histopathological examination revealed that subchronic (3 months) exposure (via oral ingestion from feed) to a 2,4-D acid concentration (2,500,000µg/L (≈11 mM)), that is more than 1000 fold higher than

concentrations of the herbicide typically encountered by humans from the environment, induced structural changes (i.e. decreased cytoplasm and cytoplasmic granules, reduced mitochondria organelles and increased nuclear density of cells) in the proximal convoluted tubule segment of the nephron (Ozaki *et al*., 2001). The nephron is the functional unit of the kidney (Silverthorn, 1998). The nephrotoxic potential of high doses of 2,4-D was also investigated by Charles and co-workers in an earlier study. Histopathological studies revealed that there was loss of the brush border in proximal tubular cells and vacuolization of all tubular cells in rats subchronically exposed to 300 mg/kg/day 2,4-D acid or 2,4-D DMA from their feed for 3 months (Charles *et al*., 1996).

Thus, taken together, the above studies have investigated the nephrotoxic effects of high 2,4-D doses (300 mg/kg) and concentrations (≈11 mM) that reflects concentrations that are more than 1000 fold higher than that typically found in the environment. Therefore, although the results from the above studies provide useful information for elucidating the adverse effects that may occur in the kidney of humans exposed to high concentrations of 2,4-D, the results are not likely to predict the nephrotoxic effects that may occur following typical, everyday exposure to low concentrations (<100 nM) of the herbicide that are usually found in the environment.

2.1.6.4 Genotoxic effects of 2,4-D

The genotoxic effects of 2,4-D have been assessed using both *in vivo* and *in vitro* experimental model systems. A review of the *in vivo* studies, followed by the *in vitro* studies is presented below. Several researchers have assessed the genotoxic effects of 2,4-D, *in vivo*, using high doses (1.7 to 200 mg/kg) of the chemical (Amer *et al*., 2001; Madrigal-Bujaidar *et al*., 2001; Venkov *et al*., 2000) that reflects concentrations that are more than 100 fold higher than that found in the environment. Chromosomal aberrations (i.e. gaps, fragments, breaks, translocations and poly-ploid metaphases) have been reported to occur in the bone marrow and spermatocytes of rats exposed (oral gavage) to 3.3 mg/kg of 2,4-D acid for 3 and 5 consecutive days (Amer *et al*., 2001). Comparative effects were also observed in rats that orally ingested 33 mg/kg of 2,4-D acid for 1 day. Significant chromosomal aberrations, including sister chromatid exchanges have also been observed in bone marrow cells that were isolated from mice

exposed (intraperitoneal injection) to 3.5 mg/kg of 2,4-D acid for 12 hrs (Venkov *et al*., 2000). Chromosomal aberrations induced by 2,4-D exposure have also been reported by researchers who used doses of the herbicide that were more than 100 fold higher than that used by the previously described studies (Madrigal-Bujaidar *et al*., 2001). Rats were orally exposed to single doses 100 and 200 mg/kg of 2,4-D acid. Dose-dependent increases in sister chromatid exchanges in isolated bone marrow and spermatogonial cells were observed.

Thus, the results from the *in vivo* studies suggest that 2,4-D doses that are equal to or greater than 3.3 mg/kg may induce genotoxic effects (i.e. chromosomal aberrations) in humans. However, humans are not likely to be exposed to such high doses, since 2,4-D concentrations in the environment are typically less than 100 nM. A review of the *in vitro* genotoxic studies will now commence.

The genotoxic effects of 2,4-D concentrations (1 μM to 45 mM) that are more than 10 fold or higher than that encountered by humans in the environment have been assessed, *in vitro* (González *et al*., 2005; Holland *et al*., 2002; Zeljezic and Garaj-Vrhovac, 2004). Concentration-dependent increases in the number of chromatid and chromosome breaks, micronuclei and nuclear buds was observed in human lymphocytes cultures exposed to concentrations (400 and 4000μg/L (≈ 2 to 20 μM of 2,4-D acid)) of commercial 2,4-D (containing 2,4-D acid) that are more than 10 fold higher than that found in the environment (Zeljezic and Garaj-Vrhovac, 2004). Concentration-dependent genotoxic effects (i.e. micronucleus proliferation, frequency) of high 2,4-D concentrations (1 μM to 1 mM) have also been observed in human whole blood or isolated lymphocyte cultures exposed to technical 2,4-D acid or commercial 2,4-D for 48 hrs (Holland *et al*., 2002). Using Chinese hamster ovary cell cultures, the genotoxic effects of high 2,4-D concentrations have also been evaluated (González *et al*., 2005). Concentration-dependent increases in the frequency of DNA-strand breaks were observed in cell cultures exposed to 2000 to 10,000 μg/L (≈ 9 to 45 μM of 2,4-D acid) of either 2,4-D acid or commercial 2,4-D (containing 2,4-D DMA) for 1.5 hrs. Concentration- and time-dependent increases in sister chromatid exchange were observed in cell cultures exposed to a similar range of 2,4-D concentrations for 24 and 36 hrs.

Thus, from the above review of 2,4-D genotoxicity studies, it can be concluded that although the genotoxic effects of 2,4-D have been assessed using various experimental models (*in vivo* (rats and mice) and *in vitro* (human whole blood, human isolated lymphocytes and Chinese hamster ovary cell cultures)), endpoints (chromosomal aberrations, micronucleus proliferation) and durations of exposure (1.5 hrs to 5 days), the studies did not assess the human genotoxic effects that may associated with exposure to low environmental concentrations (<100 nM) of 2,4-D. the above *in vivo* and *in vitro* studies used 2,4-D doses (1.7 to 200 mg/kg) and concentrations (1 μM to 45 mM), respectively, that reflects concentrations of the herbicide that are more than 10 fold higher than that typically found in the environment, and hence to which humans are not likely exposed to over acute or chronic periods of time.

2.1.6.5 Carcinogenic effects of 2,4-D

Several *in vivo* studies have assessed the carcinogenic effects of 2,4-D by using high concentrations (>1 μM) of the herbicide (Amer *et al*., 2001; Ge *et al*., 2002; Holland *et al*., 2002; Madrigal-Bujaidar *et al*., 2001; Ozaki *et al*., 2001; Venkov *et al*., 2000). Results from these studies suggest that overt 2,4-D concentrations (<1 μM) may induce carcinogenesis in humans. For instance, 2,4-D has been reported to induce significant increases in replicative indices of lymphocyte cultures isolated from whole blood obtained from twelve spray applicators (mean urine level of 2,4-D acid was 240 μg/L (≈ 1 μM)) exposed solely to the herbicide for 3 months (Holland *et al*., 2002). In another *in vivo* study, southern blot analysis demonstrated that exposure to high 2,4-D doses (daily oral dose of 1.7 x 10^6 μg/L (≈ 8mM) of 2,4-D acid through feed for a period of 6 days) induced DNA hypomethylation of the proto-oncogene, *c-myc*, in hepatocytes of mice (Ge *et al*., 2002). An inverse relationship exists between DNA methylation and transcription of genes (Ge *et al*., 2002), therefore, hypomethylation of the proto-oncogene, *c-myc* suggests possible increased transcription of the gene, with subsequent translation. The alteration of the regulation of proto-oncogenes is one mechanism involved in the activation of proto-oncogenes to oncogenes (Kumar *et al*., 1997). Oncogenes are genes that are involved in the process of carcinogenesis by inducing uncontrolled cell proliferation (King and Stansfield, 1997; Kumar *et al*., 1997).

Furthermore, the dysregulation of the *myc* gene is known to be involved in Burkitt's lymphoma (a B-cell tumor) (Kumar *et al*., 1997).

The carcinogenic potential of high 2,4-D concentrations have also been illustrated in an *in vivo* subchronic toxicity study, where rats were orally exposed to high concentrations of 2,4-D acid (1.7×10^4, 8.5×10^4, 2.5×10^5, 1.2×10^6, 2.5×10^6 μg/L (≈ 80 μM, 380 μM, 1 mM, 6 mM and 11 mM)) through their feed for a period of 3 months (Ozaki *et al*., 2001). Results from histopathological studies demonstrated that exposure to 2,4-D induced a concentration-dependent increase in hyperplasia of cells of the outer medulla. Pathological hyperplasia is suspected to lead to cancerous proliferation (Mitchell and Cotran, 1997).

In addition to the above studies and since genetic damage is known to play an integral role in carcinogenesis (Kumar *et al*., 1997), the results from the previously reviewed *in vivo* genotoxicity studies (Amer *et al*., 2001; Madrigal-Bujaidar *et al*., 2001; Venkov *et al*., 2000) (see section 2.1.6.4) also suggest that 2,4-D may induce carcinogenesis in human bone marrow, germ cells and lymphocytes. However, these studies tested high 2,4-D doses (≥3.3 mg/kg) that do not reflect environmental 2,4-D concentrations that humans are exposed to. Thus, all of the above studies have failed to address the carcinogenic potential of low 2,4-D concentrations in humans.

The results from previously reviewed *in vitro* studies (González *et al*., 2005; Holland *et al*., 2002; Zeljezic and Garaj-Vrhovac, 2004) (see section 2.1.6.4) that also tested high 2,4-D concentrations (1 μM to 45 mM) and illustrated that exposure to the herbicide may induce genotoxicity in humans, also suggest that 2,4-D may induce carcinogenesis in human lymphocytes and germ cells at those high concentrations. However, humans are not typically exposed to such high environmental concentrations of 2,4-D. Thus taken all together, previous *in vivo* and *in vitro* studies have tested the carcinogenic potential of 2,4-D using doses (≥3.3 mg/kg) or concentrations (1 μM to 45 mM) that do not reflect concentrations of the herbicide that are typically found in the environment, and hence to which humans may not be exposed to. Thus, the results from these studies may not accurately predict the carcinogenic potential of low environmental concentrations of the herbicide in humans.

2.1.6.6 Neurotoxic effects of 2,4-D

The neurotoxicity of 2,4-D has also been evaluated. However, similar to all of the above reviewed studies, researchers failed to assess the neurotoxic effects of low environmental concentrations of the herbicide. Pregnant rats were chronically exposed, *in vivo*, to high dietary 2,4-D acid (70 mg/kg/day) from gestation day (GD) 16 to postpartum day (PD) 23 (Bortolozzi *et al*., 2004). Following weaning (PD 23), offspring were divided into 2 groups, where one group was fed 2,4-D treated diets, and the other was fed 2,4-D free diets until PD 90. The results of rapid filtration assays illustrated that 2,4-D disrupted the dopamine (DA) neurotransmitter system of the brain, reflected by increased density of DA D_2-type receptors in the prefrontal cortex, striatum and cerebellum areas of the brain. These effects were irreversible in rats that were exposed to 2,4-D until PD 90. The results from the study are intriguing and illustrate that high doses of the widely used herbicide may induce irreversible neurotoxic effects. However, doses as high as those are not reflective of true environmental levels of 2,4-D, and hence to which humans are exposed, thus the results may not reflect the neurotoxic potential of 2,4-D concentrations that are typically found in the environment.

The neurotoxic effects of only high environmental 2,4-D concentrations (1 and 2mM) have been evaluated, *in vitro* (Rosso *et al*., 2000) Immunofluorescence and microtubule polymerization assays illustrated that exposure to 2,4-D acid for 24 hrs induced a concentration-dependent inhibition of neurite extension and reduced microtubule assembly in human cerebellar granule cell cultures, respectively.

Thus, taken all together, results from the above *in vivo* and *in vitro* 2,4-D studies, suggest that high doses (70 mg/kg/day) or concentrations (1 and 2 mM) of 2,4-D may induce neurotoxic effects (disruption of neurotransmitter systems, inhibition of neurite extension and microtubule assembly) in humans. However, doses or concentrations as high as those reflects concentrations that are at least 1000 fold higher than that typically encountered by humans in the environment, therefore, the results from the above studies does not elucidate the neurotoxic potential of true environmental concentrations of 2,4-D.

2.1.6.7 Immunotoxic effects of 2,4-D

The immunotoxicity of 2,4-D has been assessed. Rats were exposed (via intraperitoneal injection) to single doses of 50, 100, 150 or 200 mg/kg of commercial 2,4-D (containing 2,4-D DMA) (de la Rosa *et al*., 2003). Flow cytometry analyses revealed decreases in pre-B and IgM^+ cell population in the bone marrow of rats exposed to 200 mg/kg for 7 days. In another study, the immune responses in the offspring of rats whose dams were exposed on GD 6 to GD 16 to dietary doses (8.5, 37 and 370 mg/kg/day of 2,4-D acid) of commercial 2,4-D (containing 2,4-D DMA) in their drinking water were evaluated (Lee *et al*., 2001). Flow cytometric analyses demonstrated that exposure to the highest dose of 2,4-D resulted in a decrease in T-lymphocyte mitogen responses and an increase in B cell population. The T cytotoxic or suppressor cell counts were also suppressed in the highest dose groups. Results suggest that high 2,4-D doses may be immunotoxic to humans. However, doses used in the above *in vivo* studies are unreflective of 2,4-D concentrations that are typically found in the environment. Thus, the studies may not accurately predict the immunotoxic effects in humans of low 2,4-D concentrations.

The immunotoxic effects of 2,4-D were assessed *in vitro*, using concentrations of the herbicide that were more than 10 fold higher than that typically encountered by humans in the environment. Exposure (2, 4, 6 and 24 hrs) to 2,4-D concentrations (0.1 to 5 mM) induced concentration- and time-dependent increases in the death of human peripheral blood lymphocytes and Jurkat T cells (Kaioumova *et al*., 2001b). Results suggest that high 2,4-D concentrations may be immunotoxic to humans. However, humans do not encounter such high 2,4-D concentrations in the environment. Thus, results from the above studies suggest that high 2,4-D doses (>8.5 mg/kg) or concentrations (0.1 to 5 mM) may induce immuntoxicity in humans, however, doses or concentrations as high as those do not reflect concentrations of the herbicide that are typically found in the environment, and hence, to which humans are exposed to. Thus, the results from the above studies may not accurately predict the adverse immune effects of low 2,4-D concentrations that are routinely encountered by humans in the environment.

2.1.6.8 Adverse reproductive effects of 2,4-D

The adverse human reproductive effects that may be induced by 2,4-D exposure have been evaluated. Via microscopic examination, a dose-dependent increase in the percentage of sperm head abnormalities (i.e. amorphous sperm head, sperm head without hook, triangular and banana shaped sperm head, small, big and double sperm heads) were observed in mice exposed daily to high doses of 33 and 82.5 mg/kg of 2,4-D acid for 3 and 5 days (Amer *et al*., 2001). The potential adverse reproductive effects of 2,4-D were also illustrated from the work conducted by a group of investigators that orally dosed mice with single doses of 50, 100 and 200 mg/kg of 2,4-D acid (Madrigal-Bujaidar *et al*., 2001). Scoring of the frequency of sister chromatid exchanges revealed that exposure to 100 and 200 mg/kg of 2,4-D acid induced a dose-dependent induction of sister chromatid exchanges in mice spermatogonial cells. Thus, while the above *in vivo* studies illustrate that 2,4-D may induce adverse reproductive effects (germ cell abnormalities) in humans, the studies tested doses of 2,4-D that reflect concentrations of the herbicide that were more than 1000 fold higher than that encountered by humans in the environment. The results of the study may not accurately predict the adverse reproductive effects of low 2,4-D concentrations in the environment.

In vitro, the adverse reproductive effects of high concentrations (≈ 9 to 45 μM) (González *et al*., 2005) of the herbicide that are not typically found in the environment have been assessed. Exposure of Chinese hamster ovary cells to 2,4-D concentrations (≈ 9 to 45 μM) induced concentration-dependent increases in the frequency of DNA-strand breaks and sister chromatid exchanges in human female reproductive organs (González *et al*., 2005).

Therefore, similar to the previous toxicity studies reviewed, the adverse reproductive effects of only high doses (≥33 mg/kg) or concentrations (≈ 9 to 45 μM) of 2,4-D have been tested in *in vivo* and *in vitro* studies, respectively. Those doses and concentrations are more than 10 fold higher than that typically encountered by humans in the environment. Thus, while the results are intriguing, they are not likely to predict the adverse human reproductive effects that may be induced following exposure to 2,4-D in the environment.

2.1.6.9 Adverse developmental effects of 2,4-D

The adverse developmental effects on various systems or organs that may be induced following 2,4-D exposure has been assessed in *in vivo* studies. Below is a review of some such studies. Using a high dose (370 mg/kg) of the herbicide, it was illustrated that the herbicide alters the development of the immune system in offsprings of rats (Lee *et al*., 2001) (see section 2.1.6.7 for details of study). Decreases in T-lymphocyte mitogen responses and increases B cell population were observed. The developmental effects in the central nervous system (CNS) induced by 2,4-D exposure have also been tested. The adverse CNS effects that were observed in offsprings (exposed to 70 mg/kg/day of 2,4-D acid) of rats in the neurotoxicity study conducted by Bortolozzi et al. (2004) (see section 2.1.6.6 for details of study) also illustrates the adverse developmental effects on the CNS of high doses of the chemical. Adverse developmental effects on the CNS of offsprings of rats have also been reported by Duffard et al. (1996). In the study, lactational exposure to 2,4-D (dams were intraperitoneally injected with 100 mg/kg of 2,4-D acid from PD 15 to 25 (period of rapid myelination) induced myelin deficit (assessed via histopathology) in the brain of rat offspring (Duffard *et al*., 1996).

In addition to the immune system and CNS, 2,4-D has also been shown to induce adverse effects on tooth development. Pregnant rats were exposed daily to concentrations (25,000, 50,000 and 100,000 µg/L (0.11, 0.23 and 0.45 mM) of commercial 2,4-D (containing 2,4-D DMA) (Alpöz *et al*., 2001) that are more than 1000 fold higher than that typically found in the environment. Rats were exposed to the herbicide in their food for 15 days prior to pregnancy and throughout pregnancy. Following birth, the offspring continued to be exposed to 2,4-D through lactation for 30 days. Histological examination revealed that exposure to 2,4-D induced dose-dependent increases in abnormal dentin formation. Exposure to 50,000 and 100,000 µg/L resulted in decreased enamel thickness.

Thus, results from the above studies suggest that high 2,4-D doses (≥70 mg/kg) or concentrations (≥25,000 µg/L) may induce adverse developmental effects on various organs or systems (CNS, immune system, and tooth) in humans. However, doses and concentrations as high as those, reflect 2,4-D concentrations that are more than 1000

fold higher than that typically encountered by humans in the environment. The results of the studies may not accurately predict the adverse development effects that may occur in humans exposed to 2,4-D from the environment.

In summary, a review of the above studies illustrates that although the toxicity of 2,4-D has been extensively investigated, the majority of *in vivo* and *in vitro* studies tested overt, unrealistic doses (1 to 600 mg/kg) or concentrations (1 μM to 45 mM) of the herbicide, that reflects concentrations that are 10 fold or higher than that encountered by humans in the environment. Ideally, *in vivo* studies should have employed 2,4-D doses within the range of 2.2×10^{-6} to 2.2×10^{-3} mg/kg, while *in vitro* studies should have tested 2,4-D concentrations within the range of 0.1 to 100 nM, or 0.022 to 22 μg/L. Testing of these doses or concentrations would have been reflective of 2,4-D concentrations (0.1 to 100 nM) that are typically encountered by humans in the environment, and thus, would have aided in elucidating the adverse human health effects that are associated with acute or chronic environmental exposure to low concentrations of the herbicide. This thesis addresses the adverse human health effects that may be associated with exposure to low concentrations of the herbicide.

2.1.7 2,4-D and gene expression

The majority of the above studies that were conducted to investigate the toxicity of 2,4-D share two factors common with classical toxicology studies; that is the use of (1) high 2,4-D concentrations (>1 mM) and (2) overt endpoints (i.e. cell death, pathological aberrations). Toxicogenomics is an emerging technique in toxicology research (Aardema and MacGregor, 2002). It is the study of alteration of gene expression patterns (Aardema and MacGregor, 2002). The alteration of gene expression pattern is considered to be a more sensitive endpoint than currently employed pathological endpoints (Aardema and MacGregor, 2002). Thus, due to its sensitivity, the alteration of gene expression pattern may be a better endpoint for assessing the toxicity of low concentrations of toxicants, such as 2,4-D, as low concentrations of toxicants may induce more subtle effects than high concentrations. Toxicogenomics is a relatively novel tool that has only begun to gain merit in 2,4-D toxicology research

within the last few years. Below is a review of some existing *in vivo* (yeast cells, rats) and *in vitro* (HepG2) experimental studies that have employed toxicogenomics (cDNA microarray analysis, 2-dimensional gel electrophoresis, RT-PCR) to assess 2,4-D toxicity.

Most recently, cDNA microarray analysis was employed to study the acute effects of 2,4-D (0.3 mM of 2,4-D acid for 15 min) on early transcriptional responses in yeast (Teixeira *et al*., 2006). Acute exposure to 2,4-D resulted in alterations in the expression patterns of 820 genes. Significant increases (at least 2 fold) in the mRNA expression levels of antioxidant oxidative stress response (glutathione), chaperones and heat shock protein (heat shock protein (Hsp) 12 and 104) genes were observed.

Toxicogenomics was also employed to evaluate the transcriptional responses to high 2,4-D exposure in rats (Badawi *et al*., 1999). Rats were exposed to a single oral dose of 375 mg/kg of 2,4-D acid for 72 hrs. The analysis (via RT-PCR) of the liver, kidney and mammary glands illustrated that 2,4-D induced the up-regulation mRNA expression of several cytochrome P450 biotransformation enzymes (i.e. CYP1A1, CYP1A2 and CYP1B1) that are involved in estrogen metabolism (Badawi *et al*., 1999). The authors explained that the increased estrogen metabolism may suggest 2,4-D – induced carcinogenesis.

In addition to the transcriptional responses, the translational responses to 2,4-D exposure have also been investigated, *in vivo* (Teixeira *et al*., 2005). Using 2-dimentional gel electrophoresis, it was illustrated that exposure (1.5 hrs) to 0.3 mM of 2,4-D induced alteration in the expression patterns of 26 proteins in yeast. Proteins, whose expression were significantly up-regulated (at least 2-fold) included those associated with stress response (i.e. Hsp12), mRNA and protein degradation (i.e. mRNA decapping enzyme).

Thus, the above *in vivo* studies illustrates that 2,4-D induces alterations in gene expression at the level of mRNA and protein accumulation. More importantly, and as illustrated in the studies conducted by Teixeira and colleagues, is the finding that alteration of gene expression, at the level of mRNA and protein accumulation can be detected following very acute (15 min or 1.5 hrs) exposure to a moderate concentration (0.3 mM) of 2,4-D. This illustrates the sensitivity of the endpoint, in contrast to more

over biological endpoints (i.e. cell death) that are typically used to assess 2,4-D toxicity. However, similar to the majority of previous studies, the above studies failed to examine the adverse effects of low 2,4-D concentrations (0.1 to 100 nM). Our laboratory was the first to take advantage of toxicogenomics to asses the adverse human health effects of low environmental 2,4-D concentrations (Bharadwaj *et al*., 2005). The hypotheses of this present study were partially derived from results reported from that study, where acute exposure (24 hrs) to 0.1 nM to 1 mM of commercial 2,4-D (containing 2,4-D DMA) induced alteration in the gene expression pattern of 238 genes, including FTL, FTH1 and PCNA. Results suggest; (1) low concentrations of 2,4-D may be cytotoxic, reflected by alteration in gene expression patterns, at the level of mRNA accumulation and (2) in contrast to overt biological endpoints (i.e. cell death), gene expression may be a more sensitive, and hence a better endpoint for assessing the adverse effects of low concentrations of toxicants

Thus, taken all together, the above studies that employed toxicogenomics to assess the toxicity of 2,4-D, illustrate that the herbicide induces alteration in gene expression at the transcriptional and translational levels. Furthermore, the study conducted by Bharadwaj et al. (2005) illustrates that the alteration of gene expression pattern is a sensitive endpoint for determination of the toxic effects of low concentrations of toxicants (i.e. 2,4-D). Additionally, all of the above studies illustrate that the alteration of gene expression patterns induced by chemical (i.e. 2,4-D) exposure may also serve to highlight cell and molecular pathways involved in toxicity. For instance, the up-regulation of FTL, FTH1 and PCNA mRNA expression (expressions are suggested to be up-regulated in response to oxidative stress) in HepG2 cells observed by Bharadwaj and co-workers suggest that ROS mediated oxidative stress may be a mechanism of 2,4-D toxicity. Therefore, in this thesis, studying the alteration in gene expression, serves as a useful tool for assessing the human toxicity that may be associated with low concentrations of 2,4-D and highlighting underlying mechanism(s) of its toxicity.

2.1.8 Suggested mechanisms of 2,4-D toxicity

Within the last several years, toxicology researchers have been diverging away from simple cause and effect toxicity studies and have begun to investigate the mechanism(s) of chemical toxicity. Several investigators have attempted to elucidate the mechanisms of 2,4-D toxicity. However, to this date, the mechanisms of 2,4-D toxicity have not yet been completely elucidated. Below is a review of some existing *in vivo* and *in vitro* studies that provides evidence that suggests that 2,4-D toxicity may be induced by various mechanisms (i.e. apoptosis, necrosis, uncoupling of oxidative phosphorylation, depression of transmembrane potential, formation of a reactive metabolite, ROS and associated oxidative stress).

2.1.8.1 Role of apoptosis in 2,4-D toxicity

Programmed cell death, or apoptosis, may play a role in 2,4-D toxicity. Apoptosis is characterized by defined cellular events including; disruption of plasma membrane, DNA fragmentation, chromatin condensation, change in cell volume and formation of apoptotic bodies (De Moliner *et al*., 2002).

Results from an *in vivo* study suggests that apoptosis may be a mechanism of 2,4-D toxicity (Kaioumova *et al*., 2001a). In this study, dose- and time-dependent apoptosis (assessed by DNA laddering pattern visualized by DNA gel electrophoresis) was evident in the thymocytes of rats exposed (via oral gavage) to single doses of 2.8, 22.8, 228 mg/kg of commercial 2,4-D (containing 2,4-D DMA) for 4, 8, 12 and 24 hrs.

The results from several *in vitro* studies that have utilized various cell models (human HepG2, blood lymphocytes, Jurkat T, cerebellar granule cells) also provide evidence to suggest that apoptosis may be a mechanism of 2,4-D toxicity. Apoptotic activity was assessed using various assays (annexin-V, DNA gel electrophoresis, flow cytometry, nicoletti, and terminal transferase dUTP nick and labeling (TUNEL)) Results from annexin-V assay revealed that exposure to 4, 8 and 16 mM of 2,4-D acid for 24 and 48 hrs induces HepG2 cell death by apoptosis (Tuschl and Schwab, 2003). Comparative results were also observed in later studies, when the authors exposed HepG2 cells to 4, 8 and 16 mM of 2,4-D acid for 24 hrs (Tuschl and Schwab, 2004) or 1 to 28 days (Tuschl and Schwab, 2005), respectively. In later studies that were

performed by the same researchers, results of apoptotic activity were supported by the results from another assay, flow cytometry (Tuschl and Schwab, 2004; 2005).

In contrast to Tuschl and Schwab, Kaioumova and colleagues used human peripheral blood lymphocytes and Jurkat T cells to assess apoptotic activity following 2,4-D exposure (Kaioumova *et al*., 2001b). Concentration- and time-dependent apoptosis was observed in cells exposed to 0.1 to 5 mM of commercial 2,4-D (containing 2,4-D DMA) for 2, 4, 6 and 24 hrs. Similar to Tuschl and Schwab, apoptotic activity was determined by the annexin-V assay. Furthermore, the authors also supported experimental results by performing additional assays (DNA gel electrophoresis and nicoletti assay) for assessing apoptotic activity.

Finally, *in vitro* experimental evidence suggesting that apoptosis may be a mechanism of 2,4-D toxicity was presented by De Moliner et al. (2002). In contrast to Tuschl and Schwab and Kaioumova and colleagues, the authors assessed 2,4-D – induced apoptotic activity in human cerebellar granule cell cultures. Results obtained from the TUNEL assay revealed that there was a non concentration-dependent increase in apoptotic activity in cells exposed to 1 or 2 mM for 24 hrs.

Thus, results from above studies that have used various experimental models (*in vivo* (rats) and *in vitro* (human HepG2, blood lymphocytes, Jurkat T, cerebellar granule cells)), assay (annexin-V, DNA gel electrophoresis, flow cytometry, nicoletti, and TUNEL), doses (2.8 to 228 mg/kg), concentrations (0.1 to 16 mM), durations of exposure (4 hrs to 28 days) and 2,4-D forms (2,4-D acid or 2,4-D DMA) suggest that apoptosis may be a mechanism of 2,4-D toxicity.

2.1.8.2 Role of necrosis in 2,4-D toxicity

Necrosis, or unprogrammed cell death is characterized by cellular swelling, organelle disruption, random and diffused DNA fragmentation and inflammation (Mitchell and Cotran, 1997) and has been suggested to be a mechanism of 2,4-D toxicity.

Results from a TUNEL assay showed that exposure to 2 mM of 2,4-D acid for 24 hrs induced necrotic activity in 72% of human cerebellar granule cells (De Moliner *et al*., 2002). Necrotic activity induced by 2,4-D exposure was also documented in a later

study, where, via the annexin-V assay, it was revealed that exposure to 4, 8 and 16 mM of 2,4-D acid for 24 and 48 hrs induced necrosis in HepG2 cell (Tuschl and Schwab, 2003).

Thus, results from the above studies that have used various *in vitro* models (human HepG2 and cerebellar granule cells), assay (annexin-V and TUNEL), concentrations (2 to 16 mM), durations of exposure (24 and 48 hrs) and a similar 2,4-D form (2,4-D acid) suggest that necrosis may be a mechanism of 2,4-D toxicity.

2.1.8.3 Alteration of cell physiology in 2,4-D toxicity

It is anticipated that 2,4-D toxicity will induce alterations in the physiological environment of the cell. It has been suggested that 2,4-D may alter cell physiology by affecting mitochondrial oxidative phosphorylation and transmembrane potential.

Two *in vitro* studies provide evidence that suggest that uncoupling of oxidative phosphorylation may play a role in 2,4-D toxicity. Both studies used rat hepatocytes as their cell model and assessed mitochondrial oxidative phosphorylation via polarographically. Mitochondria isolated from rat hepatocytes were exposed to 0.1 to 4.0 mM of 2,4-D acid (Zychlinski and Zolnierowicz, 1990). Exposure to 1.5 to 4 mM induced a concentration-dependent increase in the uncoupling of mitochondrial oxidative phosphorylation. Evidence suggesting that uncoupling of oxidative phosphorylation may be a mechanism of 2,4-D toxicity was also provided Palmeira and co-workers (Palmeira *et al*., 1994). Mitochondria that was isolated from rat hepatocytes was exposed to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 mM of 2,4-D acid. Similar to Zychlinski and Zolnierowicz, Palmeira et al. (1994) reported a concentration-dependent increase in the uncoupling of mitochondrial oxidative phosphorylation following 2,4-D exposure.

Thus, the above *in vitro* studies that used rat hepatocytes, 2,4-D acid, various concentrations of 2,4-D (0.1 to 4 mM) and assessed mitochondrial oxidative phosphorylation via polarographically, provide experimental evidence that suggest that the uncoupling of mitochondrial oxidative phosphorylation may be a mechanism of 2,4-D toxicity.

Three *in vitro* studies have provided evidence that suggest that 2,4-D reduces transmembrane potential using primary rat hepatocytes (Palmeira *et al*., 1994), human HepG2 (Tuchl and Schwab, 2003) and Jurkat-T (Kaioumova *et al*., 2001b) cells as their cell models. Transmembrane potential was assessed by calculation of membrane distribution of tetraphenylphosphonium and 5,5',6',6'-tetrachloro-1,1',3,3-tetraethybenimidazolycarbocyanine iodide (JC-1). Results (obtained from calculation of membrane distribution of tetraphenylphosphonium) from one study revealed that mitochondrial membrane potential was reduced in primary rat hepatocytes exposed to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 mM of 2,4-D acid (Palmeira *et al*., 1994). A concentration-dependent effect was observed at concentrations greater than 0.1mM 2,4-D acid. Results obtained from the JC-1 fluorescent assays also provide evidence to support the hypothesis that 2,4-D represses mitochondrial membrane potential (Kaioumova *et al*., 2001b; Tuschl and Schwab, 2003). A concentration-dependent depression of mitochondrial membrane potential in HepG2 cells exposed to 4, 8 and 16 mM of 2,4-D acid for 6 hrs has been reported (Tuchl and Schwab, 2003). A time-dependent reduction in mitochondrial transmembrane potential has been observed following exposure of Jurkat-T cells to 1mM of a commercial formulation of 2,4-D (containing 2,4-D DMA) for 2, 4, 6 and 24 hrs (Kaioumova *et al*., 2001b).

Thus, the above *in vitro* studies that used rat hepatocytes, human HepG2 and Jurkat-T cells, different 2,4-D forms (2,4-D acid or DMA), various concentrations (0.1 to 1 mM) and assessed mitochondrial transmembrane potential via tetraphenylphosphonium or JC-1 probe detection, provide experimental evidence that suggest that reduction of mitochondrial transmembrane potential may be a mechanism of 2,4-D toxicity. Therefore, taken all together, results from the all of the above *in vitro* studies, suggest that uncoupling of mitochondrial oxidative phosphorylation and reduction of mitochondrial transmembrane potential may be mechanisms by which 2,4-D alters cell physiology during toxicity.

2.1.8.4 Formation of a reactive metabolite in 2,4-D toxicity

The metabolism of 2,4-D in humans is an area of great controversy. Some researchers have argued that in humans, 2,4-D is not metabolized to any reactive

metabolites and is essentially eliminated unchanged (Gregus *et al*., 1999; Sauerhauff *et al*., 1977; Van Ravenzwaay *et al*., 2003). In contrast some researchers have illustrated that 2,4-D may form non-toxic conjugates in humans (Griffin *et al*., 1997; Sauerhauff *et al*., 1977). This suggests that in humans, 2,4-D may be metabolized to toxic reactive metabolites and that conjugation with endogenous compounds, such as glutathione (GSH), facilitates detoxification of such metabolites in humans. However, experimental evidence suggesting that 2,4-D may be metabolized in humans to toxic reactive metabolite(s) is scarce and limited to the results obtained an *in vitro* chemical synthesis study (Li *et al*., 2003). Results from this study illustrate that 2,4-D may be metabolized to form an acyl-CoA thioester, 2,4-dichlorophenoxyacetyl-*S*-acyl-CoA (2,4-D-CoA). *In vitro* chemical stability studies revealed that the reactive metabolite is stable at human physiologic pH (pH 7.4) and temperature (37°C). *In vitro* reaction studies illustrated that 2,4-D-CoA formed adducts with nucleophilic groups on human serum albumin (HSA). Furthermore, it was illustrated *in vitro*, that 2,4-D-CoA binds covalently to hepatic proteins in freshly prepared rat hepatocytes. Results from high performance liquid chromatography assays revealed that synthetic 2,4-D-CoA was also able to transacylate the cysteine sulfhydryl group of GSH to form 2,4-D-*S*-acyl-glutathione (2,4-D-SG). The results suggest that the novel metabolite may be an electrophilic reactive metabolite that may be detoxified via transacylation with GSH or induce toxicity via covalent binding to nucleophilic groups on biomolecules, such as proteins, to result in possible toxic effects, *in vivo*. Covalent binding to target molecule(s) is one mechanism of action of a toxicant (Gregus and Klaassen, 2001).

2.1.8.5 Role of ROS and oxidative stress in 2,4-D toxicity

Several investigators who have used both *in vivo* and *in vitro* experimental models have provided evidence that suggest that ROS mediated oxidative stress may play a role in 2,4-D toxicity. The *in vivo*, followed by the *in vitro* studies will be reviewed.

Using electron paramagnetic resonance spectroscopy, it was illustrated that 2,4-D induces ROS production in yeast cells (Teixeira *et al*., 2004). A concentration-dependent increase in the accumulation of the hydroxyl radical ($OH^{\cdot}$), a ROS, was

observed in yeast cells that were exposed to 0.45, 0.55 and 0.65 mM of 2,4-D acid for 15 min. Results suggest that 2,4-D may directly induce ROS production, *in vivo*. The ROS subsequently induces toxicity by the mechanism of oxidative stress.

Several *in vitro* studies (human erythrocytes, primary rat hepatocytes) have tried to determine if ROS mediated oxidative stress may be mechanisms of 2,4-D toxicity. However, these studies failed to directly measure ROS production, *in vitro*. Instead ROS mediated oxidative stress was assessed by measuring the activity of antioxidant enzymes, levels of glutathione (GSH) and presence of lipid peroxidation. Antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px)) and GSH are involved in the detoxification of ROS (Gregus and Klaassen, 2001; Kehrer, 1993; Kohen and Nyska, 2002). Lipid peroxidation results from ROS – induced oxidative stress (Kohen and Nyska, 2002).

The effects of 2,4-D on the activity of several antioxidant enzymes and GSH have been investigated (Bukowska, 2003). Human erythrocytes were exposed to 0.045, 0.22, 0.45, 1.12 mM of 2,4-D acid for 1 hr. Spectrophotometric analyses revealed that there was a significant concentration-dependent decrease in SOD activity in erythrocytes exposed to 0.22 to 1.12 mM of 2,4-D. Analyses also revealed that there was a significant concentration-dependent increases or decreases in GSH-Px activity and GSH levels, respectively, in erythrocytes exposed to 0.45 and 1.12 mM of 2,4-D. Similar effects of 2,4-D exposure on GSH levels were also observed in an earlier study (Palmeira *et al*., 1995). Fluorescent measurements revealed concentration-and time-dependent decreases in GSH levels in primary rat hepatocytes exposed to 1, 5 and 10 mM of 2,4-D acid for 0 to 200 min. Results suggest that 2,4-D exposure may induce ROS production in a concentration-dependent manner, and that increasing ROS concentration may exhaust GSH supplies, *in vitro*.

Lipid peroxidation is also a valuable endpoint for determining if exposure to a chemical induces oxidative stress. The effect of 2,4-D exposure on lipid peroxidation has been investigated (Duchnowicz and Koter, 2003). Human erythrocytes were exposed to 1 mM of 2,4-D acid for 1 hr. Results from the thiobarbituric acid reactive substance (TBARS) assay revealed significant increases in malondialdehyde (MDA) levels. MDA is an end product of lipid peroxidation (Palmeira *et al*., 1995). Similar effects were also

observed in primary rat hepatocyte cell cultures (Palmeira *et al*., 1995). A concentration- and time-dependent increase in MDA levels were observed in hepatocytes that were exposed to 1, 5 and 10 mM of 2,4-D acid for 0 to 200 min.

Thus, results from both *in vivo* (yeast) and *in vitro* (human erythrocytes, primary rat hepatocytes) studies that have used the same 2,4-D acid form, various endpoints (ROS production, activity of antioxidant enzymes, levels of GSH and presence of lipid peroxidation), concentrations (0.045 to 10 mM) and durations of exposure (15 to 200 min) suggest that ROS and associated oxidative stress may be mechanisms of 2,4-D toxicity. However, with the exception of the study performed by Teixeira and colleagues, a flaw of all other studies is that the studies failed to illustrate that 2,4-D directly induces ROS production in *in vitro* experimental models. Thus, to the best of our knowledge, this thesis is the first to examine if 2,4-D directly induces ROS production in human cell cultures (HepG2 and HEK293 cells), *in vitro*.

Reviewed above, the results from several studies that used *in vivo* (yeast cells) or *in vitro* (human HepG2, erythrocytes, Jurkat-T cells, primary rat hepatocytes) experimental models suggest that apoptosis, necrosis, alterations of cell physiology, reactive metabolites, ROS and oxidative stress may be mechanisms of 2,4-D toxicity. However, as previously mentioned, the mechanism(s) of 2,4-D toxicity is a relatively novel research area that is still evolving. Thus, one objective of the current study was to determine a possible mechanism of 2,4-D cytotoxicity, by investigating ROS production, *in vitro*. The production of ROS was investigated in this thesis based on previous cDNA microarray analyses that illustrated increased expression of FTL, FTH1 and PCNA mRNA in response to 2,4-D exposure (Bharadwaj *et al*., 2005) and previous *in vivo* and *in vitro* studies that provides evidence that suggest that FTL, FTH1 and PCNA mRNA and protein expression is increased in response to ROS mediated oxidative stress (Balajee *et al*., 1999; Balla *et al*., 1992; Epsztejn *et al*., 1999; Holmes *et al*., 2002; Lin and Girotti, 1997; Orino *et al*., 2001; Regan *et al*., 2002; Savio *et al*., 1998). A review of such studies is presented in the following sections.

2.2 Ferritin

2.2.1 Ferritin: structure and function

Ferritin is a stable ubiquitous (Corsi *et al*., 2002; Ponka *et al*., 1998; Rogers and Munro, 1987; Rogers *et al*., 1990), multimeric (Cairo *et al*., 1995) iron-storage protein (Crichton *et al*., 2002; Hagen *et al*., 2002; Harrison and Arosio, 1996; Kwak *et al*., 1995; Ponka *et al*., 1998; Rogers and Munro, 1987; Rogers *et al*., 1990; Sánchez *et al*., 2005; Vidal *et al*., 2004). The protein is found in a wide range of species that includes vertebrates, invertebrates, plants, fungi and bacteria (Leibold and Guo, 1992). The maintenance of cellular iron homeostasis is a principal role of ferritin (Harrison and Arosio, 1996; Kwak *et al*., 1995). Approximately 25% of the total body iron stores are contained in ferritin (Leibold and Guo, 1992) and its putative insoluble (Arosio and Levi, 2002) derivative product, hemosiderin (Arosio and Levi, 2002; Leibold and Guo, 1992). Hemosiderin is an aggregate of iron and protein found in lysosomes (Leibold and Guo, 1992). Ferritin's molecular structure is highly conserved in all organisms (Harrison and Arosio, 1996) and is mainly a cytosolic protein (Arosio and Levi, 2002; Corsi *et al*., 2002). However, in vertebrates, a minor portion of ferritin is also present in serum and secretory fluids (Arosio and Levi, 2002). Recently, a novel type of ferritin has been found in human mitochondria (Levi *et al*., 2001). This novel ferritin is referred to as mitochondrial ferritin (MtF) (Drysdale *et al*., 2002; Levi *et al*., 2001; Levi and Arosio, 2004). According to researchers, MtF is structurally similar to cytosolic ferritins, and also bears ferroxidase activity, suggesting that MtF may also be involved in iron detoxification in the mitochondria (Levi *et al*., 2001) (see below for review of ferritin ferroxidase activity and association between ferritin and iron detoxification). However, in contrast to cytosolic ferritins, MtF are homopolymers (Levi *et al*., 2001).

Mammalian ferritin is a hollow sphere (Chaincone *et al*., 2004) which is made up of 24 subunits, consisting of FTL and FTH1 polypeptides (Cairo *et al*., 1995; Hagen *et al*., 2002; Harrison and Arosio, 1996; Kwak *et al*., 1995; Leibold and Guo, 1992; Lo and Hurta, 2000; Rogers and Munro, 1987; Rogers *et al*., 1990; Thompson *et al*., 2003; Vidal *et al*., 2004). Among mammals, the FTL subunits are 82-88% identical, while the FTH1 subunits are 95% identical (reviewed by Leibold and Guo, 1992). Ferritin is able to sequester 4500 iron atoms per ferritin molecule (Harrison and Arosio, 1996; Sánchez

et al., 2005), as insoluble (Harrison and Arosio, 1996) ferric oxy-hydroxide (Chiancone *et al*., 2004; Harrison and Arosio, 1996; Ponka *et al*., 1998). However, under normal cellular conditions, ferritin is only 20% saturated with iron (Reif, 1992).

There are approximately 25 iso-ferritins of varying FTL and FTH1 subunit ratios (i.e. $H_{24}L_0$, $H_{23}L_1$, $H_{22}L_2$, to H_0L_{24}) within the ferritin molecule (Harrison and Arosio, 1996). The ratio of the FTL (MW: 19kDa (Leibold and Guo, 1992; Rogers and Munro, 1987; Rogers *et al*., 1990; Thompson *et al*., 2003;) and FTH1 (Molecular Weight (MW): 21kDa (Leibold and Guo, 1992; Rogers and Munro, 1987; Rogers *et al*., 1990; Thompson *et al*., 2003) subunits within the ferritin molecule vary depending on the tissue in which it is expressed (Kwak *et al*., 1995; Thompson *et al*., 2003). For example, the brain and heart consist of H-rich ferritins that have a lower FTL to FTH1 subunit ratio within ferritin molecules (Harrison and Arosio, 1996). H-rich ferritins in these organs normally consist of less iron/molecule (i.e. 1000 iron atoms per ferritin molecule (Harrison and Arosio, 1996)). In contrast, in iron-storing organs such as the liver and spleen there is a higher FTL to FTH1 subunit ratio within ferritin molecules (Harrison and Arosio, 1996). For instance, liver ferritins (FTH1 to FTL subunit ratios of $H_{2\text{-}3}L_{22\text{-}21}$) have a relatively high iron content of 1500 iron atoms per ferritin molecule (Harrison and Arosio, 1996). The existence of variations of FTL and FTH1 subunit ratios allow for ferritins to have different and independent iron-storage and oxidizing capacity (Arosio and Levi, 2002). For instance, the presence of a higher FTL to FTH1 subunit ratio allows for increased iron-storage capability of liver ferritins (Arosio and Levi, 2002).

The FTH1 subunit, which is largely responsible for the biological activity of mammalian ferritin (Corsi *et al*., 2002), bears the molecule's ferroxidase enymatic activity (Corsi *et al*., 2002; Hagen *et al*., 2002; Kwak *et al*., 1995). Ferroxidase allows for the catalytic oxidation of the soluble (Corsi *et al*., 2002) toxic form (Fe^{2+}) of iron (Corsi *et al*., 2002; Hagen *et al*., 2002; Harrison and Arosio, 1996; Kwak *et al*., 1995; Thompson *et al*., 2003;) into the non-toxic ferric form (Fe^{3+}) (Ponka *et al*., 1998). The ferric form (Fe^{3+}) of iron is subsequently stored as an insoluble (Harrison and Arosio, 1996) ferric oxy-hydroxide molecule (Chiancone *et al*., 2004; Harrison and Arosio, 1996; Ponka *et al*., 1998). In contrast, the FTL subunit confers protein stability (Hagen

et al., 2002; Kwak *et al*., 1995) and nucleation of the iron core (Corsi *et al*.,2002; Ponka *et al*., 1998).

2.2.2 Regulation of ferritin gene expression

Gene expression of FTL and FTH1 is primarily and tightly regulated at the translational level (Arosio and Levi, 2002; Cairo *et al*., 1995; Corsi *et al*., 2002; Crichton et al., 2002; Leibold and Guo, 1992). Gene expression is regulated in response to alterations in intracellular iron availability (Crichton *et al*., 2002) through specific interactions between the cytosolic iron regulatory proteins (IRPs) (Crichton *et al*., 2002) and the iron responsive element (IRE) (Arosio and Levi, 2002) situated in the 5'-untranslated region (UTR) of the FTL and FTH1 mRNA (Cairo *et al*., 1995; Harrison and Arosio, 1996; Ponka *et al*., 1998).

In mammals, two IRPs have been identified, IRP-1 and IRP-2 (Crichton *et al*., 2002). The proteins, IRP-1 and IRP-2 have been described to act as intracellular iron sensors (reviewed by Papanikolaou and Pantapoulos, 2005). Similar homology exists between IRP-1 and the mitochondrial enzyme, aconitase (Ponka *et al*., 1998). In mammals, aconitase is an enzyme that contains a [4Fe-4S] cluster and is involved in the citric acid cycle (Ponka *et al*., 1998). A 61% amino acid sequence similarity exists between IRP-1 and IRP-2 (Ponka *et al*., 1998). However, in contrast to IRP-1, IRP-2 lacks aconitase activity, and therefore only possesses RNA-binding capabilities (Ponka *et al*., 1998). The IRP-2 protein binds to IREs with similar affinity as IRP-1, but iron regulation by IRP-2 is mediated by specific proteolysis (Ponka *et al*., 1998). The IREs are structural motifs that are located in the 5'-UTR of the FTL and FTH1 mRNA (Cairo *et al*., 1995; Harrison and Arosio, 1996; Papanikolaou and Pantapoulos, 2005; Ponka *et al*., 1998). These responsive elements are approximately 30 nucleotides in length and fold and form a loop and a stem (reviewed by Papanikolaou and Pantapoulos, 2005). The IREs are able to recognize and hence provide a binding site for the IRPs (Leibold and Guo, 1992; Papanikolaou and Pantapoulos, 2005).

In mammalian cells, iron homeostasis is maintained by balancing the uptake of iron with its intracellular storage and utilization (Crichton *et al*., 2002). This balance is achieved primarily at the level of protein synthesis (Crichton *et al*., 2002). Ferritin and

transferrin are the major proteins involved in maintaining iron homeostasis (Leibold and Guo, 1992). Transferrin functions to transport iron between its sites of absorption, storage and use (Ponka *et al*., 1998). In the presence of increased intracellular iron concentration, IRP-1 possesses aconitase activity and binds the IRE with low affinity, resulting in increased translation of FTL and FTH1 mRNA (Crichton *et al*., 2002; Ponka *et al*., 1998). The absence of the association between IRP-1 and the IRE (located in the 3'-untranslated region) of the transferrin receptor mRNA results in degradation of transferrin receptor mRNA (reviewed by Ponka *et al*., 1998). Following intracellular expansion of the labile iron pool, IRP-2 is degraded (reviewed by Crichton *et al*., 2002; Ponka *et al*., 1998). In contrast, during periods of decreased intracellular iron concentration, IRP-1 does not possess aconitase activity (Crichton *et al*., 2002; Ponka *et al*., 1998). Therefore, IRP-1 binds the IRE with high affinity, with subsequent decreased translation of FTL and FTH1 mRNA and increased translation of transferrin receptor mRNA (Ponka *et al*., 1998).

The increased expression of FTL and FTH1 gene at the translational level effectively reduces the bioavailability of excess intracellular iron (Arosio and Levi, 2002). Transition metals, such as iron, are extremely important in the generation of ROS (Kehrer, 1993), due to its active participation in the Fenton and Haber-Weiss chemistry (Biemond *et al*., 1988; Chiancone *et al*., 2004; Harrison and Arosio, 1996; McCord, 1998; Papanikolaou and Pantopoulos, 2005). The ROS are known mediators of oxidative stress (reviewed by Valko *et al*., 2007). Thus, increased iron sequestration as a result of the up-regulation of iron-storage proteins, such as ferritin, may provide protection to cells and tissues from oxidative stress.

It has now been suggested that during oxidative stress conditions, ferritin gene expression may in fact be regulated secondary to iron release by heme oxygenase-1 (HO-1) protein (Gonzales *et al*., 2002; Vile and Tyrell, 1993; Vile *et al*., 1994). The HO-1 protein is responsible for the catabolization of heme into carbon monoxide, biliverdin (rapidly converted to bilirubin) and free iron (Otterbein *et al*., 2003; Unno *et al*., 2007). It has been illustrated *in vitro*, that oxidative stress conditions (ultraviolet A (UVA) rays or H_2O_2) induces the immediate release of heme from microsomal hemeproteins (i.e. cytochrome P450) in primary human skin fibroblasts (FEK4) (Kvam

et al., 1999). In the presence of ROS, heme may be released from hemeproteins as a result of ROS – induced protein oxidation and degradation. Proteins are biological targets for ROS – induced oxidation, damage and degradation (Davies, 1987; Davies *et al*., 1987a; 1987b; 1987c). Therefore, during oxidative stress conditions, the chain of events responsible for induction of ferritin gene expression may be as follows; (1) increased release of heme from hemeproteins, (2) increased gene expression of HO-1, (3) increased catabolization of heme to carbon monoxide, biliverdin and free iron and (4) increased ferritin gene expression. Thus, increased expression of the ferritin protein ultimately provides protection during oxidative stress conditions, by limiting the availability of free iron to the Fenton and Haber-Weiss reactions and thereby reducing further generation of ROS. A review of some studies that presents evidence that suggest that the up-regulation of ferritin gene expression may be a cytoprotective mechanism against oxidative stress is presented below.

2.2.3 Ferritin and oxidative stress

The sequestration of free iron by ferritin, which subsequently reduces iron availability to the iron-catalyzed Fenton and Haber-Weiss reactions, is regarded as a protective mechanism against cellular oxidative stress (Balla *et al*., 1992). Several studies conducted on various *in vitro* models (i.e. mouse cortical astrocytes, porcine aortic endothelial, murine erythroleukemia, immortalized human HeLa and HepG2 cells) provides evidence that suggest that the increased sequestration of labile iron as a result of the up-regulation of ferritin expression is a protective response to oxidative stress (Balla *et al*., 1992; Cairo *et al*., 1995; Epsztejn *et al*., 1999; Orino *et al*., 2001; Regan *et al*., 2002).

In vitro studies conducted on mice cortical astrocytes illustrates that ferritin expression increases in response to oxidative stress (Regan *et al*., 2002). Ferritin protein expression was increased following exposure to 5 μM of hemoglobin (Hb) or 3 μM of hemin. Both Hb and hemin are direct sources of iron. Western blot analyses revealed that exposure to 5 μM of hemoglobin for 2, 4 and 8 hrs induced a time-dependent increase in ferritin protein synthesis in astrocytes. Results suggest that an up-regulation of ferritin protein expression may be a protective response to increased intracellular iron.

The sequestration of free iron by ferritin protects against oxidative stress by reducing iron availability to the Fenton and Haber-Weiss reactions and thus, further generation of ROS production. In the same study, results from cytotoxicity assay (measurement of lactate dehydrogenase (LDH) activity) revealed that pre-treatment of astrocytes with 1, 2 or 4 mg/L of apoferritin (ferritin that lacks iron (Harrison and Arosio, 1996)) provided concentration-dependent protection against cell death following exposure to a toxic concentration (30 μM) of hemin. Furthermore, astrocytes pre-treated with 30 μM of Hb or 3 μM of hemin for 24 hrs were protected from hemin-induced oxidative injury in a concentration-dependent manner. The results suggest that pre-treatment with apoferritin, Hb or hemin induced ferritin protein expression, which resulted in sequestration of free iron and reduced ROS production and associated oxidative stress upon hemin challenge.

Enhanced ferritin protein synthesis in response to increased intracellular iron concentration and possible increased ROS production and potential oxidative stress has also been observed by Balla and co-workers. Ferritin immunoassay illustrated that exposure to 1.0, 2.5, 5.0 and 10 μM of hemin induced a concentration-dependent increase in ferritin protein synthesis in porcine aortic endothelial cells. Pre-treatment of cells with 5 μM of hemin or 100 μM of hydrogen peroxide (H_2O_2) resulted in a concentration-dependent reduction of ferritin synthesis in cells exposed to 1.0, 2.5, 5.0 and 10 μM of hemin (Balla *et al*., 1992). Results suggest that treatment or pre-treatment of cells with mediators (i.e. hemin or H_2O_2) of ROS production induces ferritin protein expression as a protective mechanism, since increased ferritin protein expression subsequently limits free iron availability of Fenton and Haber-Weiss chemistry and reduces further ROS production and oxidative damage.

Further evidence to suggest that the increased expression of ferritin may be a protective response to increased ROS production and oxidative stress has also been presented by several other research groups (Cairo *et al*., 1995; Epsztejn *et al*., 1999; Orino *et al*., 2001). These studies showed that increased ferritin expression was associated with decreased ROS production. For instance, Orino and colleagues exposed HeLa cells to 125 or 250 μM of H_2O_2 for 8 hrs (Orino *et al*., 2001). Western blot analyses illustrated that exposure to H_2O_2 resulted in an increase in both FTL and FTH1

mRNA and protein expression. Additionally in H_2O_2 challenged cells, over expression of FTL and FTH1 cDNA in transfected cells reduced the accumulation of intracellular ROS (assessed using 2,7'-DCFH-DA). The results illustrates that the up-regulation of ferritin may be in fact a protective response to ROS – induced oxidative stress. Similar increases in FTL and FTH1 mRNA and protein expression were also observed in the liver of mice exposed to the pro-oxidant, phorone for 3 or 6 hrs (Cairo *et al*., 1995). Decreased ROS production (assessed using 2',7'-carboxy-dichlorofluorescein) following iron loads and oxidant challenges in murine erythroleukemia cell cultures transfected with FTH1 protein subunit have also been reported (Epsztejn *et al*., 1999).

Thus, all of the above studies present evidence that suggest that increased ferritin mRNA or protein expression may be a protective response against iron-mediated increases in ROS production and associated oxidative stress. Therefore, the up-regulation of FTL and FTH1 mRNA expression that was observed in HepG2 cells exposed to 0.1 nM to 1 mM 2,4-D for 24 hrs (Bharadwaj *et al*., 2005) may have been a protective response to 2,4-D induced ROS production and associated oxidative stress. Thus, taken all together, the above studies presents evidence that provides the necessary rationale in this thesis for investigating ROS production in HepG2 and HEK293 cells exposed to 2,4-D and also allows for the suggestion that increased expression of FTL and FTH1 mRNA and protein expression highlights ROS mediated oxidative stress as a mechanism of 2,4-D toxicity.

2.3 Proliferating cell nuclear antigen

2.3.1 PCNA: structure and function

Originally referred to as cyclin (Mathews *et al*., 1984; Moris and Mathews, 1989), PCNA is a homotrimeric ring-shaped protein (Brand *et al*., 1994; Prosperi, 2006; Schurtenberger *et al*., 1998; Wyman and Botchan, 1995). It is an acidic nuclear protein (Mathews *et al*., 1984) that plays an integral role in nucleic acid metabolism (Kelman, 1997), including DNA replication, repair and cell cycle regulation (Kelman, 1997; Schurtenberger *et al*., 1998).

The homotrimeric PNCA structure is composed of three identical monomers (Maga and Hübscher, 2003) with a molecular weight of 28.7kDa (Brand *et al*., 1994).

However, when the protein is resolved by SDS-PAGE, it migrates with an apparent molecular weight of 36kDa (Brand *et al*., 1994). The proliferating antigen, which was first identified in the sera of some patients with systemic lupus erythematosus (SLE) is a member of the DNA sliding clamp family (Kelman and O'Donnell, 1995; Maga and Hübscher, 2003; Warbick, 2000; Wyman and Botchan, 1995). The DNA sliding clamps are defined as ring-shaped proteins that indirectly bind to DNA by encircling and forming a topological link with the double helix molecule (Hingorani and O'Donnell, 2000). Additional members of the DNA sliding clamp family include; *Escherichia Coli* (*E.coli*) DNA polymerase III β subunit (Kelman and O'Donnell, 1995) and bacteriophage T4 gene45 protein (Wyman and Botchan, 1995). The DNA sliding clamp members are highly evolutionary conserved (Warbick, 2000). Thus, PCNA homologues are found in eukaryotes, archaea, bacteriophages and some viruses (Warbick, 2000), as well as in the cells of humans, mouse, hamster and bird (Almendral *et al*., 1987).

2.3.2 Regulation of PCNA gene expression

The expression of this highly conserved protein is regulated at the transcriptional level (Almendral *et al*., 1987). The expression level of PCNA protein is greatly increased in the S phase of the cell cycle (Bravo and Celis, 1980; Celis and Celis, 1985) and is synthesized during the proliferation of normal cells, transformed cells and tumors (Almendral *et al*., 1987; Bravo and Macdonald-Bravo, 1985). In contrast, the expression level of the proliferating antigen is reduced in non-dividing cells (Almendral *et al*., 1987; Bravo and Macdonald-Bravo, 1985).

2.3.3 PCNA and oxidative stress

Due to its key role in DNA replication, PCNA has become a novel tool, as a cell proliferation marker in toxicology studies aimed at determining the cell and molecular mechanisms of toxicity and/or carcinogenicity of compounds of interest (Dietrich, 1993). However, some *in vivo* (rats) and *in vitro* (human fibroblast cells) experimental studies have utilized PCNA protein expression as a potential marker of oxidative DNA damage (Balajee *et al*., 1999; Holmes *et al*., 2002; Savio *et al*., 1998).

An *in vivo* study was conducted that was aimed at determining if oxidative stress plays a role in peroxisome proliferation-induced carcinogenicity in isolated rat hepatocytes (Holmes *et al*., 2002). The authors used three proteins as markers of oxidative stress; DNA polymerase beta (Polβ), apurinic/apyrimidinic endonuclease (Ref-1) and PCNA. The proteins; Polβ, Ref-1 and PCNA are all involved in the repair of oxidative DNA damage (reviewed by Holmes *et al*., 2002). Rats were exposed to WY 14,643, a peroxisome proliferator. Peroxisome proliferators (i.e 2,4-D) are a structurally diverse group of compounds (Ge *et al*., 2002) that may induce hypertrophy of tissue peroxisomes and pronounced increases in the tissue levels of many peroxisomal enzymes (reviewed by Holmes *et al*., 2002). A concentration-dependent increase in Polβ and Ref-1 expression was observed in the liver of rats exposed to 5,000 or 50,000 µg/L of WY 14,634 for 6 days. Western blot analyses illustrated that PCNA protein expression was up-regulated 5 fold in rats exposed to 500,000 µg/L of WY 14,634 for 34 days. The results suggest that in addition to increased cell proliferation, increased PCNA protein expression may have been a protective response against oxidative DNA damage, since PCNA is known to be involved in DNA repair.

Results from two *in vitro* studies that were conducted a few years earlier also suggest that increased PCNA protein expression indicate chemical insult via the mechanism of oxidative stress. Immunofluorescent and western blot studies analyses provide experimental evidence that suggest that increased PCNA protein expression may be a response to oxidative DNA-damage (Balajee *et al*., 1999). Human fibroblast cell cultures were exposed to ultraviolet C (UVC) or H_2O_2. Both UV and H_2O_2 are known to induce a variety of oxidative DNA lesions (Balajee *et al*., 1999). Exposure to 2.5 mM of H_2O_2 for 15 min induced a 4- to 5-fold increase in PCNA-DNA complex, while exposure to 10 J/m^2 UV for 30 min induced a 9-fold increase in PCNA-DNA complex formation in the nucleus. Results suggest that up-regulation of PCNA protein expression may be a protective response to oxidative DNA damage, where, PCNA protein may play a role in one or more repair pathways of induced oxidative DNA lesions.

An additional immunofluorescent study also produced results that suggest that PCNA protein expression is up-regulated in response to oxidative DNA damage (Savio

et al., 1998). Compared to Balajee and co-workers, in the study, human fibroblasts cells were exposed to 10 fold or less H_2O_2 (0 to 200 μM) for a longer duration of time (1 hr). Exposure induced a rapid concentration-dependent increase in the nuclear binding of PCNA in cells. Results suggest that up-regulation of PCNA may be in response to oxidative DNA damage induced by the known DNA oxidizing agent, H_2O_2.

The results from the above studies suggest that increased PCNA protein expression may be a protective response against ROS – induced oxidative DNA damage. Therefore, the up-regulation of PCNA mRNA expression that was observed in HepG2 cells exposed to 0.1 nM to 1 mM of 2,4-D for 24 hrs (Bharadwaj *et al*., 2005) may have been a protective response to 2,4-D – induced intracellular ROS production and associated oxidative DNA damage in cells. Thus, taken all together, the above studies present evidence that provides the necessary rationale in this thesis for investigating ROS production in HepG2 and HEK293 cells exposed to 2,4-D and also allows for the suggestion that increased expression of PCNA mRNA and protein expression indicates ROS mediated oxidative stress as a mechanism of 2,4-D toxicity.

2.4 Reactive oxygen species

2.4.1 Introduction to ROS

Reactive oxygen species, commonly referred to as ROS and oxygen free radicals (OFR) are chemically unstable, independent (Kehrer, 1993) species that contain one or more unpaired electrons (Halliwell, 1987; Kehrer, 1993) in their outer orbital (Gregus and Klaassen, 2001). Some examples of ROS include the superoxide anion ($O_2^{\cdot-}$) and the noxious hydroxyl radical ($OH^{\cdot}$) (Gregus and Klaassen, 2001). These reactive species are produced from both endogenous and exogenous sources (reviewed by Kohen and Nyska, 2002). In humans, endogenous sources of ROS may include white blood cells (neutrophils, eosinophils, basophils), enzymes directly producing ROS (nitric oxide synthase), enzymes indirectly producing ROS (xanthine oxidase), mitochondrial metabolism and diseases (i.e. ischemic processes) (reviewed by Kohen and Nyska, 2002). Food, UV radiation, pollutants, xenobiotics and toxins are some examples of exogenous sources of ROS (reviewed Kohen and Nyska, 2002).

2.4.2 Relationship between iron, ROS and oxidative stress

The production of ROS may be mediated by various metals including iron, copper and chromium (reviewed by Valko *et al*., 2006). For the purposes of this thesis, in order to allow for a better understanding of the relationship between ferritin, iron, ROS and oxidative stress; only the association between iron and ROS will be reviewed. Intracellularly, an increase in iron levels may lead to an increase in ROS production via Haber-Weiss chemistry (Biemond *et al*., 1988; Chiancone *et al*., 2004; Harrison and Arosio, 1996; McCord, 1998; Papanikolaou and Pantopoulos, 2005). In the Haber-Weiss reaction, the reaction between $O_2^{\cdot -}$ and H_2O_2, described as a 'lethal mixture' (Harrison and Arosio, 1996), results in the formation of the very reactive $OH^{\cdot}$ species (Halliwell, 1987; Thomas *et al*., 1985).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad \textbf{(Fenton Reaction)} \quad \textbf{(2.1)}$$

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \quad \textbf{(2.2)}$$

$$H_2O_2 + O_2^{\cdot -} \rightarrow OH^- + OH^{\cdot} + O_2 \quad \textbf{(Haber-Weiss Reaction)} \quad \textbf{(2.3)}$$

Once produced, in order to achieve a more stable electron status, ROS readily combine with the unpaired electrons of other molecules (Kehrer, 1993), such as biological molecules (i.e. DNA, lipids and proteins) (reviewed Kohen and Nyska, 2002) leading to tissue damage (Kehrer, 1993). The damage of biological molecules by ROS results in a condition referred to as oxidative stress (reviewed by Valko *et al*., 2007). Oxidative stress is defined as a disturbance in the balance between prooxidants and antioxidants, in which there is the increased presence of prooxidants, in contrast to antioxidants (reviewed by Kehrer, 1993).

Cells have developed a tremendous antioxidant defense system against ROS damage, which include Vitamin C, E, superoxide dismutase, catalase, GSH conjugation system and several others that aid in the detoxification of ROS (reviewed by Kehrer, 1993; Kohen and Nyska, 2002). Recently, the increase in transcription of cytoprotective genes (ferritin) in response to oxidative stress has also been described as an antioxidant response (Tsuji *et al*., 2000). Previously reviewed, the up-regulation of ferritin mRNA

and subsequent protein expression limits the availability of free iron to the Fenton reaction, thus protecting the cells and tissues from the damaging effects of further generation of noxious ROS production and associated oxidative stress. Thus, in this present study, increases in ferritin mRNA or protein expression in HepG2 and HEK293 cells may be an antioxidant response to 2,4-D induced ROS production and associated oxidative stress.

3. METHODS

3.1 Experimental design

In vitro studies were conducted using human cell cultures exposed once to increasing concentrations of a technical grade or commercial formulation of 2,4-D. All exposure experiments consisted of untreated control and treated 2,4-D cell cultures. Cell viability was determined following 6, 24, 48 and 72 hrs of incubation with 0 to 10 mM of technical or commercial 2,4-D. An oxidant (H_2O_2) (Halliwell, 1987 and Zhou *et al*., 2004) was used as a positive control in ROS studies. The measurement of ROS production was performed following 1.3 hrs of incubation with 0 to 400 µM of H_2O_2, or 2 to 6 hrs of incubation with 0 to 1 mM of commercial 2,4-D. Following 24 hrs of exposure to 0 to 1 mM of commercial 2,4-D, RNA and protein were extracted for RT-PCR and western blot assays, respectively.

3.2 Culturing, subculturing and counting of cells

Two human cell lines (American Type Culture Collection (ATCC)) were used in this study; human hepatocellular carcinoma (HepG2) and human embryonic kidney (HEK293) cells. The HepG2 cells are epithelial carcinoma cells derived from the liver (ATCC, 2004). The HEK293 are epithelial cells derived from the kidney and transformed with human adenovirus 5 DNA (ATCC, 2004; Thomas and Smart, 2005).

Cell cultures were maintained according to ATCC recommendations. Frozen stocks of HepG2 and HEK293 cells were stored at a density of 1 x 10^7 cells/mL in a solution of fetal bovine serum (FBS) (Sigma-Aldrich Canada Ltd) (90% (v/v)) and dimethylsulfoxide (DMSO) (EM Science) (10% (v/v)). One mL cell suspensions were stored in 2 mL cryovials (VWR Scientific) in a cryo biological storage tank (Thermolyne) containing liquid nitrogen. Frozen cells were thawed as needed for conducting experimental procedures. Cells were thawed by placing the sealed cryovial in a styrofoam container containing warm water for approximately 1 min. Thawed cells

were placed in a sterile 50 mL centrifuge tube (VWR Scientific) with approximately 10 mL of complete growth media (Dulbecco's minimum essential medium (DMEM) containing 2 mM L-Glutamine, 4 mM nonessential amino acids and 126 mM Earle's salts (Invitrogen Corporation) supplemented with 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 10,000 U/mL penicillin – 10,000 µg/mL streptomycin (Invitrogen Corporation) and 10% (v/v) FBS. Cell suspensions were then centrifuged at 1000 x *g* for 10 min at room temperature using a Fisher Scientific IEC PR-6000 centrifuge to ensure removal of all DMSO from cells. Following centrifugation, media was removed by gentle aspiration using a pipette and discarded. Cells were then re-suspended by gentle pipetting in 15mL of complete growth media and placed in 75 cm^2 Falcon culture flasks (VWR Scientific). Total volume in 75 cm^2 Falcon culture flasks was 15 mL. Unless otherwise stated, cell cultures were always maintained in a sterile air environment, at 37ºC, 95% O_2, 5% CO_2. Media on HepG2 and HEK293 cell cultures was renewed 2-3 times per week. In order to maintain similar culturing conditions for HepG2 and HEK293 cells, in contrast to ATCC recommendations to culture HEK293 cells in media supplemented with 10% (v/v) Heat-inactivated horse serum, in this present study HEK293 cells were grown in media supplemented with 10% (v/v) FBS.

Cells were subcultured when they reached approximately 80-85% cell confluency (attained approximately 3-4 days for HepG2 and HEK293), as determined by microscopic examination using an Olympus compound microscope at 4X magnification, cell cultures were subcultivated. Cells were subcultured in 75 cm^2 Falcon culture flasks. Media was removed by gentle aspiration and discarded. 5 mL of 0.25% (w/v) trypsin-EDTA solution (Invitrogen Corporation) was added to the flasks and cell cultures were incubated for 5-10 min, followed by the addition of 10 mL of complete growth media to the flask. Cell suspensions were placed in 50 mL centrifuge tubes and centrifuged at 1000 x *g* for 10 min at room temperature to ensure removal of all trypsin-EDTA solution from cells. After centrifugation, supernatants were discarded and the cells were re-suspended in complete growth media by gentle pipetting. In order to ensure that cells were passaged into the culture flasks at equal cell density; cells were counted (described below), before being plated in the culture flasks.

Cells were dispersed from culture flasks using trypsin-EDTA as described above. Following the removal of trypsin-EDTA solution, cells were re-suspended in complete growth media by gentle pipeting. For cell counting, 1:1 mixtures of cell suspension (40 µL) and 0.4% (w/v) trypan blue stain (40 µL) (Invitrogen Corporation) were made. Cell mixtures were pipetted to ensure complete suspension of cells. 20 µL aliquots of cell mixtures were added to a Bright Line® hemacytometer. Cells were counted using an Ernest Leitz Wetzlar compound microscope at 10X magnification. The Bright Line® hemacytometer consists of a total of 9 grids. Cell numbers were calculated using an average of the cell numbers counted in 5 out of 9 grids on the hemacytometer. HepG2 and HEK293 cells were subcultured at a ratio of 1:2 and 1:4, respectively. HepG2 and HEK293 cells were used at passage numbers 15 and 6, respectively for cell viability, ROS (positive control only), RNA and protein studies. HepG2 and HEK293 cells were used at passages 19 and 9, respectively for ROS assay following treatment with 2,4-D.

3.3 Preparation of chemical solutions for cell treatments

3.3.1 2,4-D

Two forms of 2,4-D were used in this study; (1) a technical grade of 2,4-D; 2,4-D dimethylamine (2,4-D DMA) concentrate; purity 67.1% (a gift from Nufarm Agriculture Inc.) and (2) a commercial formulation of 2,4-D; C.I.L. Dandelion Killer (containing 2,4-D DMA)(Nu-Gro Corporation). The 2,4-D treatment concentrations used in the study were; **0.1 nM** (2,4-D DMA: 0.026 µg/L; 2,4-D acid: 0.022 µg/L), **1 nM** (2,4-D DMA: 0.26 µg/L; 2,4-D acid: 0.22 µg/L), **10 nM** (2,4-D DMA: 2.60 µg/L; 2,4-D acid: 2.20 µg/L), **100 nM** (2,4-D DMA: 26 µg/L; 2,4-D acid: 22 µg/L), **1 µM** (2,4-D DMA: 260 µg/L; 2,4-D acid: 220 µg/L), **10 µM** (2,4-D DMA: 2.6×10^3 µg/L; 2,4-D acid: 2.2×10^3 µg/L), **100 µM** (2,4-D DMA: 2.6×10^4 µg/L; 2,4-D acid: 2.2×10^4 µg/L) and **1 mM** (2,4-D DMA: 2.6×10^5 µg/L; 2,4-D acid: 2.2×10^5 µg/L). Additional 2,4-D treatment concentrations of **5 mM** (2,4-D DMA: 1.3×10^6 µg/L; 2,4-D acid: 1.1×10^6 µg/L) and **10 mM** (2,4-D DMA: 2.6×10^6 µg/L; 2,4-D acid: 2.2×10^6 µg/L) were used only in cell viability studies. Using initial stock (1M) solutions of either technical or commercial 2,4-D, serial dilutions were performed to obtain solutions of 2,4-D that contained 10X desired 2,4-D treatment concentrations. The 2,4-D solutions were

prepared in complete growth media for cell viability, mRNA and protein studies. The 2,4-D solutions were prepared in sterile water for the ROS assay. The solutions of 2,4-D were prepared less than 24 hrs before use, sterile filtered using Acrodisc® 0.2 µM syringe filters (Pall German Laboratory) and stored at 4ºC, protected from light.

3.3.2 H_2O_2

The H_2O_2 treatment concentrations used in this study were **100 µM**, **200 µM**, **300 µM** and **400 µM**. Stock solutions of H_2O_2 were made in sterile water to contain 10X desired H_2O_2 treatment concentrations. The H_2O_2 treatments were prepared less than 30 min before use and stored at 4ºC, protected from light.

3.4 Plating and treatment of cells prior to performing assays

3.4.1 Plating and treatment of cells prior to performing cell viability assay

The HepG2 and HEK293 cells were passaged and counted as previously described (see section 3.2) and plated into NUNC™ black 96-multiwell plates (VWR Scientific). The HepG2 cells were plated at a density of 30,000 cells/well. In contrast, since HEK293 cells were larger, these cells were plated at a density of either 30,000 or 15,000cells/well for 6 and 24 hrs or 48 and 72 hrs cell viability studies, respectively. Cells were incubated. Cell cultures were treated with 2,4-D following 48 hrs after the initial plating to allow cultures to attain at least 55-60% cell confluency. Media was removed from cells and 200 µL (untreated control cell cultures) or 180 µL (treated cell cultures) aliquots of fresh complete growth media was added, followed by the addition of 20 µL media containing either technical or commercial 2,4-D. Total well volume was 200 µL for untreated control and treated cell cultures. Gentle pipetting using a multichannel pipette was used to ensure the thorough mixing of 2,4-D treatments with complete growth media, without displacing cells.

3.4.2 Plating and treatment of cells prior to performing ROS assay

3.4.2.1 Plating and treatment of cells with H_2O_2 prior to performing ROS assay

Cells were passaged and counted as described above (see section 3.2) and plated into NUNC™ black 96-multiwell plates at a density of 30,000 cells/well; a density similar to that previously used by Wu *et al* (1997). Cells were incubated. At approximately, 55-60% cell confluency, media was removed and discarded from cells using a multichannel pipette and cells were washed twice with 200 µL aliquots of 1X Dulbecco's phosphate buffered saline (DPBS) (Ca^{2+}, Mg^{2+} and phenol red-free) (Invitrogen Corporation). Two hundred µL (untreated control cell cultures) or 180 µL (treated cultures) aliquots of D-PBS was added to cells, followed by the addition of 20 µL of H_2O_2 stock treatments. Total well volume was 200 µL for untreated control and treated cell cultures. Gentle pipetting using a multichannel pipette was used to ensure the thorough mixing of H_2O_2 treatments with D-PBS, without displacing cells. Cells were incubated.

3.4.2.2 Plating and treatment of cells with 2,4-D prior to performing ROS assay

HepG2 and HEK293 cell cultures were passaged, counted and maintained as described above (see section 3.2) prior to 2,4-D treatments. Two hundred µL (untreated control cell cultures) or 180 µL (treated cultures) aliquots of D-PBS was added to cells in each well, followed by the addition of 20 µL of commercial 2,4-D stock treatments. Total well volume was 200 µL for untreated control and treated cell cultures. Gentle pipetting using a multichannel pipette was used to ensure the thorough mixing of 2,4-D treatments with D-PBS, without displacing cells. Cells were incubated.

3.4.2.3 Plating of cells and treatment with 2,4-D prior to RNA and protein extraction for RT-PCR and western blot assays

HepG2 and HEK293 cells were passaged and counted as described above (see section 3.2) and plated into 9 individual NUNC™ 100 x 20 mm culture dishes (VWR Scientific) at a density of 1 x 10^7 cells/culture dish for either RNA or protein studies. At

approximately 80-85% cell confluency (attained approximately 4-5 days for HepG2 and 3 days for HEK293 cells), media from cells were removed and discarded and cells were replenished with 10 mL (untreated control cell cultures) or 9 mL (all cell cultures, excluding untreated control cell cultures) of fresh complete growth media, followed by 1 mL of 2,4-D treatment stock solutions. Final volume in cell culture dishes was 10 mL for untreated control and treated cell cultures. Culture dishes were then gently swirled clockwise for 30 sec to ensure thorough mixture of 2,4-D treatments with complete growth media, without displacing cell cultures. Plated cells were incubated for 24 hrs prior to RNA and protein extraction.

3.5 Assays

3.5.1 Cell viability

Cell viability was determined fluorometrically using an *in vitro* toxicology assay kit, containing the dye, Resazurin (Sigma-Aldrich Canada Ltd). The basis of the assay is the reduction of Resazurin. Cell viability is determined as a function of metabolic activity. Viable cells have the ability to reduce the oxidized (blue) form of Resazurin to a fluorescent (red) intermediate, therefore indicating the magnitude of the toxic effect of test compounds (Sigma-Aldrich Canada Ltd.)

Cell viability assays were performed as outlined by the manufacturer's protocol (Sigma-Aldrich Canada Ltd protocol references: Dutka *et al*., 1983; King, 1984; Liu, 1981; Strotmann *et al*., 1993) with minor modifications. Cell cultures were incubated with 20 µL of the Resazurin dye (final concentration 10% (v/v)) in the last 2.5 hrs prior to the end of the respective incubation period (6, 24, 48, and 72 hrs). Total well volume was 220 µL for untreated control and treated cell cultures. Following incubation, fluorescence was measured using a Dynex Technologies Fluorolite 1000 fluorometer reader, at excitation and emission wavelengths of 546 nm and 590 nm, respectively. Cell viability assays were performed in replicates of 6 for untreated control and treated cell cultures.

3.5.2 ROS

The production of ROS was determined according to Seth et al. (2004), Wan et al. (2003) and Wu et al. (1997) with minor modifications. The production of ROS in cells was determined fluorometrically using 2',7'-DCFH-DA (Sigma-Aldrich Canada Ltd). The compound, 2',7'-DCFH-DA is a non-ionic, non-polar molecule that is permeable to the cell membrane (Bass *et al*., 1983). Subsequent to its entry into the cell, the molecule is enzymatically hydrolyzed by intracellular esterases to the non-permeable molecule, 2',7'-dichlorofluorescin (DCFH), where in the presence ROS, DCFH is rapidly oxidized to the highly fluorescent molecule, 2',7'-dichlorofluorescein (DCF) (Bass *et al*., 1983). The fluorescent signal is directly proportional to ROS production (Osseni *et al*., 1999, 2000).

3.5.2.1 Determination of ROS production in cells exposed to H_2O_2

In order to avoid photo-oxidation of 2'7'-DCFH-DA, all procedures of this assay were performed in the dark. All 2'7'-DCFH-DA solutions were prepared immediately before use and any unused portions were discarded. Initially, a 5 mM stock solution of 2',7'-DCFH-DA was prepared in 95% ethanol (Wu *et al*., 1997) and stored at 4ºC (Seth *et al*., 2004), protected from light, until needed. The 5 mM 2',7'-DCFH-DA stock solution was diluted to 1 mM in D-PBS, immediately before use. At the end of the H_2O_2 incubation period (20 min), cell cultures were incubated with 50 µL of 1 mM 2',7'-DCFH-DA (final concentration 2',7'-DCFH-DA 200 µM; final concentration ethanol 3.8% (v/v)) (Wu *et al*., 1997) for an additional 1 hr (Seth *et al*., 2004). Total well volume was 250 µL for untreated control and treated cell cultures. Fluorescence was subsequently measured using a Fluorolite 1000 fluorometer reader (Dynex Technologies), at excitation and emission wavelengths of 485 nm and 535 nm, respectively. ROS assays were performed in replicates of 6 for untreated control and treated cell cultures.

3.5.2.2 Determination of ROS production in cells exposed to 2,4-D

Following 1 hr incubation with 2,4-D, HepG2 and HEK293 cells were incubated with 50 µL of 1mM 2',7'-DCFH-DA as described above and fluorescence measured

(see section 3.4.2a). Fluorescence was measured every hour for a total of 5 hrs. Cell cultures were incubated in the intervals between fluorescent measurements. ROS assays were performed in replicates of 8 for untreated control and treated cell cultures.

3.5.3 mRNA analysis

3.5.3.1 RNA extraction and purification

Following 2,4-D incubation, media was removed and discarded, and cells were gently washed twice with sterile 1X phosphate buffered saline (PBS) (pH 7.4) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) (Sambrook and Russell, 2001). Trizol reagent (Invitrogen Corporation) was used to extract RNA from HepG2 and HEK293 cells. RNA extraction was performed as outlined by the manufacturer's protocol (Invitrogen Corporation protocol reference: Chomczymski and Sacchi, 1987) with minor modifications. To avoid RNA degradation by Ribonuclease (RNase) enzymes (Ambion, 2007), all reagents that were used in RNA extraction process were prepared using RNase-free water supplemented with autoclaved 0.1% (v/v) diethyl pyrocarbonate (DEPC) (Sigma-Aldrich Canada Ltd), an RNase enzyme inhibitor (Leonard *et al*., 1970; Solymosy *et al*., 1968). Cells were directly lysed by addition of 1 mL Trizol reagent to each culture dish. Samples were incubated for 5 min at room temperature. Samples from each plate were transferred to individual sterile 15 mL centrifuge tubes, mixed with 200 µL of chloroform, shaken vigorously 15 sec and incubated for 3 min at room temperature. Samples were then centrifuged using a Beckman Coulter™ centrifuge at 11,500 x *g* for 15 min at 2 to 8ºC. Following centrifugation, the aqueous phases containing RNA were transferred to new sterile 15 mL centrifuge tubes, mixed with 500 µL of isopropyl alcohol and incubated for 10 min at room temperature to allow RNA precipitation. Samples were centrifuged at 11,500 x *g* for 10 min at 4ºC. The RNA pellets were washed with 1 mL of 75% ethanol (v/v) (prepared in RNase-free water) by gently mixing, followed by centrifugation at 7,500 x *g* for 5 min at 2 to 8ºC. The ethanol was removed and the RNA pellets were allowed to air dry for 10 min. The RNA pellets were dissolved in 3 mL of a solution containing tris-ethylene diamine tetraacetic Acid (TE) (pH 8.0) (10 mM Tris-HCL), 1 mM EDTA (Sambrook and Russell, 2001)) 300 µL 3 M NaAc (pH 5.2) and 7.5 mL 100% ethanol),

equally aliquotted into sterile 1.5 mL microcentrifuge tubes and stored at -80ºC. The RNA was quantified by measuring the absorbance at 260 nm using a Bio-Rad SmartSpec™ Plus Spectrophotometer.

Contaminating genomic DNA was removed from quantified RNA samples to eliminate false positive results in RT-PCR (Ambion, Inc., 2006) by DNase I enzymatic digestion using DNase I (Fermentas Canada Inc.) as outlined in the manufacturer's protocol (Fermentas Canada Inc. protocol references: Anderson, 1981; Kienzl *et al*., 1996; Kunitz, 1950; Sambrook and Russell, 2001; Wiame *et al*., 2000). Five µg pellets of RNA were obtained by centrifugation of stored RNA precipitates in sterile, RNase-free 1.5 mL microcentrifuge tubes using a Fisher Scientific accuSpin™ Micro R at 16,060 x *g* for 15 min at 4ºC were air dried for 10 min and dissolved in 45 µL of RNase-free water. Samples were mixed with 5 µL of 10X reaction buffer (100 mM Tris-HCL (pH 7.5 at 25ºC), 25 mM $MgCl_2$, 1 mM $CaCl_2$) (Fermentas Canada Inc.) and 5 µL of DNase I (1 U/µL) (Fermentas Canada Inc.) and incubated for 30 min at 37ºC. Total reaction volume was 55 µL. Reactions were mixed with 5 µL of 25 mM EDTA and incubated for 10 min at 65ºC.

Samples were subsequently purified using an RNeasy® Mini Kit (Qiagen Inc.) according to the manufacturer's protocol. The kit was supplied with RNeasy® lysis RLT (containing guanidine thiocyanate) and wash RPE (supplied as a concentrate) buffer, mini spin columns (containing silica-gel membrane) and 2 mL collection tubes. The RNA sample volumes were adjusted to 100 µL with RNase-free water. 350 µL of buffer RLT was added to the samples and mixed thoroughly by pipetting. Two hundred and fifty µL of 100% ethanol was added to the samples and mixed thoroughly by pipetting. Samples (total volume 700 µL) were applied to mini spin columns, placed in 2 mL collection tubes and centrifuged for 16,060 x *g* for 1 min at 4ºC. Mini spin columns were transferred to new 2 mL collection tubes and spun twice with 500 µL of buffer RPE at 16,060 x *g* for 15 sec. Fifty µL of RNase-free water was then added to columns, and purified RNA was eluted by centrifugation at 16,060 x *g* for 1 min. Purified RNA samples were used for cDNA synthesis.

3.5.3.2 cDNA synthesis

First strand cDNA synthesis was performed using RevertAid™ M-MuLV Reverse Transcriptase (M-MuLV RT, Fermentas Canada Inc.) as outlined in the manufacturer's protocol (Fermentas Canada Inc. protocol references: Gerard and D'Alessio, 1993; Sambrook and Russell, 2001; Verma, 1981). A negative (–) Reverse Transcriptase control (-RT control, consisting of all cDNA synthesis reagents with the exception of M-MuLV RT) was included in the cDNA synthesis procedure. First strand cDNA synthesis was performed according to the following stepwise reactions; **(1)** 0.5 µL (0.5 µg) of oligonucloetide $(dT)_{18}$ (GE Healthcare) was added to 5 µg of purified RNA samples (dissolved in 50 µL of RNase-free water) and reaction mixtures (total reaction volume of each sample 55.5 µL) were incubated at 70ºC for 5 min and placed on ice for 5 min, **(2)** 20 µL of 5X reaction buffer (250 mM Tris-HCL(pH 8.3 at 25ºC), 250 mM KCL, 20 mM $MgCl_2$, 50 mM DL-Dithiothreitol (DTT)) (Fermentas Canada Inc.), 20 µL of 5 mM 4 deoxyribonucleoside triphosphate (dNTP) mix (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP) (Fermentas Canada Inc.), 0.5 µL (20 U) of RiboLock™ ribonuclease inhibitor (Fermentas Canada Inc.), 9 µL (–RT control) or 8 µL (all samples, excluding –RT control) of RNase-free water was added to the reaction mixtures (total reaction volumes were 100 µL (-RT control) or 99 µL (all samples, excluding –RT control). Reaction mixtures were incubated at 37ºC for 5 min, **(3)** 0 µL (–RT control) or 1 µL (all samples, excluding –RT control) of M-MuLV RT (200 U/µL) was added and the reaction mixtures (total reaction volume of all samples, including –RT control was 100 µL) were incubated at 42ºC for 60 min and **(4)** cDNA synthesis reactions were stopped by incubation of the mixtures at 70ºC for 10 min and chilled on ice for 10 min.

To remove any contaminating RNA from Single-Stranded (SS) cDNA samples, samples were treated with an RNase A/T1 enzyme mix (Fermentas Canada Inc.) as outlined by the manufacturer's protocol (Fermentas Canada Inc. protocol reference: Ausbel, 1994). Two µL of RNase A/T1 enzyme mix (2 mg/mL RNase A, 5000 U/mL RNase T1) was added to cDNA samples and incubated at 37ºC for 30 min (total reaction volume was 102 µL).

cDNA samples were subsequently purified by phenol/chloroform extraction (Sambrook and Russell, 2001) with minor modifications. One hundred µL of phenol (pH 8.0) /chloroform (1:1) was added to cDNA samples (total reaction volume of each samples was 202 µL) and mixed thoroughly by vortexing. cDNA samples were centrifuged at 16,060 x *g* for 5 min at 4ºC. Aqueous phases were transferred to sterile, 1.5 mL centrifuge tubes, and samples were extracted again with phenol/chloroform (1:1) as described above. Samples were next extracted with 100 µL of chloroform/isoamyl alcohol mixture (19:1) and centrifuged as above. Aqueous phases were transferred to sterile, 1.5 mL centrifuge tubes and mixed with one-third volume of 7.5 M NH_4Ac and 2.5 volumes of 100% ethanol (total volume of each sample was 385 µL). Samples were then allowed to precipitate at -20ºC for 1 hr, and then centrifuged at 16,060 x *g* for 15 min at 4ºC. The cDNA pellets were air dried at room temperature for 10 min, dissolved in 100 µL of sterile water, and stored at -20ºC. The cDNA was quantified by measuring the absorbance at 260nm using a Bio-Rad SmartSpec™ Plus Spectrophotometer.

3.5.3.3 Design of primers for use in RT-PCR

Primers used for RT-PCR were designed empirically according to Bio-Rad Laboratories Real-Time PCR application guide in order to optimize RT-PCR conditions (Bio-Rad Laboratories, 2005). Primers were designed to; (1) avoid GC (Guanidine and Cytosine) repeats in excess of 3 consecutive base pairs (bp), (2) contain an overall GC content of 50-60%, (3) have a melting temperature (T_m) between 50ºC-65ºC and (4) contain terminal G or C nucleotides. Additionally, primers were designed to result in amplification of cDNA sequences (amplicons) between 75-200bp.

Designed primers were synthesized by Invitrogen Corporation. One mM stock solutions of all primers were made in TE (pH 8.0) (10 mM Tris-HCL, 1 mM EDTA) (Sambrook and Russell, 2001) and stored at -20ºC. Ten µM forward or reverse primer working solutions were made immediately before use by dilution in sterile water.

Ferritin light polypeptide (FTL):

Ferritin light polypeptide forward primer (FTL-F) and ferritin light polypeptide reverse primer (FTL-R) were designed using a human ferritin light polypeptide cDNA sequence (National Center for Biotechnology Information (NCBI) accession number: BC018990).

FTL-F primer: $^{5'}$cga tga tgt ggc tct gga ag$^{3'}$

FTL-R primer: $^{5'}$gga cat caa gaa gcc agc tg$^{3'}$

% GC in FTL-F: 55%

% GC in FTL-R: 55%

T_m of FTL-F: 62ºC

T_m of FTL-R: 62ºC

Amplicon length: 140 bp

Ferritin heavy polypeptide 1 (FTH1):

Ferritin heavy polypeptide forward primer (FTH1-F) and ferritin heavy polypeptide 1 reverse primer (FTH1-R) were designed using a human ferritin heavy polypeptide 1 cDNA sequence (NCBI accession number: BC000857).

FTH1-F primer: $^{5'}$cta cgc ctc cta cgt ttc cc$^{3'}$

FTH1-R primer: $^{5'}$ggt tct gca gct tca tca g$^{3'}$

% GC in FTH1-F: 55%

% GC in FTH1-R: 53%

T_m of FTH1-F: 62ºC

T_m of FTH1-R: 58ºC

Amplicon length: 140 bp

Proliferating cell nuclear antigen (PCNA):

Proliferating cell nuclear antigen forward primer (PCNA-F) and proliferating cell nuclear antigen reverse primer (PCNA-R) were designed using a human proliferating cell nuclear antigen cDNA sequence (NCBI accession number: BC062439).

PCNA-F primer: $^{5'}$gcc tgc tgg gat att agc tc$^{3'}$

PCNA-R primer: $^{5'}$ca tac tgg tga ggt tca cgc$^{3'}$

% GC in PCNA-F: 55%

% GC in PCNA-R: 53%

T_m of PCNA-F: 62ºC

T_m of PCNA-R: 62ºC

Amplicon length: 150 bp

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):

GAPDH gene (reference gene) was used as the internal control in RT-PCR assays. Glyceraldehyde-3-phosphate dehydrogenase forward primer (GAPDH-F) and glyceraldehyde-3-phosphate dehydrogenase reverse primer (GAPDH-R) were designed using a human glyceraldehyde-3-phosphate dehydrogenase cDNA sequence (NCBI accession number:NM_002046).

GAPDH-F primer: $^{5'}$cca ggg ctg ctt tta act ctg$^{3'}$

GAPDH-R primer: $^{5'}$cg ttc tca gcc ttg acg gtg$^{3'}$

% GC in GAPDH-F: 52%

% GC in GAPDH-R: 60%

T_m of GAPDH-F: 64ºC

T_m of GAPDH-R: 64ºC

Amplicon length: 138 bp

3.5.3.4 RT-PCR assays

The RT-PCR assays were performed using a 2X iQ™ SYBR® Green Supermix solution (Bio-Rad Laboratories) according to the manufacturer's protocol. The RT-PCR assays were performed in white 48-multiwell PCR plates (Bio-Rad Laboratories). Assays for the target genes (FTL, FTH1, PCNA) and reference gene (GAPDH) included reaction mixtures for **(1)** –RT control, **(2)** untreated control samples, **(3)** 2,4-D treated samples, **(4)** cDNA blank (containing all RT-PCR components, excluding primers), **(5)** SYBR Green I blank (containing all RT-PCR components, excluding cDNA template and primers) and **(6)** primer blank (containing all RT-PCR components, excluding cDNA template).

Master mixtures for RT-PCR assays (containing all reaction components, excluding cDNA templates) of target genes were obtained by the addition 775 µL of iQ®

SYBR Green Supermix (final concentrations of all components indicated below), 542.5 µL of sterile water, 38.75 µL (final concentration indicated below) of forward primer and 38.75 µL (final concentration indicated below) of reverse primer followed by the thorough vortexing of mixtures. Forty five µL of the master mixture was added to the respective wells (each well contained 25 µL (1X) iQ™ SYBR® Green Supermix (50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.2 mM dNTP, iTaq DNA polymerase (1.25 U), 3 mM $MgCl_2$, SYBR Green I, 10 nM fluoroscein, and stabilizers, 125 nM forward primer, 125 nM reverse primer, 17.5 µL of sterile water). Five µL (25 ng) of cDNA template was added to the wells (final reaction volume in each well was 50 µL) and mixed using a multichannel pipette. Master mixtures for RT-PCR assays of the reference gene were prepared as described above for the target genes.

Reaction mixtures for cDNA blank, SYBR Green I blank, and primer blank were prepared individually. The cDNA blank (total reaction volume 50 µL) consisted of 25 µL (1X) of iQ® SYBR Green Supermix, 5 µL (25 ng) of control cDNA template and 20 µL of sterile water. The SYBR Green I blank (total reaction volume 50 µL) consisted of 25 µL (1X) of iQ® SYBR Green Supermix and 25 µL of sterile water. Primer blank (total reaction volume 50 µL) consisted of 25 µL (1X) of iQ® SYBR Green Supermix, 1.25 µL (125 nM) of forward primer, 1.25 µL (125 nM) of reverse primer and 22.5 µL of sterile water.

The RT-PCR assays were performed using a Bio-Rad MiniOpticon™ System. The PCR cycle protocol used in each reaction was: hot start at 95ºC for 3 min, denaturation at 95ºC for 45 sec, annealing at 56ºC for 45 sec, and extension at 72ºC for 2 min, plate read (reading of fluorescence), for 30 cycles, followed by production of a melting curve between 55-90ºC, where fluorescence was read at every 0.2ºC temperature increments held for 2 sec.

Target mRNA expression levels in each sample was calculated relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH, using the Pfaffl method (Pfaffl, (2001)). The Pfaffl method is a formula that is used to calculate relative gene expression when the efficiencies of reactions being compared are not the same (Bio-Rad Laboratories).

The Pfaffl method:

Ratio = $[(E_{target})^{\Delta C_T, \text{ target (calibrator-test)}}] / [(E_{ref})^{\Delta C_T, \text{ ref (calibrator-test)}}]$

E_{target}: Efficiency of the reaction in amplification of the target cDNA sequence

E_{ref}: Efficiency of the reaction in amplification of the internal control cDNA sequence

C_T: the cycle at which a sample's fluorescence trace crosses the threshold line

ΔC_T: difference between 2 C_T values

Calibrator: control sample

Test: test sample

RT-PCR assays were performed in replicates of 3 for one set of exposure experiments for untreated control and treated cell cultures.

3.5.4 Protein analysis

3.5.4.1 Protein extraction

Following 2,4-D incubation, cells were harvested as outlined by Sambrook and Russell. (2001) with minor modifications. Media was removed and discarded and cells were gently washed twice with sterile PBS solution. Cell culture dishes were placed on ice, and cells were directly lyzed by addition of 1mL of lysis buffer (10 mM Hepes (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 0.1 mM EDTA, 2 mM DTT, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)). Cells from each culture dish were collected using rubber policemen and cell homogenates were transferred to individual sterile 1.5 mL microcentrifuge tubes. Samples were sonicated on ice for approximately 30 sec at a power level of 7 (amplitude: 247 microns) using a Sonifier® Cell Disruptor. Following sonication, samples were then centrifuged at 16,060 x *g* for 15 min at 4ºC and supernatants were transferred to sterile 1.5 mL microcentrifuge tubes and stored at -80ºC. Protein was quantified using the Bradford assay (Bradford, 1976). Absorbance was measured at 595nm using a Bio-Rad SmartSpec™ Plus Spectrophotometer.

3.5.4.2 Western blot analysis

Denaturing SDS-PAGE and subsequent protein transfer to nitrocellulose membrane was performed using Bio-Rad's Mini-Protean 3 cells and Trans-Blot® SD

Semi-Dry Transfer cell apparatus according to Sambrook and Russell, 2001 with minor modifications. Protein aliquots (10-30 μg as indicated in figure legends) were mixed with one-fifth volume of 5X Laemmli buffer (0.0625 M Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) Bromophenol Blue) (Laemmli, 1970) and denatured at 95 to 100ºC for 10 min, then chilled on ice for 1 min. Samples were centrifuged at 16,060 x *g* for 1 min at 4ºC and chilled on ice, then loaded onto SDS-polyacrylamide gels (15% (w/v) acrylamide for ferritin and 12% (w/v) acrylamide for PCNA detection). Detection of GAPDH and Hsp72 were performed after ferritin or PCNA antibodies were stripped off transferred blots as described below. 5 uL of PageRuler™ prestained protein ladder (Fermentas Canada Inc.) was included in the outer left lane of all gels. Gels for ferritin or PCNA were initially electrophoresed at 90V for 30 min, in order to allow proteins to stack, followed by electrophoresis at 120V for 3 hrs and 2 hrs, in order to allow for ferritin and PCNA protein resolution, respectively.

In preparation for the transfer of proteins to 0.45 μM nitrocellulose membranes (Bio-Rad Laboratories), pieces of blotting paper (Ahlstrom) and nitrocellulose membrane (cut to gel area, approximately 55 cm^2) were soaked for 30 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) (Towbin *et al*., 1979). Following the electrophoresis of proteins, the gels were assembled for transfer as follows; gels were stacked on 2 layers of blotting paper, 1 layer of 0.45 μM pure nitrocellulose membrane and covered with 2 additional layers of blotting paper. A glass rod was gently, but firmly rolled over the gel stacks to ensure complete removal of trapped air bubbles. Proteins were transferred using a Bio-Rad Trans-Blot® SD Semi-Dry Transfer Cell at 20V for 1 hr.

Following transfer to nitrocellulose membranes, membranes were blocked with 10-15 mL of blocking solution. The blocking solution was prepared according to Towbin et al. (1979) with minor modifications; 5% (w/v) Nestle Carnation skim milk in TBST (20 mM Tris-HCL (pH 7.5), 500 mM NaCl, 0.1% (v/v) Tween 20) at 4ºC overnight. All primary and secondary antibody incubations were performed in 5% milk as described above.

For detection of FTL and FTH1, membranes were incubated with primary anti-ferritin antibody (rabbit polyclonal to Ferritin, catalog number: ab7332; Novus Biologicals) (1:10,000) at 4ºC overnight. For the detection of PCNA, membranes were incubated with primary anti-PCNA antibody (mouse monoclonal to PCNA, catalog number: SC-56, Santa Cruz Biotechnologies, Inc.) (1:1,000) at 4ºC overnight. For the detection of Hsp72, the membranes were incubated with a primary anti-Hsp70 (Hsp72) antibody (rabbit polyclonal to Hsp70 (Hsp72), catalog number: SPA-812, Stessgen Bioreagents) (1:10,000) at 4ºC overnight. For the detection of GAPDH, membranes were incubated with primary anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.) (1:5,000) at 4ºC overnight. Primary antibodies were removed and membranes were washed 3 times (1 x 5min; 1 x 15min; 1 x 5min) in 5% milk and incubated with the respective secondary antibodies.

Ferritin and Hsp72 were detected using goat anti-rabbit-secondary antibody-Horseradish Peroxidase Conjugate (catalog number: 170-6516, Bio-Rad Laboratories Ltd.) (1:4,000) and (1:5000) at room temperature for 1.5 hrs, respectively. PCNA and GAPDH were detected using donkey anti-mouse-secondary antibody-Horse Radish Peroxidase (catalog number: SA1-100, Affinity Bioreagents) (1:5000) at room temperature for 1.5 hrs. Secondary antibodies were removed and membranes were washed 3 times (1 x 5 min; 1 x 15 min; 1 x 5 min) in 5% skim milk, followed by 2 washes (2 x 5 min) in TBST and 1 wash (1 x 5 min) in TBS (20 mM Tris-HCL (pH 7.5), 500 mM NaCl).

The washed membranes were incubated with Western Lightning™ Chemiluminescence Reagent *PLUS* (Perkin Elmer LAS, Inc.) according to the manufacturer's protocol. Reagent was prepared immediately prior to chemiluminescence in sterile 15 mL centrifuge tubes by mixing 5 mL of enhanced luminol and 5 mL oxidizing reagent followed by gentle shaking. The mixture was applied onto membranes, incubated in the mixture for 1 min, then blotting paper was used to remove excess chemiluminescence reagent from membranes. All blots were exposed to KODAK BioMax MS Film and developed using a Summit Quality Control Processor X-ray film developer.

For re-blotting, (performed before detection of GAPDH and Hsp72) membranes were washed at room temperature for 5 min in TBS and stripped using *Re-Blot Plus* strong antibody stripping solution (supplied as a 10X stripping solution, Chemicon International Inc.) according to the manufacturer's protocol. The membranes were incubated by gentle agitation at room temperature for 20 to 25 min with 10 mL of 1X *Re-Blot Plus* Strong Antibody Stripping solution (1:10 dilution in sterile water). Membranes were incubated with 10-15 mL of 5% skim milk blocking solution at 4ºC overnight. The blocked membranes were then used for detection of GAPDH and Hsp72.

Western analyses were performed in replicates of 3 for one set of exposure experiments for untreated control and 2,4-D treated cell cultures.

3.5.4.3 Densitometry

Protein expression was quantified using densitometry. Briefly, exposed films were scanned using an Epson perfection 4990 photo scanner. Scanned images were cropped using Adobe® Photoshop® CS2. Densitometry was performed using National Institute of Health (NIH) Image J. Protein expression was quantified by measuring the mean density of the entire target and GAPDH protein expression band for untreated control and treated samples. Mean background density value was subtracted from all untreated control and treated sample protein mean density values. Target protein expression level in each sample was calculated relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

3.6 Statistical analysis

Statistical analyses were performed using SPSS® 14.0 for windows.

To determine if treatments induced statistically significant effects, compared to untreated control cell cultures, analyses were performed using one-way analysis of variance (ANOVA), followed by a two-sided Dunnett's post-hoc test. Results were considered significant if $P<0.05$. To determine if treatments induced statistically significant effects between exposure time points or between treatment groups within the same cell line, analyses were performed using one-way ANOVA, followed by a Tukey honestly significant difference (Tukey HSD) post-hoc test. Results were considered

significant if $P<0.05$. To determine if there were statistically significant differences in the treatment effects between (1) different 2,4-D forms within the same cell line, or (2) 2 cell lines, analyses were performed using unpaired student t-tests. Results were considered significant if $P<0.05$.

3.7 Figure production

All figures were produced using Microsoft Office® Excel and Power Point software.

4. RESULTS

4.1 Introduction

In the present study, two human cell lines; HepG2 and HEK293 cells were used to evaluate the toxicity of 2,4-D. HepG2 cells were chosen for use in this study because they have been employed in a vast array of *in vitro* toxicology studies and retain both Phase I and II hepatic biotransformation enzymes (Merch-Sundermann *et al*., 2004), allowing for a more realistic metabolism of 2,4-D *in vitro*. The use of HepG2 cells also helped to verify results of cDNA microarray analyses performed on HepG2 cells by Bharadwaj et al. (2005).

In humans, 82.3% of 2,4-D is excreted in the kidney mainly unchanged (Sauerhoff *et al*., 1977). The HEK293 cells (renal cells), were chosen as an additional *in vitro* cell model to assess the cell specificity of 2,4-D – induced cytotoxicity.

Cell cultures were exposed to either a technical grade or a commercial formulation of 2,4-D to determine if cytotoxic effects are enhanced by the presence of 'inert ingredients' in the 2,4-D commercial formulation or are a result of the active ingredient; 2,4-D. Cells were exposed for 6 to 72 hrs depending on the assay used to evaluate toxicity. A commercial formulation containing 2,4-D DMA was used because 2,4-D amine and ester commercial formulations make up approximately 90-95% of global 2,4-D use (reviewed by Charles *et al*., 2001) and amine formulations are more hydrophilic (Charles *et al*., 2001; PMRA, 2005) and less volatile (WHO, 1984) than ester formulations.

The HepG2 and HEK293 cell cultures were exposed to concentrations of 2,4-D ranging from 0.1 nM to 10 mM. This particular range of 2,4-D concentrations was chosen because, it included; (1) environmentally realistic concentrations of 2,4-D that have been found in Canadian groundwater [i.e. 0.07 µg/L, 2.67 µg/L (Grover *et al*., 1997) and 29 µg/L (reviewed by Environment Canada, 1991; WHO, 2003)], (2) Canada's established IMAC guideline for 2,4-D in drinking water [100 µg/L (Environment Canada, 1991)] and (3) toxic concentrations of 2,4-D (1 to 10 mM).

4.2 The effects of 2,4-D on cell viability

Cell viability assays were performed to determine if exposure to 2,4-D at environmental concentrations would induce toxic effects in human cell cultures. Cell viability was determined fluorometrically using an *in vitro* toxicology assay kit, containing the dye, Resazurin. The present cell viability assay provides a rapid and efficient method for determination of the magnitude of cytotoxicity induced by test compounds/chemicals.

Cells were exposed to 2,4-D for 6 to 72 hrs to determine if toxicity was more pronounced with longer durations of exposure. The concentrations; 5 and 10mM were expected to induce more pronounced cytotoxicity, compared to low concentrations (0.1 nM to 1 mM), and thus was used as a positive control to determine the efficacy of the Resazurin assay for assessing 2,4-D – induced cytotoxicity. Cell viability was calculated as a percent (%) of the fluorescence of untreated control cell cultures, using the formula:

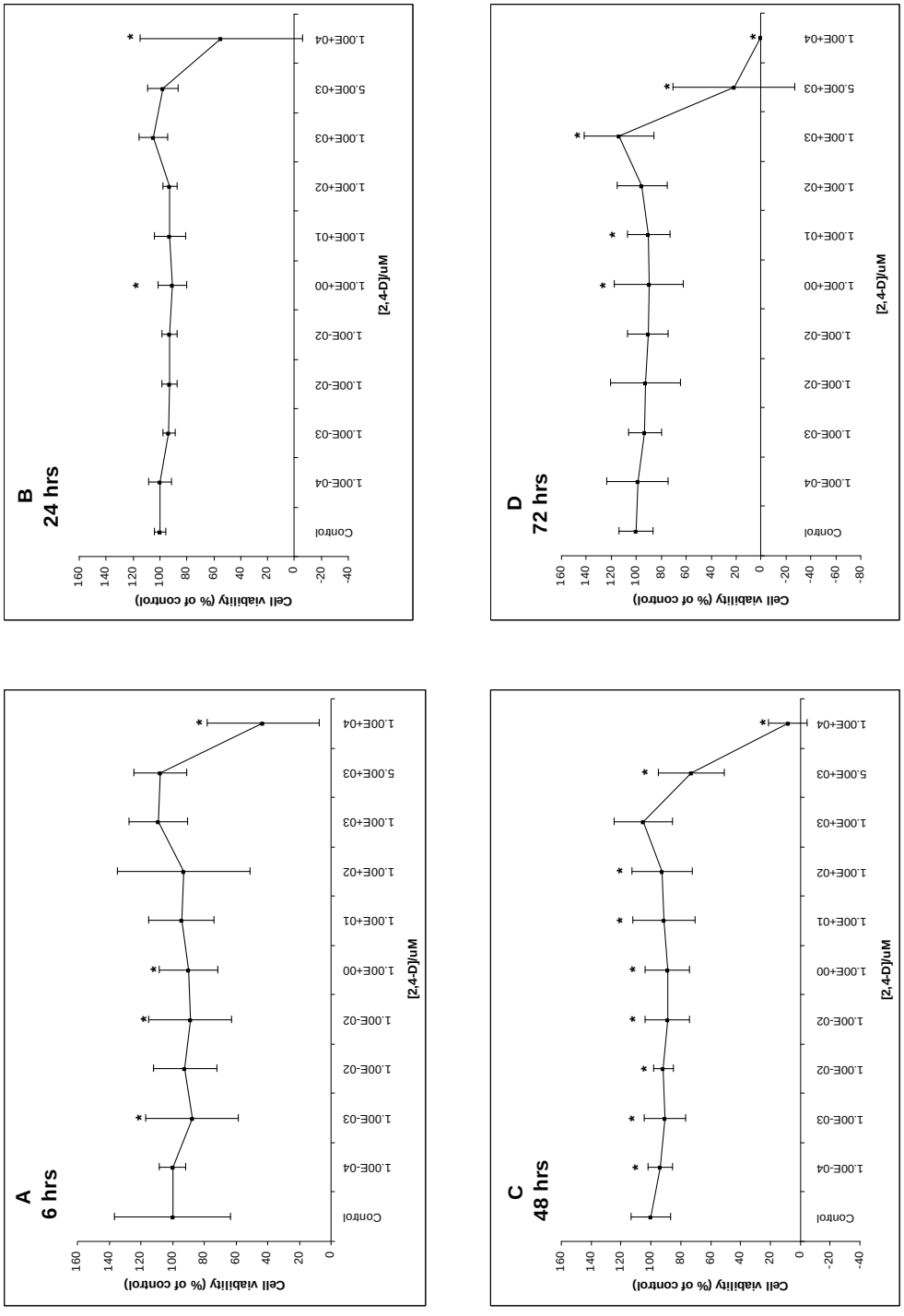
$$\% \text{ cell viability} = \frac{\text{(mean treated sample fluorescence)}}{\text{(mean control fluorescence)}} \times 100\% \qquad \textbf{(4.1)}$$

Cell viability results were presented as the mean % cell viability ± standard error of the mean (SEM).

4.2.1 The effects of 2,4-D on HepG2 cell viability

As seen in figures 1 (A) to (D), exposure to technical 2,4-D decreased viability in exposed cells. A decrease in cell viability was observed at all durations of exposure (6 to 72 hrs). However, effects appeared to be limited (mean decreases in viability < 20%) at lower environmental concentrations (0.1 to 100 nM) and more pronounced (mean decreases in viability > 40%) at higher concentrations (5 and 10 mM). A statistical significant (P<0.0.5) decrease (6%) in viability was initially manifested at 0.1 nM following 48 hrs of exposure. Exposure to concentrations of 1 nM to 100 µM induced significant decreases in viability following 6, 24 and 48 hrs of exposure. Increasing

Figure 1: The effects of technical 2,4-D on viability of exposed HepG2 cells. Cells were exposed to 0 to 10mM of 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean ± standard error of the mean (SEM) from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant ($P<0.05$) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HepG2 cells exposed to the following concentrations and respective durations of exposure: **(A)** 1nM: $P = 0.006$; 100nM: $P = 0.016$; 1µM: $P = 0.041$; 10mM: $P = 0.0001$. **(B)** 1µM: $P = 0.015$; 10mM: $P = 0.0001$. **(C)** 0.1nM: $P = 0.032$; 1nM: $P = 0.0001$; 10nM: $P = 0.002$; 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.001$; 100µM: $P = 0.009$, 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(D)** 1µM: $P = 0.031$; 10µM: $P = 0.035$; 1mM: $P = 0.001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$.


A
6 hrs
B
24 hrs
C
48 hrs
D
72 hrs
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04

concentrations (over the range of concentrations of 0.1 nM to 100 μM) and durations of exposure did not alter the magnitude of observed decreases in cell viability. A statistically significant (P<0.05) increase (14%) in cell viability was observed following exposure to 1 mM for 72 hrs. Exposure to 5 or 10 mM induced statistically significant (P<0.05) decreases in cell viability at all durations of exposure (6 to 72 hrs). Exposure to 5 mM induced significant decreases of 27 and 78% in cell viability following 48 and 72 hrs of exposure, respectively. Significant decreases (57, 46, 92 and 100%) in viability were observed following exposure to 10 mM for 6 to 72 hrs of exposure, respectively.

The results of figure 1 were compiled into one graph for comparison of the effects of exposure to 0.1 nM to 1 mM of technical grade 2,4-D for 6 to 72 hrs. As seen in figure 2, in contrast to 5 and 10 mM, exposure to 0.1 nM to 100 μM for 6, 24, 48 and 72 hrs induced limited cytotoxic effects, reflected by small decreases in cell viability. Similar effects in cell viability were observed over the concentration range of 0.1 nM to 100 μM. More pronounced decreases in cell viability were not observed with longer durations of exposure. Exposure to 1 mM for similar durations of treatments increased cell viability.

Figure 3 illustrates the effect on viability of HepG2 cell cultures exposed to increasing concentrations of commercial 2,4-D for (A) 6, (B) 24, (C) 48 and (D) 72 hrs. Decreases in cell viability were observed at all durations of exposure. Effects appeared to be small (mean decreases in viability < 20%) at lower environmental concentrations (0.1 to 100 nM) and increased (mean decreases in viability > 40%) at higher concentrations (5 and 10 mM). A statistical significant (P<0.0.5) decrease (11%) in viability was initially manifested at 0.1 nM of 2,4-D following 6 hrs of exposure. Exposure to 1 nM to 100 μM induced significant decreases in viability following 6, 24 and 48 hrs of exposure. Declines in cell viability were not exacerbated with exposure to increasing concentrations over this range of concentrations or with increasing times of exposure. Increases (7, 6 and 20%) in cell viability were observed following exposure to 1 mM for 24 to 72 hrs, respectively. The effects were statistically significant (P<0.05).

Figure 2: Comparison of the effects of technical 2,4-D on viability of HepG2 cells exposed for 6, 24, 48 and 72 hrs. Cells were exposed to 0 to 10mM of 2,4-D for 6, 24, 48 and 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) test. Statistically significant ($P<0.05$) differences between exposure periods are denoted by the symbols a, b and c for the following comparisons: [a] 6 hrs vs. 24 hrs; [b]24 hrs vs. 48 hrs; [c] 48 hrs vs. 72 hrs. Significant differences between exposure periods were observed in HepG2 cells exposed to the following concentrations: 0.1nM: [a] $P = 0.0001$; [c] $P = 0.001$. 1nM: [a] $P = 0.0001$. 10nM: [a] $P = 0.0001$. 100M: [a] $P = 0.0001$. 1µM: [a] $P = 0.0001$. 10µM: [a] $P = 0.0001$. 100µM: [a] $P = 0.0001$. 1mM: [a] $P = 0.0001$; [c] $P = 0.001$. 5mM: [a] $P = 0.0001$; [b] $P = 0.001$; [c] $P = 0.0001$. 10mM: [b] $P = 0.0001$.

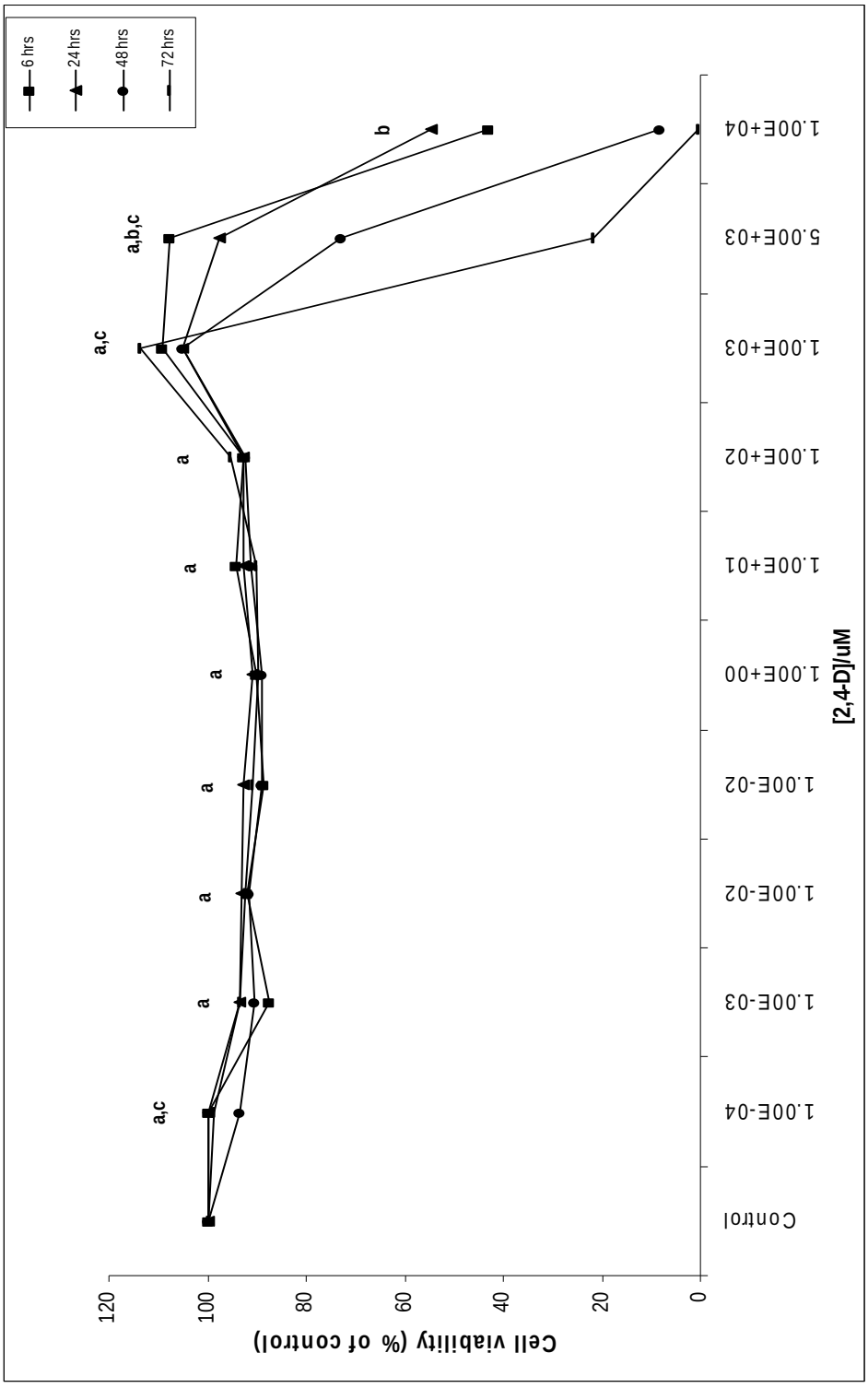
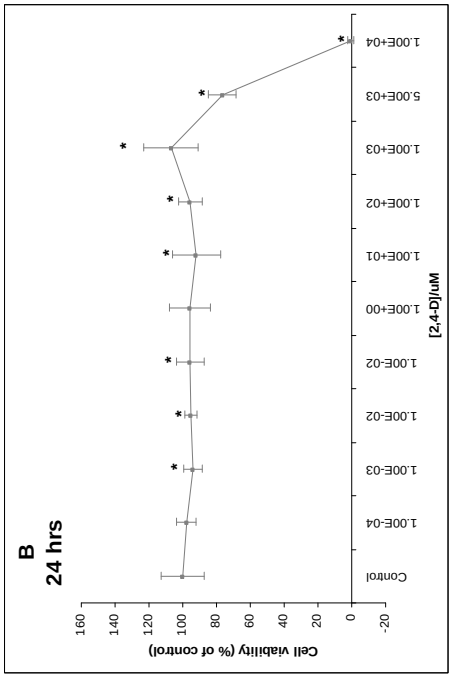
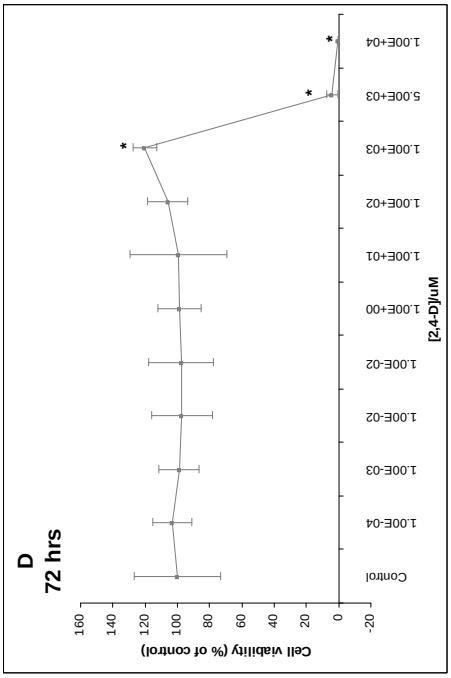
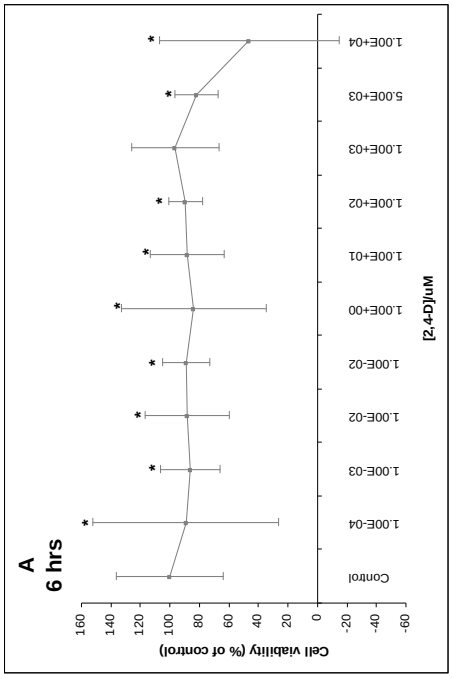
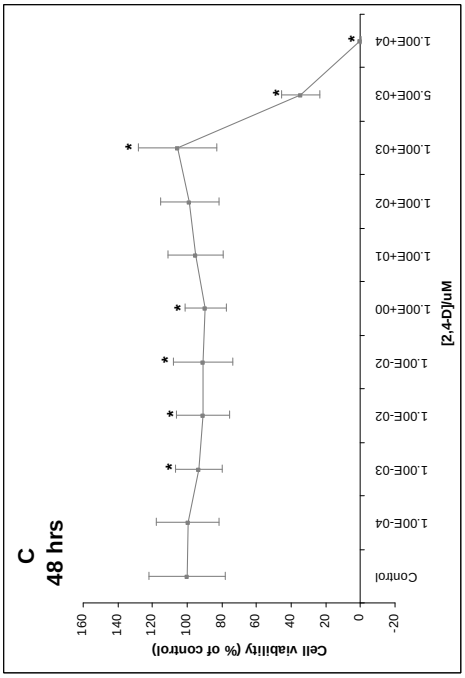

6 hrs
24 hrs
48 hrs
72 hrs
Cell viability (% of control)
120
100
80
60
40
20
0
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM
a,c
a
a
a
a
a
a
a,c
a,b,c
b

Figure 3: The effects of commercial 2,4-D on viability of exposed HepG2 cells. Cells were exposed to 0 to 10mM of 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean ± standard error of the mean (SEM) from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant ($P<0.05$) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HepG2 cells exposed to the following concentrations and respective durations of exposure: **(A)** 0.1nM: $P = 0.037$; 1nM: $P = 0.003$; 10nM: $P = 0.017$; 100nM: $P = 0.031$; 1µM: $P = 0.0001$; 10µM: $P = 0.021$; 100µM: $P = 0.043$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(B)** 1nM: $P = 0.003$; 10nM: $P = 0.033$; 100nM: $P = 0.050$; 10µM: $P = 0.0001$; 100µM: $P = 0.047$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(C)** 1nM: $P = 0.006$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1mM: $P = 0.028$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(D)** 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$.


B
24 hrs
Cell viability (% of control)
160
140
120
100
80
60
40
20
0
-20
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM


D
72 hrs
Cell viability (% of control)
160
140
120
100
80
60
40
20
0
-20
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM


A
6 hrs
Cell viability (% of control)
160
140
120
100
80
60
40
20
0
-20
-40
-60
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM


C
48 hrs
Cell viability (% of control)
160
140
120
100
80
60
40
20
0
-20
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM

Statistically significant (P<0.05) decreases in cell viability were observed following exposure to 5 or 10 mM. Exposure to 5 mM induced significant decreases of 18, 24, 65 and 96% in cell viability following 6 to 72 hrs of exposure, respectively. Exposure to 10 mM for 48 and 72 hrs was lethal to all cells; inducing a 100% decrease in cell viability.

The results of figure 3 were compiled into one graph for comparison of the effects of exposure to 0.1 nM to 1 mM of commercial 2,4-D for 6 to 72 hrs. As seen in figure 4, in contrast to 5 and 10 mM, exposure to 0.1 nM to 100 μM for all durations of exposure induced limited cytotoxic effects, reflected by small decreases in cell viability. A similar decline in cell viability was observed in cells treated over the concentrations range of 0.1 nM to 100 μM. More pronounced decreases in cell viability were not observed with longer durations of exposure. Exposure to 1 mM for 24, 48 and 72 hrs increased cell viability.

4.2.2 Comparison of the effects of technical or commercial 2,4-D on HepG2 cell viability

Figure 5 illustrates that exposure to either form of 2,4-D appears to elicit similar effects on HepG2 cell viability for all durations of exposure (6 to 72 hrs). Decreases in viability were observed following exposure to 0.1 nM to 100 μM of either technical or commercial 2,4-D for 6 to 72 hrs. More pronounced decreases in viability were not observed with increasing concentrations or times to either forms of 2,4-D. Mean decreases in viability were observed following exposure to 0.1 nM to 100 μM for 6 (technical: 9%, commercial formulation: 12%); 24 (technical: 8%, commercial formulation: 5%); 48 (technical: 9% and commercial formulation: 7%) and 72 hrs (technical: 7%, commercial formulation: 2%), respectively. In contrast to technical 2,4-D, exposure to commercial 2,4-D induced a statistically significant (P<0.05) decrease in viability at the lowest concentration (0.1 nM) at an earlier time point (technical grade: 48 hrs; commercial formulation: 6hrs). Increases in cell viability were observed upon exposure to 1 mM of either technical or commercial 2,4-D for 24 to 72 hrs. However, statistically significant (P<0.05) differences between the effects were only observed following exposure to 1 mM at 24 and 48 hrs.

Figure 4: Comparison of the effects of commercial 2,4-D on viability of HepG2 cells exposed for 6, 24, 48 and 72 hrs. Cells were exposed to 0 to 10mM of 2,4-D for 6, 24, 48 and 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) test. Statistically significant ($P<0.05$) differences between exposure periods are denoted by the symbols a, b and c for the following comparisons: [a] 6 hrs vs. 24 hrs; [b]24 hrs vs. 48 hrs; [c] 48 hrs vs. 72 hrs. Significant differences between exposure periods were observed in HepG2 cells exposed to the following concentrations: 0.1nM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.005$. 1nM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 10nM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 100nM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 1µM: [a] $P = 0.0001$; [b] $P = 0.0001$. 10µM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 100µM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 1mM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.027$. 5mM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 10mM: [a] $P = 0.0001$.

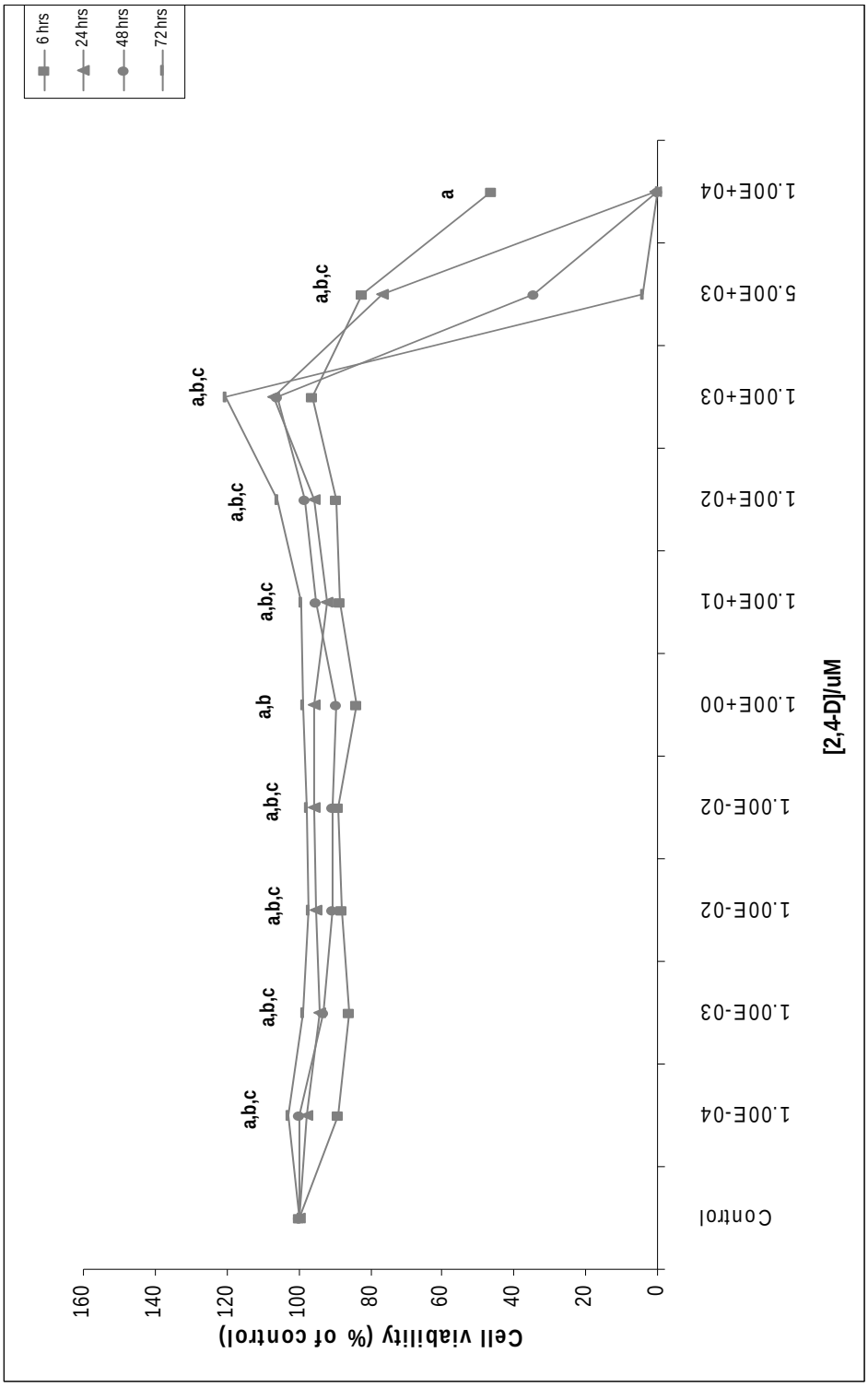
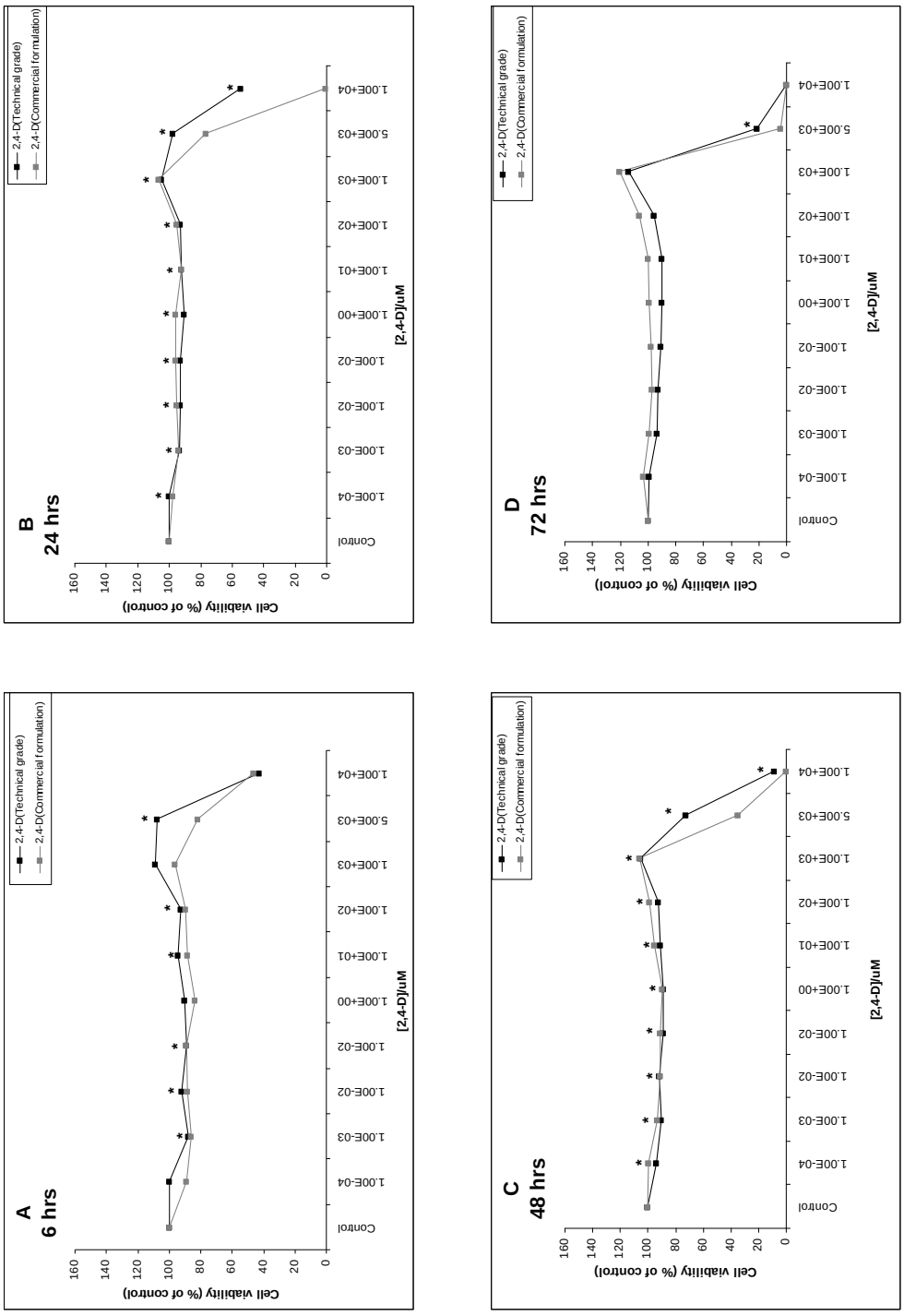

6 hrs
24 hrs
48 hrs
72 hrs
Cell viability (% of control)
0
20
40
60
80
100
120
140
160
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM
a,b,c
a,b,c
a,b,c
a,b,c
a,b
a,b,c
a,b,c
a,b,c
a

Figure 5: Comparison of the effects of technical or commercial 2,4-D on viability of exposed HepG2 cells. Cells were exposed to 0 to 10mM of technical grade or commercial 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using unpaired student t-tests. Statistically significant ($P<0.05$) differences between 2,4-D forms are denoted by the symbol *. Significant differences between 2,4-D forms were observed in HepG2 cells exposed to the following concentrations: **(A)** 1nM: $P = 0.002$; 10nM: $P = 0.005$; 100nM: $P = 0.0001$; 10µM: $P = 0.011$; 100µM: $P = 0.023$; 5mM: $P = 0.0001$. **(B)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1µM: $P = 0.013$; 10µM: $P = 0.0001$; 100µM: $P = 0.0001$; 1mM: $P = 0.001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(C)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.0001$; 100µM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(D)** 5mM: $P = 0.003$.


A
6 hrs
B
24 hrs
C
48 hrs
D
72 hrs
2,4-D(Technical grade)
2,4-D(Commercial formulation)
Cell viability (% of control)
[2,4-D]uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
0
20
40
60
80
100
120
140
160

As seen from figures 5 (A) to (D) exposure to 5 and 10 mM of either technical or commercial 2,4-D for durations of 6 to 72 hrs induced similar viability effects. A pronounced decline in viability was observed with increasing concentrations and durations of exposure to 5 and 10 mM of either forms of 2,4-D.

Thus, the similar pattern of cell viability effects induced by either forms of 2,4-D suggests that toxicity was directly induced by 2,4-D and that the presence of 'inert ingredients' in the commercial formulation did not enhance the toxicity of 2,4-D to a great degree.

4.2.3 The effects of 2,4-D on HEK293 cell viability

As seen in figures 6 (A) to (D), a 6 to 72 hr exposure to technical 2,4-D decreased cell viability. The effects appeared to be limited (mean decreases in viability < 20%) at lower environmental concentrations (0.1 to 100 nM) and exacerbated (mean decreases in viability > 40%) at higher concentrations. A mean decrease of approximately 30% in cell viability was observed over the concentration range of 0.1 to 100 nM following 6 hrs of exposure. A significant decrease (16%) in viability was initially manifested at 0.1 nM following 48 hrs of exposure. Similar reductions in cell viability were observed over the concentration range of 0.1 nM to 1 mM and durations of exposure.

Statistically significant ($P<0.05$) decreases in viability were observed following exposure to 5 or 10 mM for 6 to 72 hrs. Decreases in cell viability were more pronounced upon exposure to 10 mM (55, 93 and 100%), compared to 5 mM (37, 71 and 97%) for 24, 48 and 72 hrs, respectively.

The results of figure 6 were compiled into one graph for comparison of the effects of exposure to 0.1 nM to 1 mM of technical grade 2,4-D for 6 to 72 hrs. As seen in figure 7, in contrast to 24, 48 and 72 hrs (mean decreases in cell viability: 5, 16 and 19%, respectively), more pronounced decreases in viability were observed in cells exposed to 0.1 nM to 1 mM for 6 hrs (mean decrease in viability: 32%). More pronounced decreases in cell viability were not observed with longer durations of exposure to concentrations of 0.1 nM to 1 mM.

Figure 6: The effects of technical 2,4-D on viability of exposed HEK293 cells. Cells were exposed to 0 to 10mM of 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean ± standard error of the mean (SEM) from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant ($P<0.05$) differences from fluorescence of untreated cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HEK293 cells exposed to the following concentrations and respective durations of exposure: **(A)** 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1μM: $P = 0.0001$; 10μM: $P = 0.0001$; 100μM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(B)** 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(C)** 0.1nM: $P = 0.004$; 1nM: $P = 0.041$; 10nM: $P = 0.017$; 100nM: $P = 0.001$; 10μM: $P = 0.001$; 100μM: $P = 0.019$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(D)** 1mM: $P = 0.010$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$.

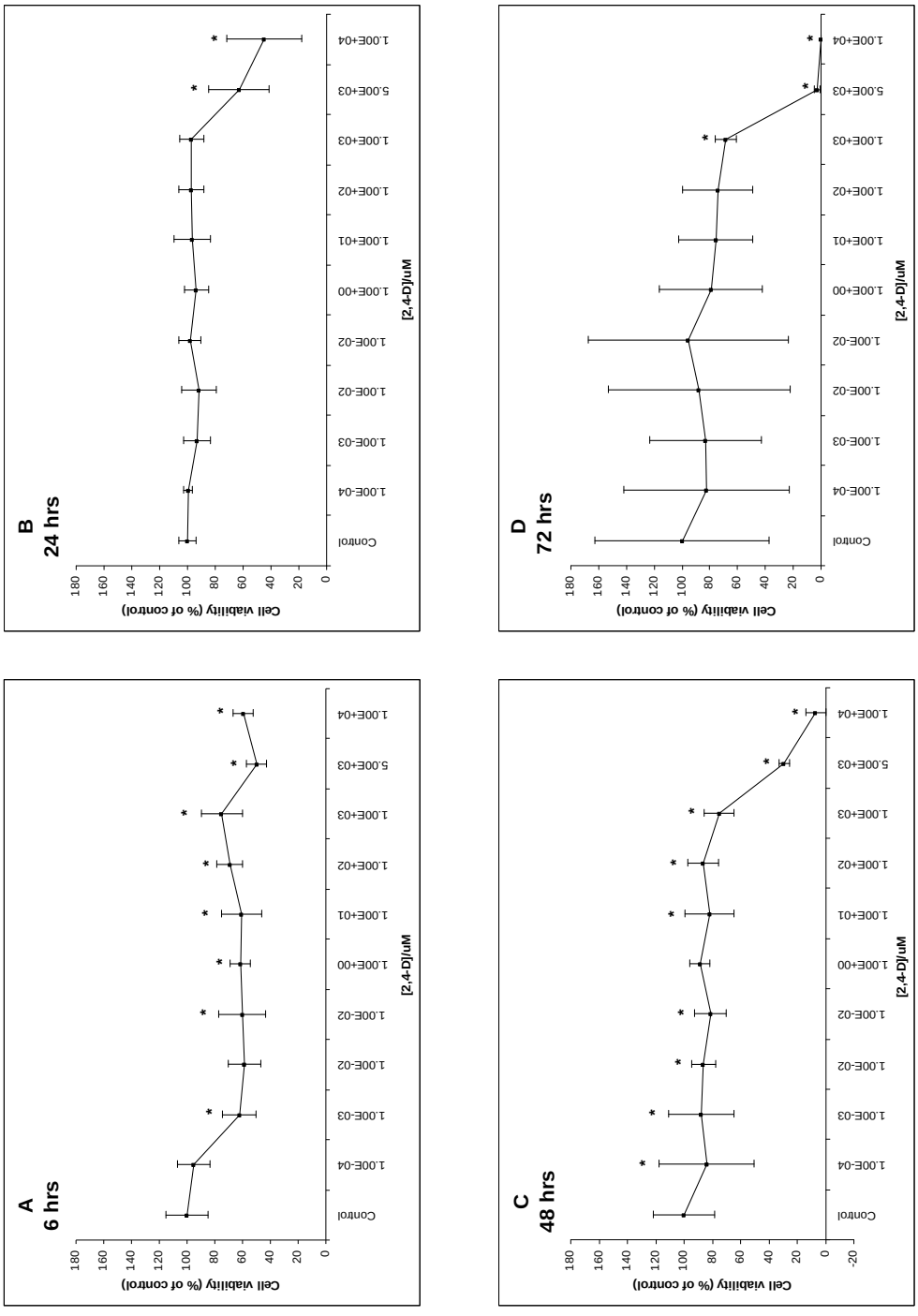

A
6 hrs
B
24 hrs
C
48 hrs
D
72 hrs
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
0
20
40
60
80
100
120
140
160
180
-20

Figure 7: Comparison of the effects of technical 2,4-D on viability of HEK293 cells exposed for 6, 24, 48 and 72 hrs. Cells were exposed to 0 to 10mM of 2,4-D for 6, 24, 48 and 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) test. Statistically significant ($P<0.05$) differences between exposure periods are denoted by the symbols a, b and c for the following comparisons: [a] 6 hrs vs. 24 hrs; [b]24 hrs vs. 48 hrs; [c] 48 hrs vs. 72 hrs. Significant differences between exposure periods were observed in HEK293 cells exposed to the following concentrations: 1nM: [a] $P = 0.002$. 10nM: [a] $P = 0.018$. 100nM: [a] $P = 0.014$; [c] $P = 0.006$. 1µM: [a] $P = 0.0001$. 10µM: [a] $P = 0.0001$. 100µM: [a] $P = 0.0001$. 1mM: [a] $P = 0.0001$; [c] $P = 0.020$. 5mM: [a] $P = 0.002$; [b] $P = 0.0001$; [c] $P = 0.0001$. 10mM: [b] $P = 0.0001$.

Cell viability (% of control)
0
20
40
60
80
100
120
140
160
180
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM
a
a
a,c
a
a
a
a,c
a,b,c
b
6 hrs
24 hrs
48 hrs
72 hrs

Figure 8 illustrates the effect on viability of HEK293 cell cultures exposed to increasing concentrations of commercial 2,4-D for (A) 6, (B) 24, (C) 48 and (D) 72 hrs. As seen in the figure, exposure to 2,4-D at all durations of exposure decreased cell viability. The viability effects appeared to be limited (mean decreases in viability < 20%) at lower environmental concentrations (0.1 to 100 nM) and exacerbated (mean decreases in viability > 40%) at higher concentrations (5 and 10 mM). A statistically significant (P<0.05) decrease (8%) in cell viability was initially manifested following exposure to 1 nM for 24 hrs. Similar decreases in cell viability were observed over the concentration range of 0.1 nM to 1 mM and at all durations of exposure. Statistically significant (P<0.05) decreases in viability were observed following exposure to 5 and 10 mM for 6 to 72 hrs. Decreases in cell viability were more pronounced upon exposure to 10 mM (70, 99, 100 and 100%), compared to 5 mM (26, 48, 74 and 97%), respectively.

The results of figure 8 were compiled into one graph for comparison of the effects of exposure to 0.1 nM to 1 mM of commercial 2,4-D for 6 to 72 hrs. As seen in figure 9, in contrast to 24, 48 and 72 hrs (mean decreases in cell viability: 11, 10 and 10%, respectively), more pronounced decreases in viability were observed in cells exposed to 0.1 nM to 1 mM for 6 hrs (mean decrease in viability: 18%). More pronounced decreases in cell viability were not observed with longer durations of exposure to concentrations of commercial 2,4-D within the range of 0.1 nM to 1 mM.

4.2.4 Comparison of the effects of technical or commercial 2,4-D on HEK293 cell viability

Figure 10 illustrates that exposure to either technical grade or commercial 2,4-D elicited similar effects on HEK293 cell viability for all durations of exposure tested. However, in contrast to technical grade 2,4-D, exposure to commercial 2,4-D induced a statistically significant (P<0.05) decrease in viability at the lowest concentration and at the earliest time point (technical grade: 0.1 nM at 48 hrs; commercial formulation: 1 nM at 24 hrs). More pronounced decreases in cell viability were not observed with increasing concentrations or times to either forms of 2,4-D. For example, mean decreases in viability observed upon exposure to either 0.1 nM to 1mM of technical or commercial 2,4-D for 6, 24, 48 and 72 hrs were: technical grade: 33%, commercial

Figure 8: The effects of commercial 2,4-D on viability of exposed HEK293 cells. Cells were exposed to 0 to 10mM of 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean ± standard error of the mean (SEM) from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant ($P<0.05$) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HEK293 cells exposed to the following concentrations and respective durations of exposure: **(A)** 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(B)** 1nM: $P = 0.017$; 10nM: $P = 0.010$; 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.038$; 100µM: $P = 0.028$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(C)** 100µM: $P = 0.014$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(D)** 5mM: $P = 0.0001$; 10mM: $P = 0.0001$.

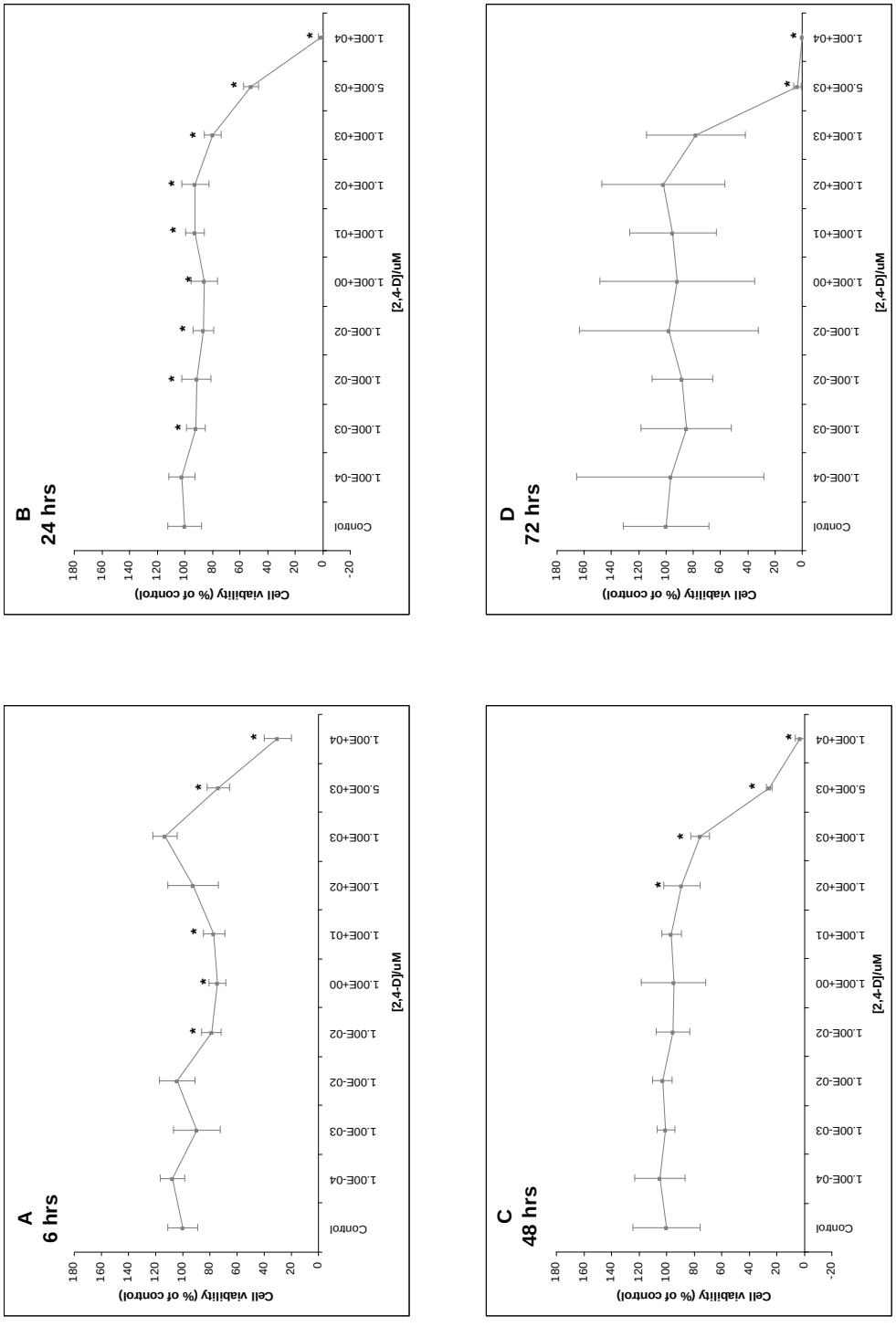
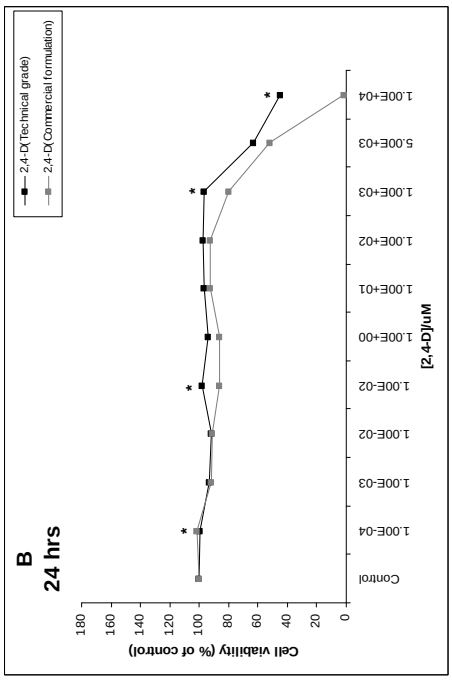
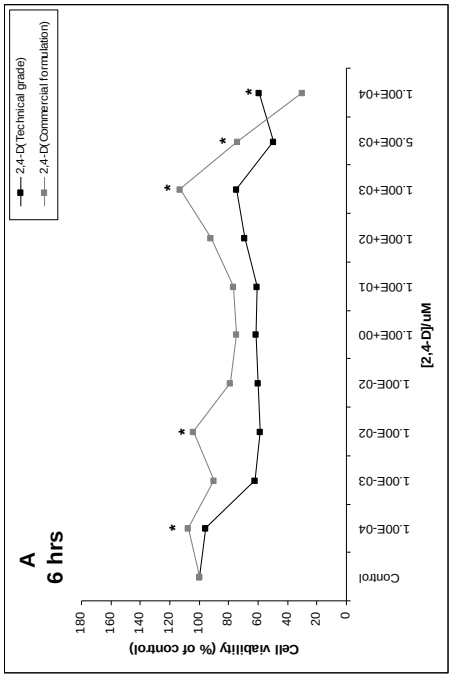

A
6 hrs
B
24 hrs
C
48 hrs
D
72 hrs
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
0
20
40
60
80
100
120
140
160
180
-20
*

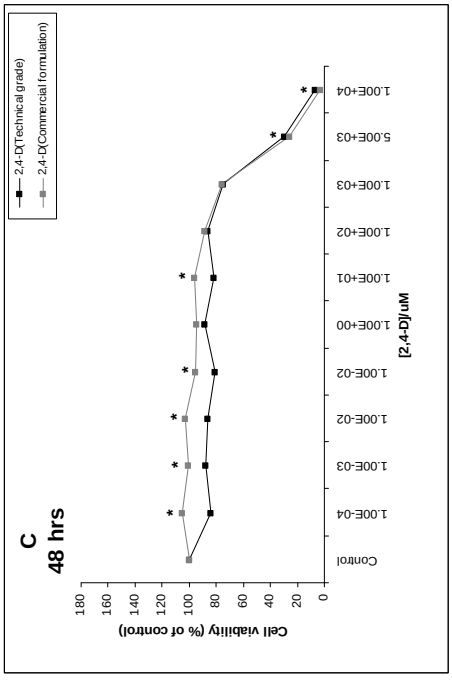
Figure 9: Comparison of the effects of commercial 2,4-D on viability of HEK293 cells exposed for 6, 24, 48 and 72 hrs. Cells were exposed to 0 to 10mM of 2,4-D for 6, 24, 48 and 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) test. Statistically significant ($P<0.05$) differences between exposure periods are denoted by the symbols a, b and c for the following comparisons: [a] 6 hrs vs. 24 hrs; [b]24 hrs vs. 48 hrs; [c] 48 hrs vs. 72 hrs. Significant differences between exposure periods were observed in HEK293 cells exposed to the following concentrations: 1nM: [a] $P = 0.0001$; [b] $P = 0.001$. 10nM: [a] $P = 0.001$; [b] $P = 0.0001$. 100nM: [a] $P = 0.021$; [c] $P = 0.019$. 1µM: [a] $P = 0.006$. 10µM: [a] $P = 0.0001$; [b] $P = 0.003$; [c] $P = 0.0001$. 100µM: [a] $P = 0.006$; [c] $P = 0.0001$. 1mM: [c] $P = 0.001$. 5mM: [b] $P = 0.0001$; [c] $P = 0.0001$. 10mM: [a] $P = 0.0001$.

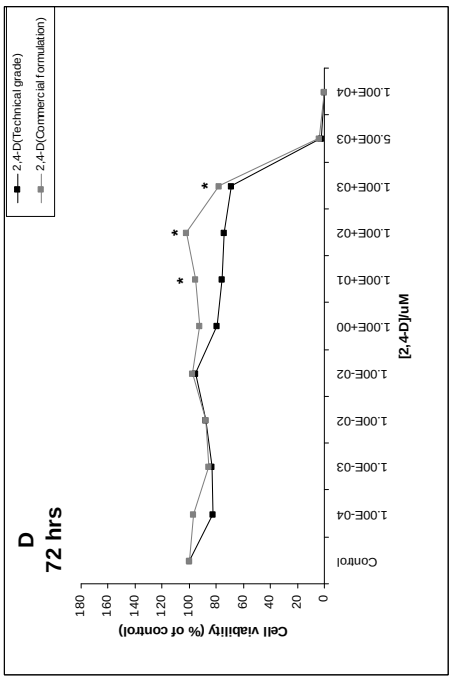
6 hrs
24 hrs
48 hrs
72 hrs
Cell viability (% of control)
0
20
40
60
80
100
120
140
160
180
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04
[2,4-D]/uM
a,b
a,b
a,c
a
a,b,c
a,c
c
b,c
a

Figure 10: Comparison of the effects of technical grade or commercial 2,4-D on viability of exposed HEK293 cells. Cells were exposed to 0 to 10mM of technical grade or commercial 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using unpaired student t-tests. Statistically significant ($P<0.05$) differences between 2,4-D forms are denoted by the symbol *. Significant differences between 2,4-D forms were observed in HEK293 cells exposed to the following concentrations: **(A)** 0.1nM: $P = 0.007$; 10nM: $P = 0.0001$; 1mM: $P = 0.011$; 5mM: $P = 0.016$; 10mM: $P = 0.0001$. **(B)** 0.1nM: $P = 0.002$; 100nM: $P = 0.049$; 1mM: $P = 0.001$; 10mM: $P = 0.001$. **(C)** 0.1nM: $P = 0.009$; 1nM: $P = 0.017$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 10µM: $P = 0.001$; 5mM: $P = 0.001$; 10mM: $P = 0.018$. **(D)** 10µM: $P = 0.004$; 100µM: $P = 0.002$; 1mM: $P = 0.043$.


A
6 hrs
2,4-D(Technical grade)
2,4-D(Commercial formulation)
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


B
24 hrs
2,4-D(Technical grade)
2,4-D(Commercial formulation)
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


C
48 hrs
2,4-D(Technical grade)
2,4-D(Commercial formulation)
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


D
72 hrs
2,4-D(Technical grade)
2,4-D(Commercial formulation)
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04

formulation: 18% (6hrs); technical grade: 5%, commercial formulation: 11% (24hrs); technical grade:16%, commercial formulation: 10% (48hrs) and technical grade: 19%, commercial formulation: 10% (72hrs), respectively. Thus, in contrast to 24, 48 and 72 hrs, exposure to either technical grade or commercial 2,4-D for 6 hrs appeared to induce greater magnitudes of cytotoxic effects, reflected by more pronounced decreases in viability.

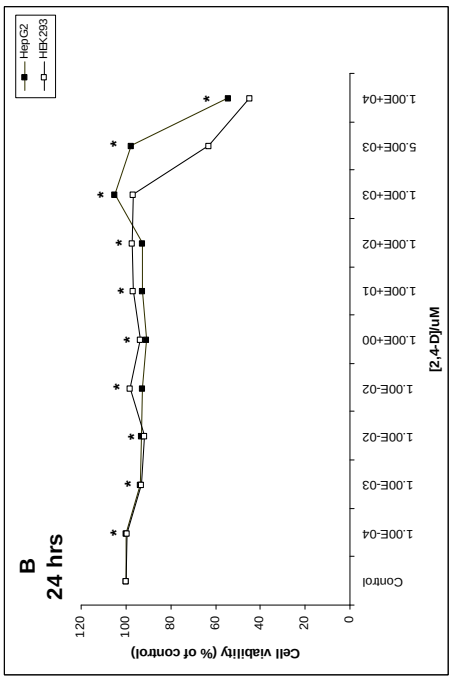
A more pronounced decrease in cell viability was observed following exposure to 10 mM (mean decrease in cell viability; technical: 72%, commercial: 93%), compared to 5 mM (mean decrease in cell viability; technical: 64%, commercial: 63%) of either forms of 2,4-D for 6 to 72 hrs. Statistically significant ($P<0.05$) differences between the viability effects were observed following exposure to 5 and 10 mM for 6 and 48 hrs; 10 mM for 24 hrs and 5 mM for 72 hrs.

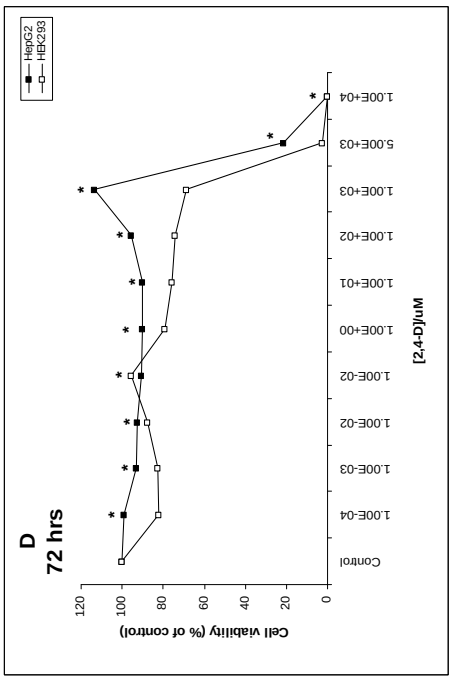
The effects on cell viability induced by either form of 2,4-D, suggests that toxicity was directly induced by 2,4-D and that the presence of 'inert ingredients' in the commercial formulation did not enhance the toxicity of 2,4-D to a great degree.

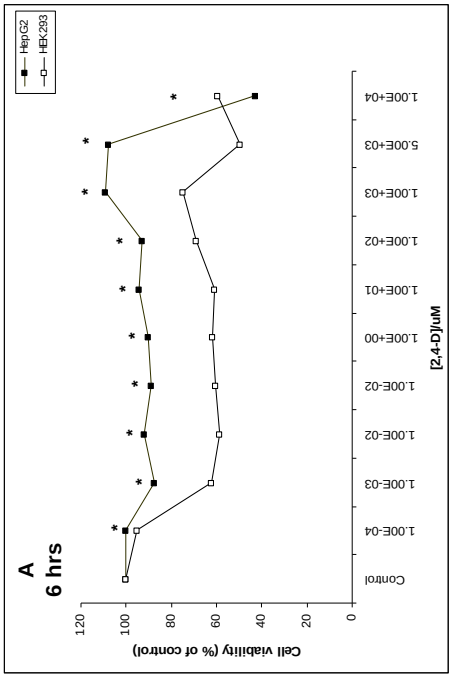
4.2.5 Comparison of the effects of technical 2,4-D on HepG2 and HEK293 cell viability

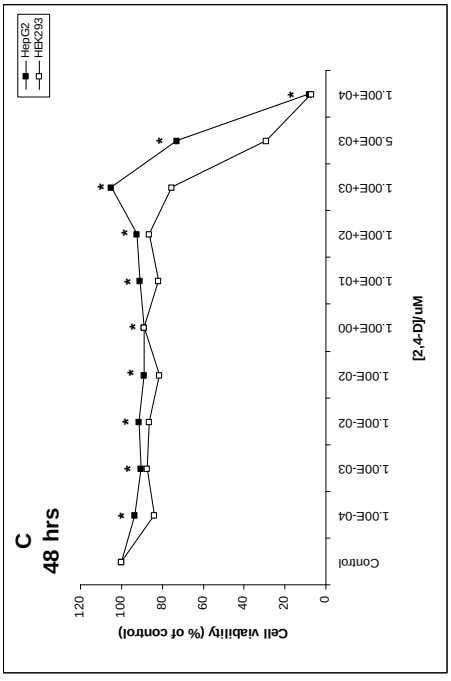
As seen from figure 11 (A) to (D), 2,4-D elicited similar effects on viability in both HepG2 and HEK293 cells at all durations of exposure (6 to 72 hrs). Significant decreases in both HepG2 and HEK293 cell viability were observed at the lowest concentrations (0.1 nM) following 48 hrs of exposure. However, in contrast to HepG2 cells, HEK293 cells appeared to be more susceptible to exposures of 0.1 nM to 100 μM for 6, 48 and 72 hrs, reflected by the more pronounced decreases in cell viability. For example exposure to 0.1 nM to 100 μM for 6 to 72 hrs induced greater magnitudes of decreases in HEK293 (33, 16 and 19%), compared to HepG2 (9, 9 and 7%) cell viability, respectively. An additional difference observed between the two cell types was the increase in cell viability observed in HepG2 cells exposed to 1 mM for 6 to 72 hrs. This was not observed in HEK293 cells similarly exposed to the same concentration and duration.

Figure 11: Comparison of the effects of technical 2,4-D on viability of exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 10mM of technical grade 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated cell cultures). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using unpaired student t-tests. Statistically significant ($P<0.05$) differences between HepG2 and HEK293 cells are denoted by the symbol *. Significant differences between HepG2 and HEK293 cells were observed upon exposure to 0.1nM to 10mM at all durations of exposure, as follows: **(A)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.0001$; 100µM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(B)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.0001$; 100µM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.002$. **(C)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1µM: $P = 0.0001$; 10µM: $P = 0.0001$; 100µM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.009$. **(D)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.006$; 1µM: $P = 0.0001$; 10µM: $P = 0.0001$; 100µM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.001$; 10mM: $P = 0.001$.


B
24 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


D
72 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


A
6 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


C
48 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04

In both HepG2 and HEK293 cells, decreases in viability were more exacerbated following 24 to 72 hrs of exposure to 10 mM (HepG2: 46, 92 and 100%; HEK293: 55, 93 and 100%), compared to 5 mM (HepG2: 2, 27 and 78%; HEK293: 37, 71 and 97%).

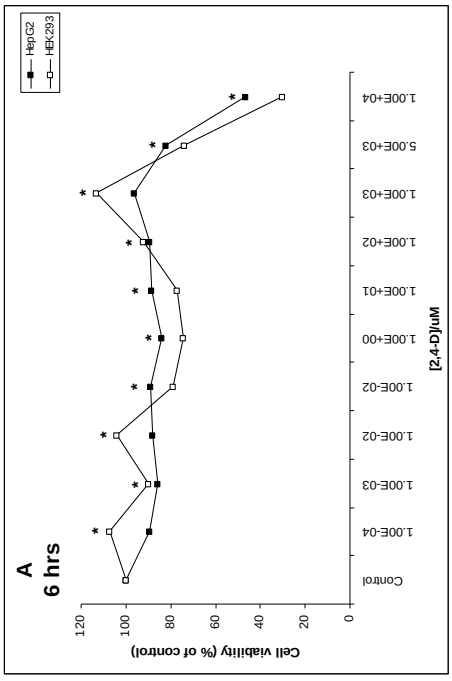
4.2.6 Comparison of the effects of commercial 2,4-D on HepG2 and HEK293 cell viability

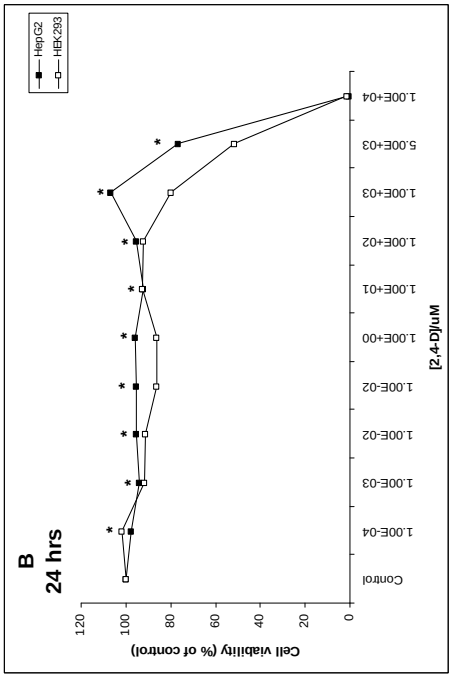
As seen from figure 12 (A) to (D), similar effects on viability were observed in HepG2 and HEK293 cells following exposure to commercial 2,4-D for 6 to 72 hrs. However, in contrast to HEK293, a significant decrease in HepG2 cell viability was initially observed at the lowest concentration and the earliest duration of exposure (HepG2: 0.1 nM at 6 hrs; HEK293: 1 nM at 24 hrs). More pronounced decreases in cell viability were observed in HEK293 (11, 10 and 10%) exposed to 0.1 nM to 100 μM for 24 to 72 hrs of exposure compared to HepG2 (5, 7 and 2%) cells, respectively.

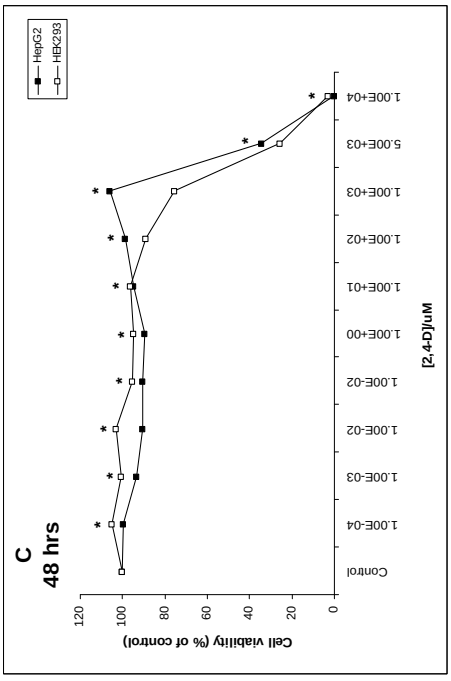
In both HepG2 and HEK293 cells, decreases in viability were more exacerbated following 6 to 72 hrs of exposure to 10mM (HepG2: 54, 99, 100 and 100%; HEK293: 70, 99, 97 and 100%), compared to 5mM (HepG2: 18, 24, 65 and 96%; HEK293: 26, 48, 74 and 97%).

Therefore, in summary, cell viability results illustrated that: (1) exposure to 0.1 nM to 1 mM of either technical or commercial 2,4-D induced similar effects on cell viability in exposed HepG2 and HEK293 cells; suggesting that 2,4-D – induced cytotoxicity may be induced directly by 2,4-D, and the presence of 'inert ingredients' in the commercial formulation has limited ability to act synergistically with 2,4-D and enhance the magnitude of toxicity to a great degree, (2) similar magnitudes of decreases in HepG2 and HEK293 cell viability were observed over the concentration range of 0.1 nM to 100 μM of either forms of 2,4-D, (3) more pronounced decreases in HepG2 and HEK293 cell viability were not observed with longer durations of exposure to either forms of 2,4-D and (4) compared to HepG2, HEK293 cells appeared to be more susceptible to the toxic effects induced by exposure to 0.1 nM to 1 mM of either forms of 2,4-D; reflected by more pronounced decreases in viability. Thus, based on the

Figure 12: Comparison of the effects of commercial 2,4-D on viability of exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 10mM of commercial 2,4-D for **(A)** 6, **(B)** 24, **(C)** 48 and **(D)** 72 hrs in complete growth media. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cell viability was determined using the fluorometric Resazurin assay. Fluorescence from a blank sample (complete growth media and Resazurin) was subtracted from all samples (untreated control and treated). Cell viability is expressed as a percent (%) of the fluorescence of untreated control cell cultures. Results are presented as the mean from 6 independent experiments. Statistical analysis was performed using unpaired student t-tests. Statistically significant ($P<0.05$) differences between HepG2 and HEK293 cells are denoted by the symbol *. Significant differences between HepG2 and HEK293 cells were observed upon exposure to 0.1nM to 10mM at all durations of exposure, as follows: **(A)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1μM: $P = 0.0001$; 10μM: $P = 0.0001$; 100μM: 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.0001$. **(B)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1μM: $P = 0.0001$; 10μM: $P = 0.0001$; 100μM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$. **(C)** 0.1nM: $P = 0.0001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.0001$; 1μM: $P = 0.0001$; 10μM: $P = 0.0001$; 100μM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.0001$; 10mM: $P = 0.005$. **(D)** 0.1nM: $P = 0.001$; 1nM: $P = 0.0001$; 10nM: $P = 0.0001$; 100nM: $P = 0.004$; 1μM: $P = 0.0001$; 10μM: $P = 0.0001$; 100μM: $P = 0.0001$; 1mM: $P = 0.0001$; 5mM: $P = 0.009$; 10mM: $P = 0.017$.


A
6 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


B
24 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04


C
48 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
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1.00E+00
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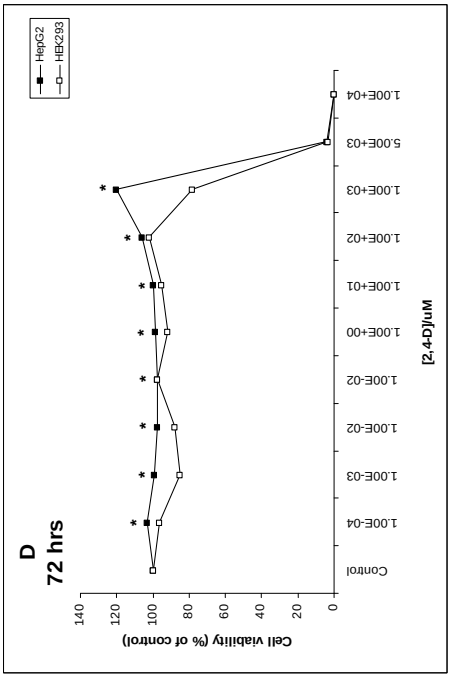
1.00E+02
1.00E+03
5.00E+03
1.00E+04


D
72 hrs
HepG2
HEK293
Cell viability (% of control)
[2,4-D]/uM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-02
1.00E+00
1.00E+01
1.00E+02
1.00E+03
5.00E+03
1.00E+04

results obtained from cell viability assays, all subsequent studies were performed using HepG2 and HEK293 cell cultures exposed to commercial 2,4-D for 24 hrs.

4.3 ROS production in cells

The ROS assays were performed using 2',7'-DCFH-DA to determine if environmental concentrations of 2,4-D induce ROS production in exposed human cell cultures. The HepG2 and HEK293 cells were exposed to H_2O_2 to assess the efficacy of the ROS assay. The compound, 2',7'-DCFH-DA, is considered to be superior for detection of intracellular ROS production (Hempel *et al*., 1999). Additionally, 2',7'-DCFH-DA provides a rapid and efficient *in vitro* method for determination of intracellular ROS production, in contrast to other conventional assays (i.e. thiobarbituric acid assay, which measures the accumulation of the lipid peroxidation end product, malondialdehyde (MDA) (reviewed by Cathcart *et al*., 1983)). It is also considered to be highly sensitive, as it is able to detect picomoles (pM) of hydroperoxides (Cathcart *et al*., 1983).

The fluorescent signal was directly proportional to ROS production. Therefore, results were presented as the mean fluorescent signal unit (FSU) ± standard error of the mean (SEM). Fluorescence was measured every hour for a total of 5 hrs to determine if more pronounced ROS production would be observed with longer durations of exposure to 2,4-D.

4.3.1 The effects of H_2O_2 on ROS production in HepG2 and HEK293 cells

As illustrated in figures 13 and 14, ROS production is increased in HepG2 and HEK293 cells following exposure to H_2O_2. Statistically significant ($P<0.05$) increases in ROS production were observed at concentrations equal to and greater than 200 μM in HepG2 cells. Concentration-dependent increases in ROS production were observed in HepG2 cells. The observed increases in ROS production between successive concentrations (100 to 400 μM) of H_2O_2 were statistically significant ($P<0.05$) in HepG2 cells. Statistically significant ($P<0.05$) increases in ROS production were observed in HEK293 cells exposed to concentrations greater and equal to 100 μM.

Figure 13: Reactive oxygen species (ROS) production in HepG2 cells exposed to hydrogen peroxide (H_2O_2). Cells were exposed to 0 to 400µM of H_2O_2 for 20 min in phosphate buffered saline (PBS) solution. The production of ROS was determined using 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA). Fluorescence from a blank sample (PBS and 2',7'-DCFH-DA) was subtracted from all samples (untreated control and treated cell cultures). Results are expressed as the mean 2',7'-dichlorofluorescein (DCF) fluorescence ± standard error of the mean (SEM) from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant (P<0.05) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from from fluorescence of untreated control cell cultures were observed in HepG2 cells exposed to the following concentrations: 200µM: P = 0.0001. 300µM: P = 0.0001. 400µM: P = 0.0001. Statistical analysis was performed using one-way ANOVA, followed by a Tukey's honestly significant difference (HSD) test. Statistically significant (P<0.05) differences in the increases in ROS production between successive increasing exposure concentrations are denoted by the symbols a, b and c for the following comparisons: [a] 100µM vs. 200µM; [b] 200µM vs. 300µM; [c] 300µM vs. 400µM. Significant differences in the increases in ROS production between successive increasing concentrations were observed in HepG2 cells as follows: 200µM: [a] P = 0.009. 300µM: [b] P = 0.0001. 400µM: [c] P = 0.0001.

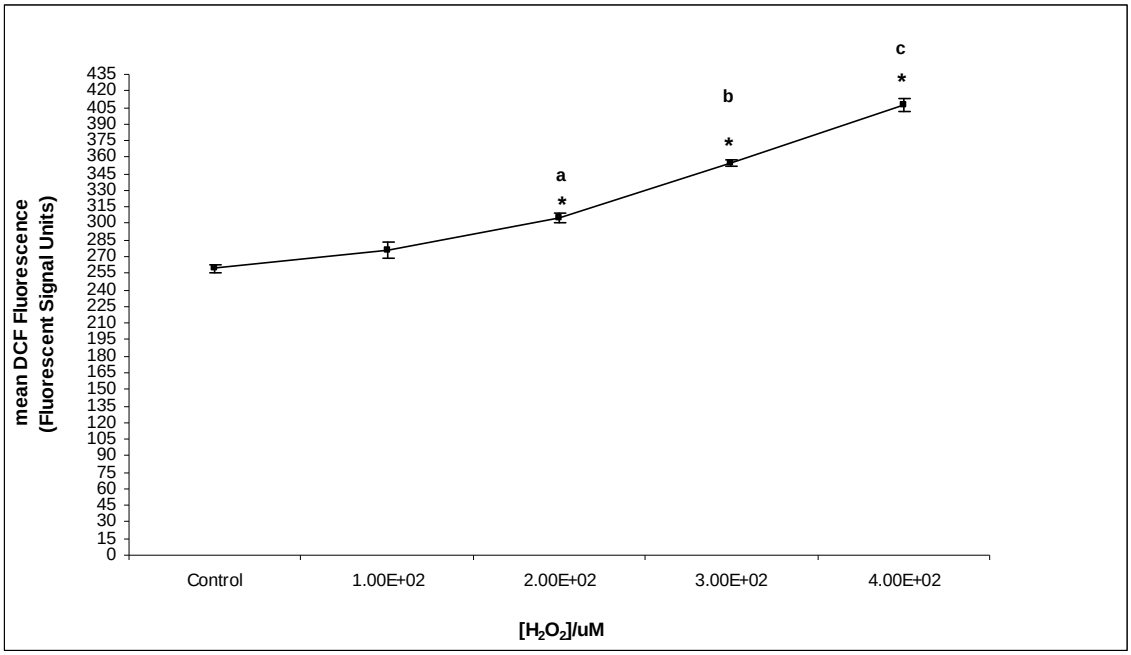
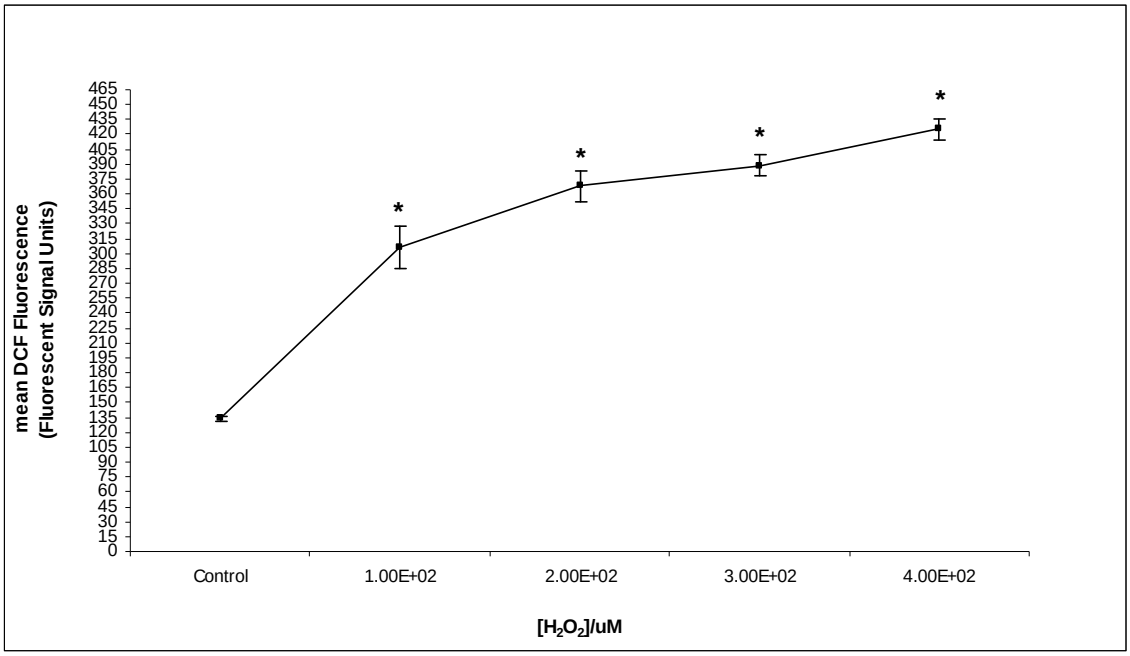

mean DCF Fluorescence
(Fluorescent Signal Units)
435
420
405
390
375
360
345
330
315
300
285
270
255
240
225
210
195
180
165
150
135
120
105
90
75
60
45
30
15
0
a
*
b
*
c
*
Control
1.00E+02
2.00E+02
3.00E+02
4.00E+02
[H2O2]/uM

Figure 14: Reactive oxygen species (ROS) production in HEK293 cells exposed to hydrogen peroxide (H_2O_2). Cells were exposed to 0 to 400μM of H_2O_2 for 20 min in phosphate buffered saline (PBS) solution. The production of ROS was determined using 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA). Fluorescence from a blank sample (PBS and 2',7'-DCFH-DA) was subtracted from all samples (untreated control and treated cell cultures). Results are expressed as the mean 2',7'-dichlorofluorescein (DCF) fluorescence ± standard error of the mean (SEM) from 6 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant (P<0.05) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HEK293 cells exposed to the following concentrations: 100μM: P = 0.0001. 200μM: P = 0.0001. 300μM: P = 0.0001. 400μM: P = 0.0001. Statistical analysis was performed using one-way ANOVA, followed by a Tukey's honestly significant difference (HSD) test. There were statistically insignificant (P < 0.05) differences in the increases in ROS production between successive increasing exposure concentrations in HEK293 cells.


mean DCF Fluorescence
(Fluorescent Signal Units)
465
450
435
420
405
390
375
360
345
330
315
300
285
270
255
240
225
210
195
180
165
150
135
120
105
90
75
60
45
30
15
0
*
*
*
*
Control
1.00E+02
2.00E+02
3.00E+02
4.00E+02
[H2O2]/uM

Increases in ROS production in HEK293 cells observed at concentrations equal to and greater than 200 μM were similar in magnitude to that observed at 100 μM.

4.3.2 The effects of increasing exposure time to PBS solution on ROS production in untreated HepG2 and HEK293 control cells

The ROS assays were performed in PBS solution. Figure 15 illustrates that in both HepG2 and HEK293 cells; ROS production increased with longer durations of exposure to PBS solution. The ROS production observed between 2 and 3 hrs, 3 and 4 hrs, 4 and 5 hrs, and 5 and 6 hrs in both HepG2 and HEK293 cells were statistically significant (P<0.05). At all exposure time points (2 to 6 hrs), ROS production in HEK293 cells was 50 to 70% statistically significant (P<0.05) less than that produced in HepG2 cells at all durations of exposure.

4.3.3 The effects of commercial 2,4-D on ROS production in cells

4.3.3.1 The effects of commercial 2,4-D on ROS production in HepG2 cells

Figure 16 illustrates that exposure to high concentrations of 2,4-D (100 μM and 1 mM) induce ROS production in HepG2 cells. Statistically significant (P<0.05) increases in ROS production were observed following exposure to 100μM and 1mM at all durations of exposure. Approximately twice the amount of ROS was produced in cells exposed to 1 mM, compared to 100 μM. The differences in ROS produced between 100 μM and 1 mM at all duration of exposure were statistically significant (P<0.05). The ROS production increased significantly (P<0.05) in cells exposed to 100 μM or 1 mM with successive exposure times (100 μM (2 to 3 hrs, 3 to 4hrs, 4 to 5hrs) and 1 mM (2 to 3 hrs, 3 to 4 hrs, 4 to 5 hrs, 5 to 6 hrs).

4.3.3.2 The effects of commercial 2,4-D on ROS production in HEK293 cells

Figure 17 illustrates that exposure to 2,4-D induces ROS production in exposed HEK293 cells. Statistically significant (P<0.05) increases in ROS production were only observed following exposure to concentrations equal to and greater than 1 μM for all

Figure 15: The effects of phosphate buffered saline (PBS) solution on reactive oxygen species (ROS) production in untreated HepG2 and HEK293 control cells. Cells were exposed to PBS for 1 hr. The HepG2 and HEK293 cells were not simultaneously exposed to PBS. The production of ROS was determined using 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA). Fluorescence from a blank sample (PBS and 2',7'-DCFH-DA) was subtracted from all samples (untreated control and treated cell cultures). Results are expressed as the mean 2',7'-dichlorofluorescein (DCF) fluorescence ± standard error of the mean (SEM) from 8 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) test. Statistically significant ($P<0.05$) differences between exposure periods are denoted by the symbols a, b, c and d for the following comparisons: [a] 2 hrs vs. 3 hrs; [b] 3 hrs vs. 4 hrs; [c] 4 hrs vs. 5 hrs; [d] 5 hrs vs. 6 hrs. Significant differences between exposure periods were observed in HepG2 and HEK293 cells, as follows: HepG2 cell cultures: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$; [d] $P = 0.0001$. HEK293 cell cultures: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$; [d] $P = 0.0001$. Statistical analysis was performed using unpaired student t-tests. Statistically significant ($P<0.05$) differences between HepG2 and HEK293 cells are denoted by the symbol **. Significant differences between HepG2 and HEK293 cells were observed at all durations of exposure, as follows: 2 hrs: ** $P = 0.0001$; 3 hrs: ** $P = 0.0001$; 4 hrs: ** $P = 0.0001$; 5 hrs: ** $P = 0.0001$; 6 hrs: ** $P = 0.0001$.

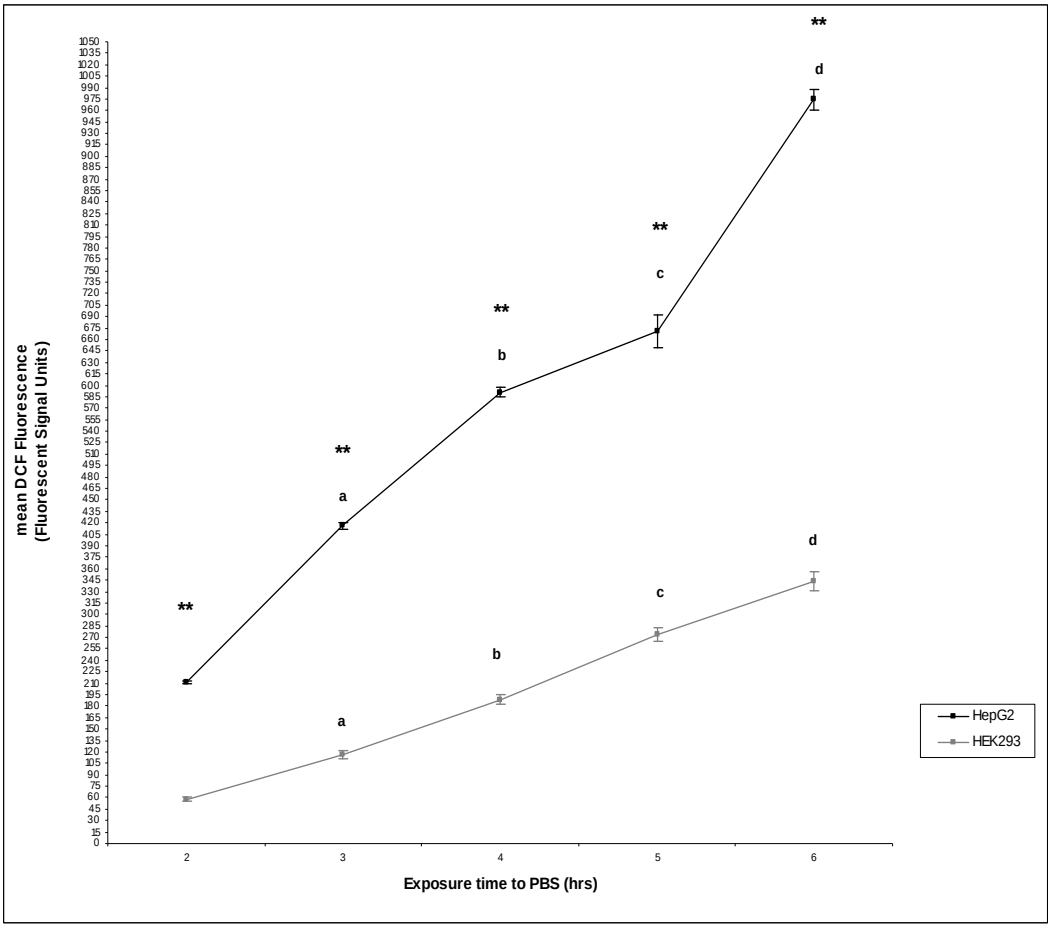

mean DCF Fluorescence
(Fluorescent Signal Units)
Exposure time to PBS (hrs)
HepG2
HEK293
**
a
b
c
d

Figure 16: Reactive oxygen species (ROS) production in HepG2 cells exposed to commercial 2,4-D. Cell were exposed to 0 to 1mM of 2,4-D in phosphate buffered saline (PBS) solution for 1 hr. The production of ROS was determined using 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA). Fluorescence from a blank sample (PBS and 2',7'-DCFH-DA) was subtracted from all samples (untreated control and treated cell cultures). Results are expressed as the mean 2',7'-dichlorofluorescein (DCF) fluorescence ± standard error of the mean (SEM) from 8 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant ($P<0.05$) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HepG2 cells exposed to the following concentrations: 100µM: 2 hrs: $P = 0.002$; 3 hrs: $P = 0.0001$; 4 hrs: $P = 0.002$; 5 hrs: $P = 0.0001$. 1mM: 2 hrs: $P = 0.0001$; 3 hrs: $P = 0.0001$; 4 hrs: $P = 0.002$; 5 hrs: $P = 0.0001$; 6 hrs: $P = 0.0001$. Statistically significant ($P<0.05$) differences in the increases in ROS production between successive increasing exposure concentrations (10µM to 1mM) are denoted by the symbols ^ and ** for the following comparisons: ^ 10µM vs. 100µM; ** 100µM vs. 1mM. Statistical analysis was performed using one-way ANOVA, followed by a Tukey's honestly significant difference (HSD) test. Significant differences in ROS production between successive increasing concentrations were observed in HepG2 cells, as follows: 2 hrs: ^ $P = 0.014$, ** $P = 0.0001$; 3 hrs: ^ $P = 0.0001$. ** $P = 0.0001$; ^ $P = 0.002$, ** $P = 0.0001$; ^ $P = 0.0001$, ** $P = 0.0001$; 6 hrs: ** $P = 0.0001$. Statistically significant ($P<0.05$) differences in the increases in ROS production between successive increasing exposure periods following exposure to 100µM and 1mM are denoted by the symbols a, b, c and d for the following comparisons: [a] 2 hrs vs. 3 hrs; [b] 3 hrs vs. 4 hrs; [c] 4 hrs vs. 5 hrs; [d] 5 hrs vs. 6 hrs. Statistical analysis was performed using one-way ANOVA, followed by a Tukey's HSD test. Significant differences in ROS production between successive increasing exposure periods were observed in HepG2 cells exposed to 100µM and 1mM as follows: 100µM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$. 1mM: [a] $P = 0.0001$; [b] $P = 0.0001$; [c] $P = 0.0001$; [d] $P = 0.0001$.

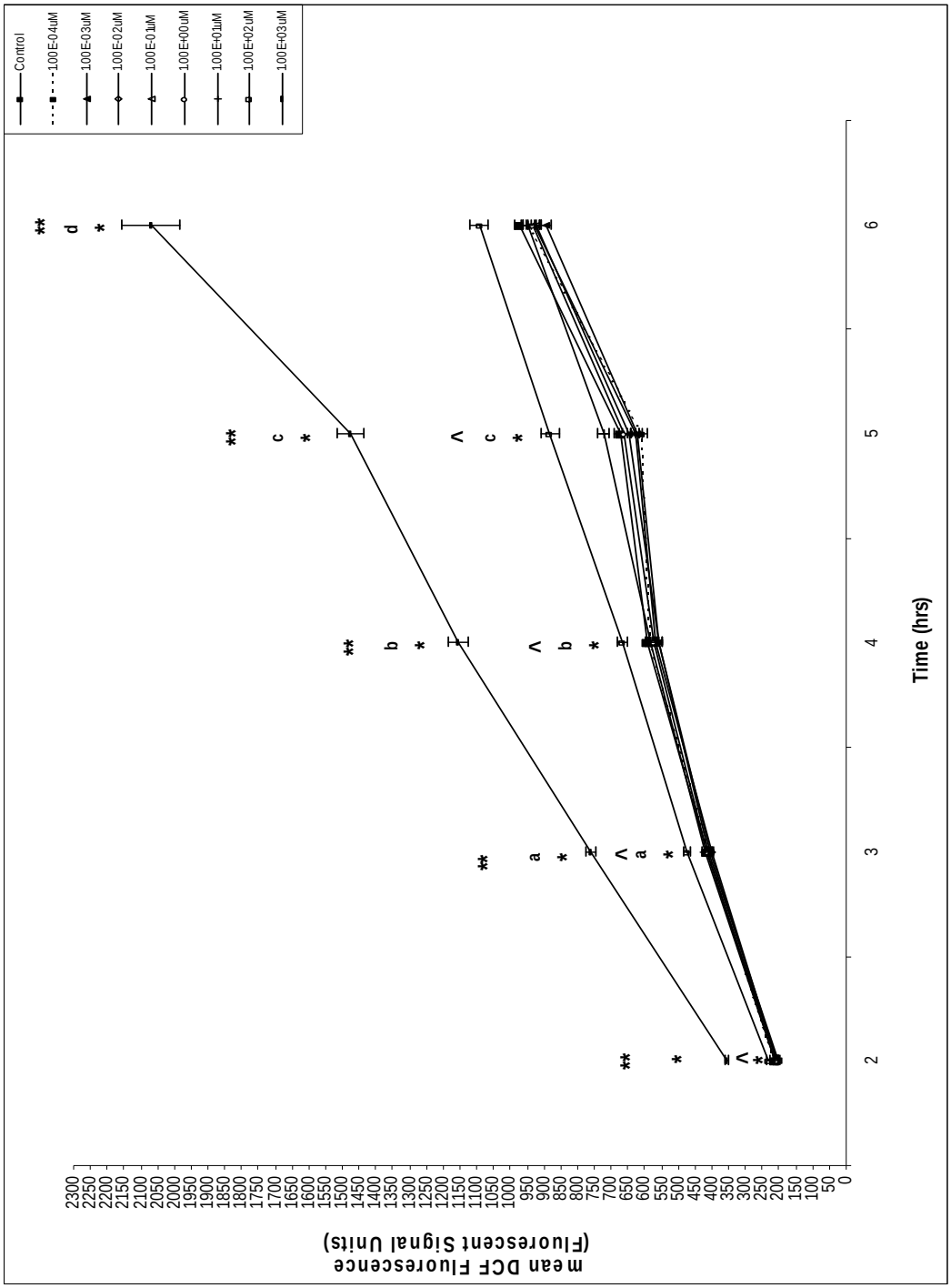
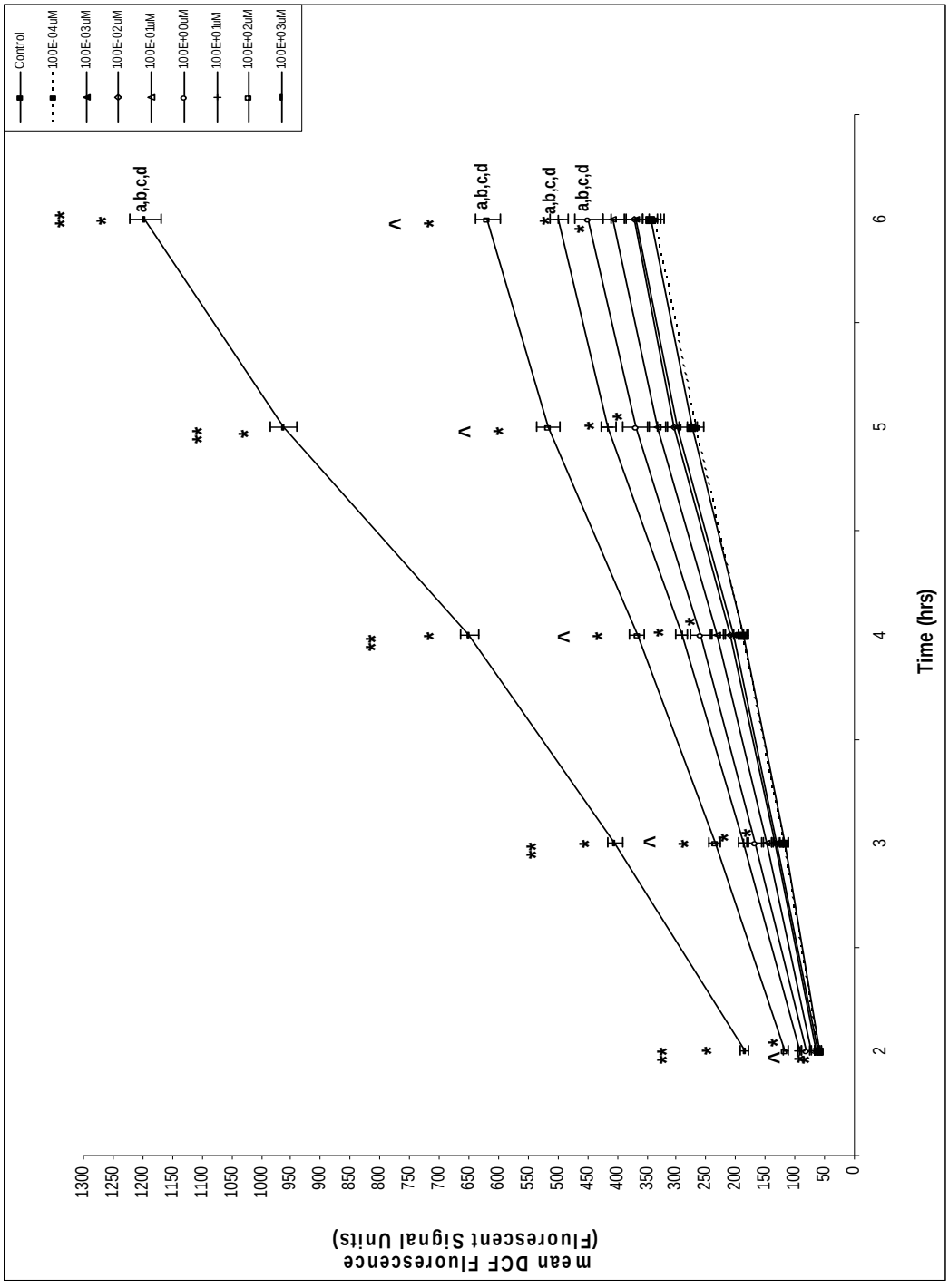

Control
100E-04uM
100E-03uM
100E-02uM
100E-01uM
100E+00uM
100E+01uM
100E+02uM
100E+03uM
mean DCF Fluorescence
(Fluorescent Signal Units)
Time (hrs)

Figure 17: Reactive oxygen species (ROS) production in HEK293 cells exposed to commercial 2,4-D. Cell were exposed to 0 to 1mM of 2,4-D in phosphate buffered saline (PBS) solution for 1 hr. The production of ROS was determined using 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA). Fluorescence from a blank sample (PBS and 2',7'-DCFH-DA) was subtracted from all samples (untreated control and treated cell cultures). Results are expressed as the mean 2',7'-dichlorofluorescein (DCF) fluorescence ± standard error of the mean (SEM) from 8 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by two-sided Dunnett's post-hoc test. Statistically significant ($P<0.05$) differences from fluorescence of untreated control cell cultures are denoted by the symbol *. Significant differences from fluorescence of untreated control cell cultures were observed in HEK293 cells exposed to the following concentrations: 1µM: 2 hrs: $P = 0.005$; 3 hrs: $P = 0.0001$; 4 hrs: $P = 0.0001$; 5 hrs: $P = 0.002$; 6 hrs: $P = 0.007$. 10µM: 2 hrs: $P = 0.0001$; 3 hrs: $P = 0.0001$; 4 hrs: $P = 0.0001$; 5 hrs: $P = 0.0001$; 6 hrs: $P = 0.0001$. 100µM: 2 hrs: $P = 0.0001$; 3 hrs: $P = 0.0001$; 4 hrs: $P = 0.0001$; 5 hrs; $P = 0.0001$; 6 hrs: $P = 0.0001$. 1mM: 2 hrs: $P = 0.0001$; 3 hrs: $P = 0.0001$; 4 hrs: $P = 0.0001$; 5 hrs: $P = 0.0001$; 6 hrs: $P = 0.0001$. Statistically significant ($P<0.05$) differences in the increases in ROS production between successive increasing exposure concentrations (1µM to 1mM) are denoted by the symbols ^ and ** for the following comparisons: ^ 10µM vs. 100µM; ** 100µM vs. 1mM. Statistical analysis was performed using one-way ANOVA, followed by a Tukey's honestly significant difference (HSD) test. Significant differences in ROS production between successive increasing concentrations were observed in HEK293 cells as follows: 2 hrs: ^ $P = 0.026$, ** $P = 0.0001$; 3 hrs: ^ $P = 0.005$, ** $P = 0.0001$; 4 hrs: ^ $P = 0.001$, ** $P = 0.0001$; 5 hrs: ^ $P = 0.005$, ** $P = 0.0001$; 6 hrs: ^ $P = 0.007$, ** $P = 0.0001$. Statistically significant ($P<0.05$) differences in the increases in ROS production between successive increasing exposure periods following exposure to 1µM to 1mM are denoted by the symbols a, b, c and d for the following comparisons. [a] 2 hrs vs. 3 hrs; [b] 3 hrs vs. 4 hrs; [c] 4 hrs vs. 5 hrs; [d] 5 hrs vs. 6 hrs. Statistical analysis was performed using one-way ANOVA, followed by a Tukey's HSD test. Significant differences in ROS production between successive increasing exposure periods were observed in HEK293 cells exposed to 1µM to 1mM, as follows: 1µM: [a] $P = 0.012$, [b] $P = 0.005$, [c] $P = 0.001$, [d] $P = 0.022$; 10µM: [a] $P = 0.0001$, [b] $P = 0.0001$, [c] $P = 0.0001$; [d] $P = 0.0001$; 100µM: [a] $P = 0.0001$, [b] $P = 0.0001$, [c] $P = 0.0001$, [d] $P = 0.0001$; 1mM: [a] $P = 0.0001$, [b] $P = 0.0001$, [c] $P = 0.006$ hrs: [d] $P = 0.0001$.


Control
100E-04uM
100E-03uM
100E-02uM
100E-01uM
100E+00uM
100E+01uM
100E+02uM
100E+03uM
mean DCF Fluorescence
(Fluorescent Signal Units)
Time (hrs)
a,b,c,d

durations of exposure. Furthermore, although ROS production appeared to increase with increasing concentrations of 2,4-D (1 μM to 1 mM); significant (P<0.05) differences in ROS production were only observed between 10 and 100 μM, and 100 μM and 1 mM. Exposure to 1 μM to 1 mM induced more pronounced increases in ROS production with longer durations of exposure. The effects were statistically significant (P<0.05).

4.3.3.3 Comparison of the effects of commercial 2,4-D on ROS production in HepG2 and HEK293 cells

Figure 18 illustrates that at all concentrations (0 to 1 mM) and times (2 to 6 hrs), 50 to 70% statistically significant (P<0.05) less ROS was produced in HEK293, compared to HepG2 cells.

4.4 The effects of commercial 2,4-D on mRNA expression in cells

The RT-PCR assays were performed to determine if exposure to environmental concentrations of 2,4-D would induce up-regulation of FTL, FTH1 and PCNA gene expression at the level of mRNA accumulation. In contrast to conventional Northern Blot assays, RT-PCR assays provide a rapid, efficient and sensitive method for determination of mRNA accumulation levels.

Target mRNA expression levels in each sample was calculated relative to the expression in control cell cultures, normalized to the expression of the reference gene, GAPDH.

4.4.1 The effects of commercial 2,4-D on FTL mRNA expression in cells

4.4.1.1 The effects of commercial 2,4-D on FTL mRNA expression in HepG2 cells

Figure 19 illustrates that exposure to 2,4-D induces alterations (< 2 fold) in FTL mRNA expression. A consistent pattern in mRNA expression was not observed over the concentrations tested. The FTL mRNA expression was repressed in cells exposed to concentrations less than 10 μM. The greatest degree of repression (0.19 fold) of mRNA expression was observed in cells exposed to 100 nM. Induction of FTL mRNA expression was observed in cells exposed to concentrations equal to and greater than

Figure 18: Comparison of reactive oxygen species (ROS) production in HepG2 and HEK293 cells exposed to commercial 2,4-D. Cells were exposed to 0 to 1mM of 2,4-D in phosphate buffered saline (PBS) solution for 1 hr. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. The production of ROS in HepG2 and HEK293 cells was determined using 2',7'-dichlorofluorescin diacetate (2',7'-DCFH-DA). Fluorescence from a blank sample (PBS and 2',7'-DCFH-DA) was subtracted from all samples (untreated control and treated cell cultures). Results are expressed as the mean 2',7'-dichlorofluorescein (DCF) fluorescence ± standard error of the mean (SEM) from 8 independent experiments. Statistically significant ($P<0.05$) differences between HepG2 and HEK293 cells are denoted by the symbol *. Significant differences between HepG2 and HEK293 cells were observed upon exposure to 0 to 1mM at all durations of exposure, as follows: control: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs: $P = 0.0001$, 6 hrs: $P = 0.0001$; 0.1nM: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs: $P = 0.0001$, 6 hrs: $P = 0.0001$; 1nM: 2hrs: $P = 0.0001$, 3hrs: $P = 0.0001$, 4hrs: $P = 0.0001$, 5hrs; $P = 0.0001$, 6hrs: $P = 0.0001$; 10nM: 2hrs: $P = 0.0001$, 3hrs: $P = 0.0001$, 4hrs: $P = 0.0001$, 5hrs: $P = 0.0001$, 6hrs: $P = 0.0001$; 100nM: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs: $P = 0.0001$, 6 hrs: $P = 0.0001$; 1μM: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs: $P = 0.0001$, 6 hrs: $P = 0.0001$; 10μM: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs; $P = 0.0001$, 6 hrs: $P = 0.0001$; 100μM: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs: $P = 0.0001$, 6 hrs: $P = 0.0001$; 1mM: 2 hrs: $P = 0.0001$, 3 hrs: $P = 0.0001$, 4 hrs: $P = 0.0001$, 5 hrs: $P = 0.0001$, 6 hrs: $P = 0.0001$.

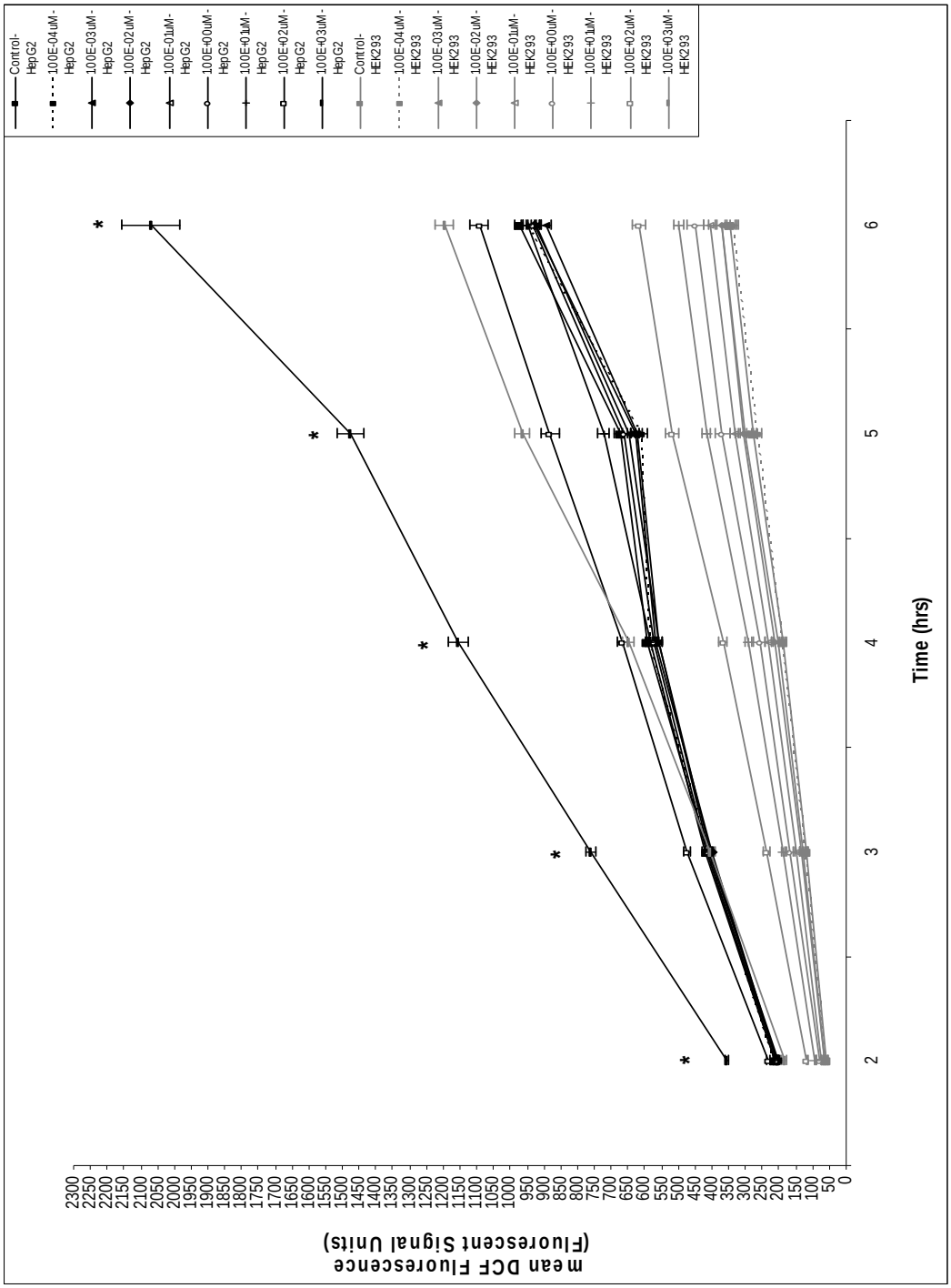
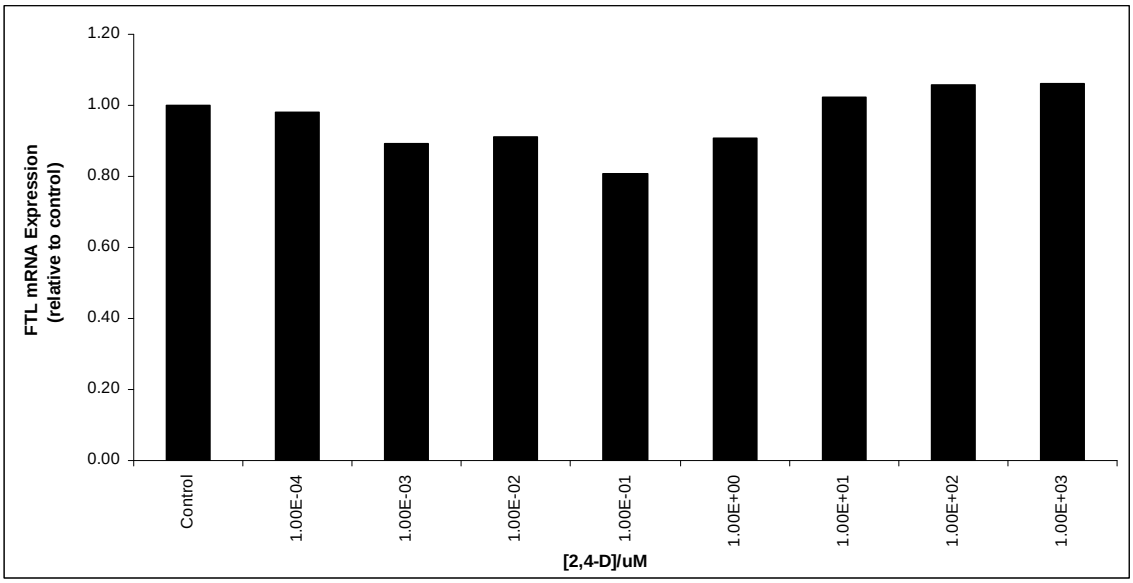

mean DCF Fluorescence
(Fluorescent Signal Units)
Time (hrs)
Control-
HepG2
100E-04uM -
HepG2
100E-03uM -
HepG2
100E-02uM -
HepG2
100E-01uM-
HepG2
100E+00uM -
HepG2
100E+01uM -
HepG2
100E+02uM -
HepG2
100E+03uM -
HepG2
Control-
HEK293
100E-04uM -
HEK293
100E-03uM -
HEK293
100E-02uM -
HEK293
100E-01uM-
HEK293
100E+00uM -
HEK293
100E+01uM -
HEK293
100E+02uM -
HEK293
100E+03uM -
HEK293

Figure 19: The effects of commercial 2,4-D on ferritin light polypeptide (FTL) mRNA expression in exposed HepG2 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).


FTL mRNA Expression
(relative to control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

10 μM. The highest magnitude of induction (1.06 fold) of FTL mRNA expression was observed in cells exposed to 100 μM or 1 mM.

4.4.1.2 The effects of commercial 2,4-D on FTL mRNA expression in HEK293 cells

Figure 20 illustrates that exposure to 2,4-D induces alterations in FTL mRNA expression. A consistent pattern in mRNA expression was not observed over the concentrations tested. The FTL mRNA expression was repressed in cells exposed to 1 nM, 100 nM and 10 μM. The highest degree of repression (0.22 fold) of mRNA expression was observed in cells exposed to 1 nM. Induction of FTL mRNA expression was observed in cells exposed to 0.1 nM, 10 nM, 1 μM, 100 μM and 1 mM. The highest degree of induction (4.33 fold) of mRNA expression was observed in cells exposed to 1 mM.

4.4.1.3 Comparison of the effects of commercial 2,4-D on FTL mRNA expression in HepG2 and HEK293 cells

Figure 21 illustrates that 2,4-D induces different effects on FTL mRNA expression in HepG2 and HEK293 cells. A consistent pattern in mRNA expression in HepG2 or HEK293 cells was not observed over the concentrations tested. With the exception of HEK293 cells exposed to 1 mM, 2,4-D induced less than 2 fold alterations in FTL mRNA expression in both HepG2 and HEK293 cells.

4.4.2 The effects of commercial 2,4-D on FTH1 mRNA expression in cells

4.4.2.1 The effects of commercial 2,4-D on FTH1 mRNA expression in HepG2 cells

Figure 22 illustrates that 2,4-D induces less than 2 fold alterations (increases and decreases) in FTH1 mRNA expression at concentrations above 0.1 nM. FTH1 mRNA expression was repressed (0.11 fold) in cells exposed to 1 nM. FTH1 mRNA expression was induced by 1.28 fold (average) in cells exposed to concentrations equal to and greater than 10 nM. A 1.58 fold increase induction of mRNA expression was observed in cells exposed to 100 μM.

Figure 20: The effects of commercial 2,4-D on ferritin light polypeptide (FTL) mRNA expression in exposed HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).

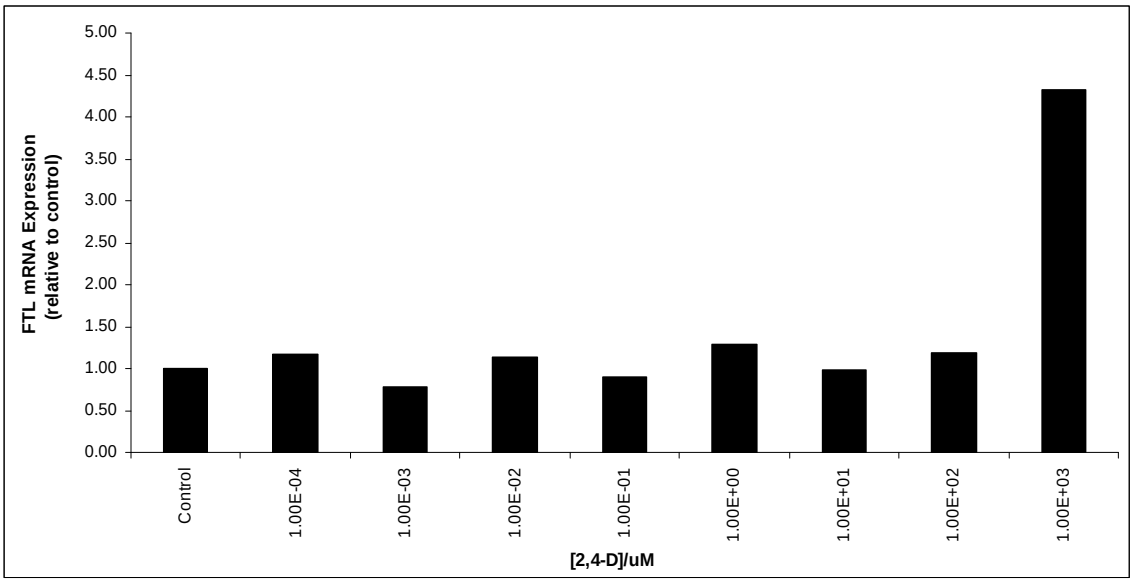

FTL mRNA Expression
(relative to control)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 21: Comparison of the effects of commercial 2,4-D on ferritin light polypeptide (FTL) mRNA expression in exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).

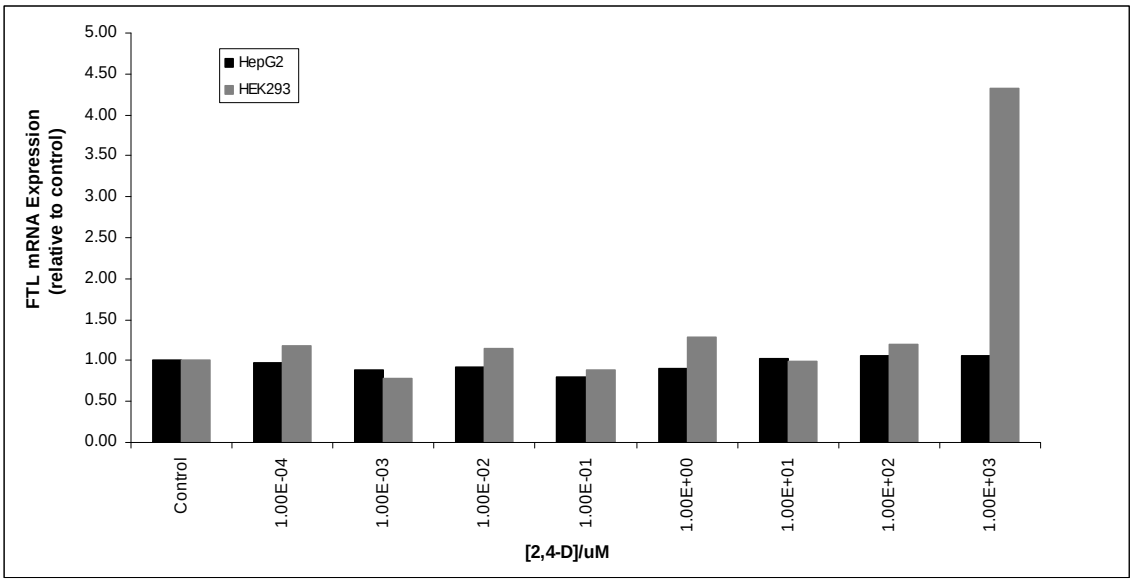
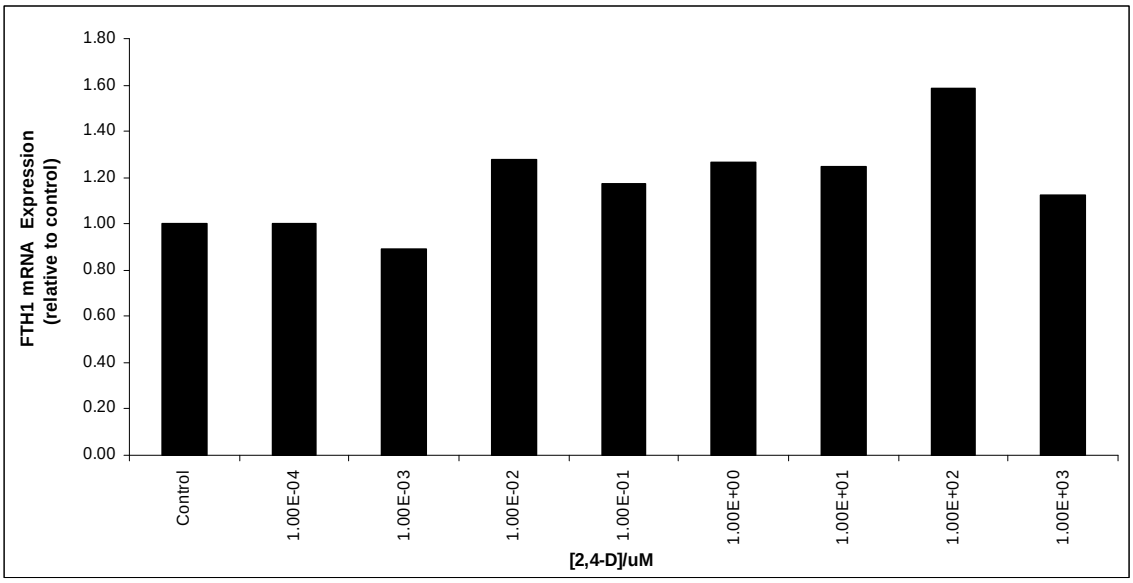

FTL mRNA Expression (relative to control)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
HepG2
HEK293
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 22: The effects of commercial 2,4-D on ferritin heavy polypeptide (FTH1) mRNA expression in exposed HepG2 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).


FTH1 mRNA Expression
(relative to control)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

4.4.2.2 The effects of commercial 2,4-D on FTH1 mRNA expression in HEK293 cells

Figure 23 illustrates that exposure to 2,4-D induces alterations (< 2 fold) FTH1 mRNA expression. A consistent pattern in mRNA expression was not observed over the concentrations tested. The FTH1 mRNA expression was repressed in cells exposed to concentrations less than 1 mM. The greatest degree of repression (0.55 fold) of mRNA expression was observed in cells exposed to 10 μM. Exposure to 1 mM induced (1.89 fold) FTH1 mRNA expression.

4.4.2.3 Comparison of the effects of commercial 2,4-D on FTH1 mRNA expression in HepG2 and HEK293 cells

Figure 24 illustrates that 2,4-D induces different effects on FTH1 mRNA expression in HepG2 and HEK293 cells. The magnitudes of alterations in mRNA expression in HepG2 and HEK293 cells were less than 2 fold. A consistent pattern in changes in mRNA expression in HepG2 or HEK293 cells was not observed over the concentrations tested.

4.4.3 The effects of commercial 2,4-D on PCNA mRNA expression in cells

4.4.3.1 The effects of commercial 2,4-D on PCNA mRNA expression in HepG2 cells

Figure 25 illustrates that exposure to 2,4-D alters (< 2 fold) PCNA mRNA expression. A consistent pattern in mRNA expression was not observed over the concentrations tested. The PCNA mRNA expression was induced in cells exposed to 0.1 nM to 100 μM. The highest degree of induction (1.79 fold) in mRNA expression was observed in cells exposed to 100 μM. Exposure to 1 mM repressed (0.47 fold) PCNA mRNA expression.

4.4.3.2 The effects of commercial 2,4-D on PCNA mRNA expression in HEK293 cells

Figure 26 illustrates that exposure to 2,4-D induces changes (< 2 fold) in PCNA mRNA expression. A consistent pattern in mRNA expression was not observed over the

Figure 23: The effects of commercial 2,4-D on ferritin heavy polypeptide (FTH1) mRNA expression in exposed HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).

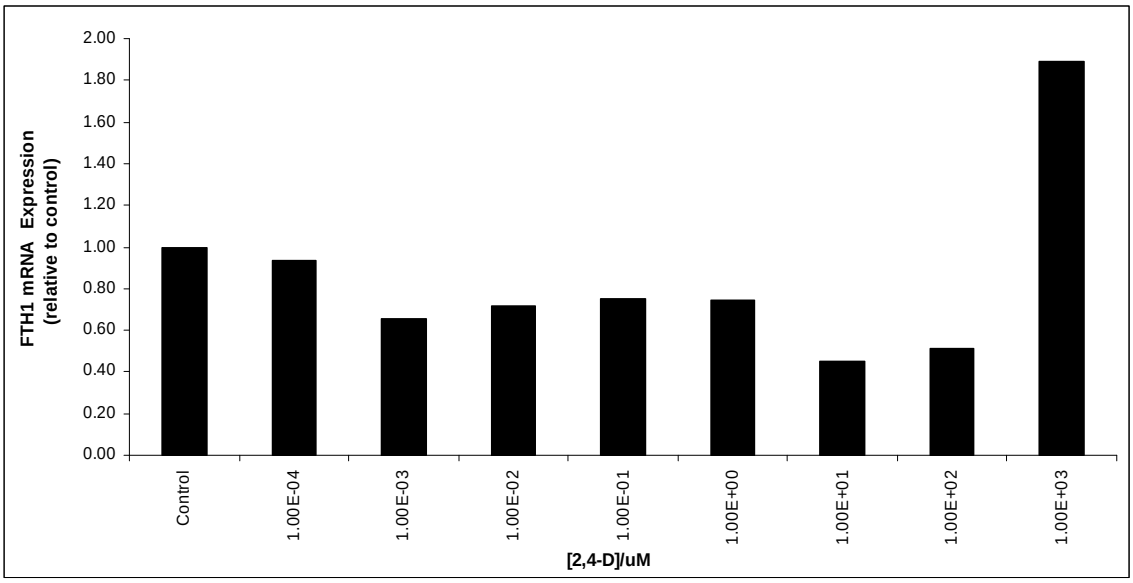

FTH1 mRNA Expression
(relative to control)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 24: Comparison of the effects of commercial 2,4-D on ferritin heavy polypeptide (FTH1) mRNA expression in exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).

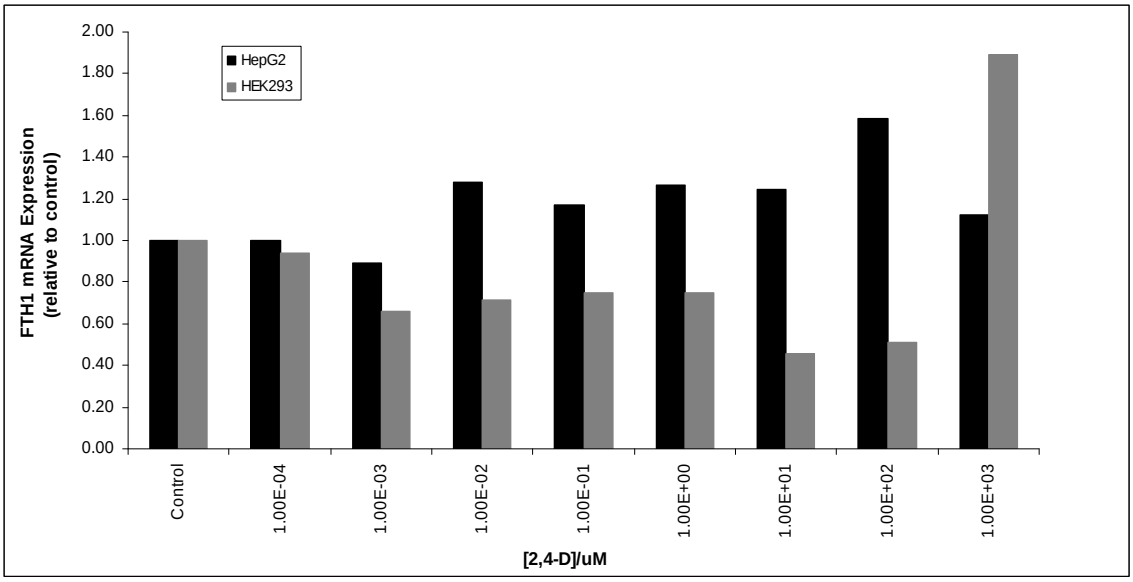

FTH1 mRNA Expression (relative to control)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
HepG2
HEK293
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 25: The effects of commercial 2,4-D on proliferating cell nuclear antigen (PCNA) mRNA expression in exposed HepG2 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).

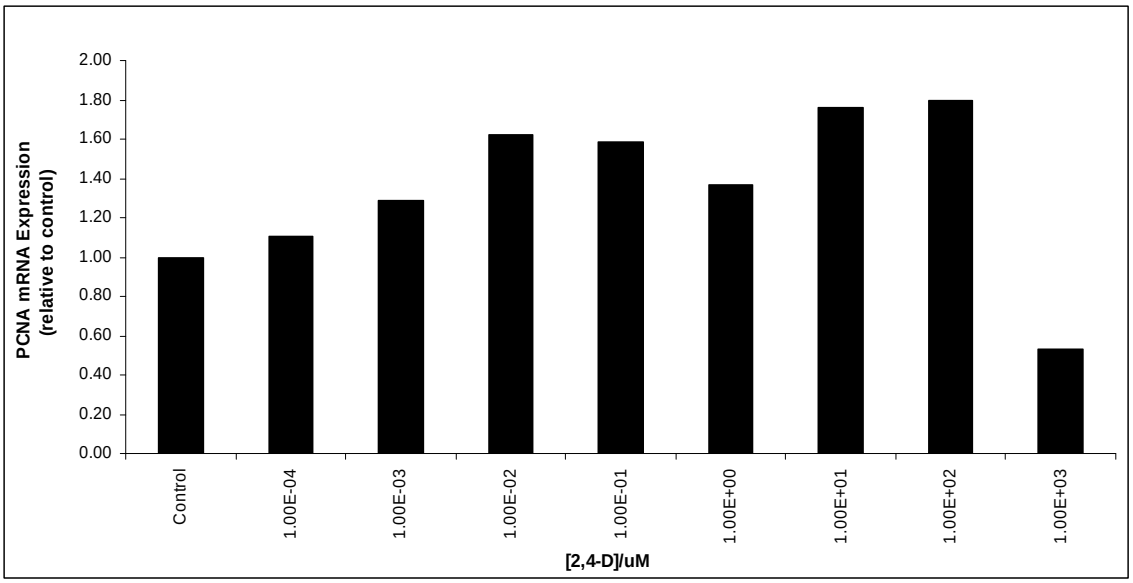
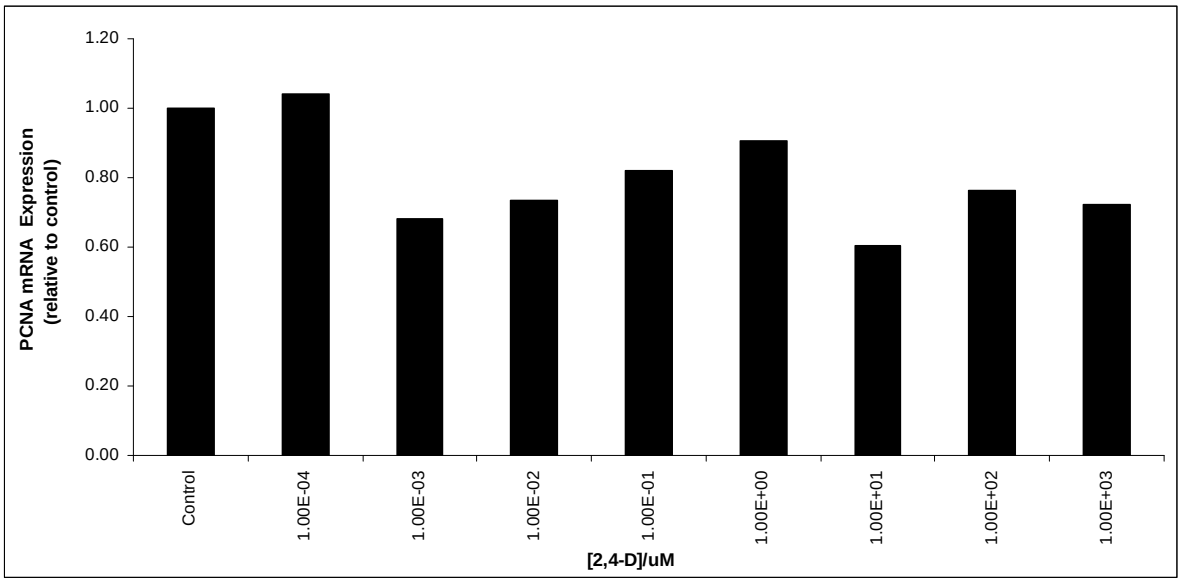

PCNA mRNA Expression
(relative to control)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 26: The effects of commercial 2,4-D on proliferating cell nuclear antigen (PCNA) mRNA expression in exposed HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).


PCNA mRNA Expression
(relative to control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

concentrations tested. The PCNA mRNA expression was induced (1.04 fold) in cells exposed to 0.1 nM. Repression of PCNA mRNA expression was observed in cells exposed to 1 nM to 1 mM. The highest degree of repression (0.40 fold decrease) of PCNA mRNA expression was observed in cells exposed to 10 μM.

4.4.3.3 Comparison of the effects of commercial 2,4-D on PCNA mRNA expression in HepG2 and HEK293 cells

Figure 27 illustrates that 2,4-D induces different effects on PCNA mRNA expression in HepG2 and HEK293 cells. The magnitudes of alterations in mRNA expression in HepG2 and HEK293 cells were less than 2 fold. A consistent pattern in mRNA expression in HepG2 or HEK293 cells was not observed over the concentrations tested.

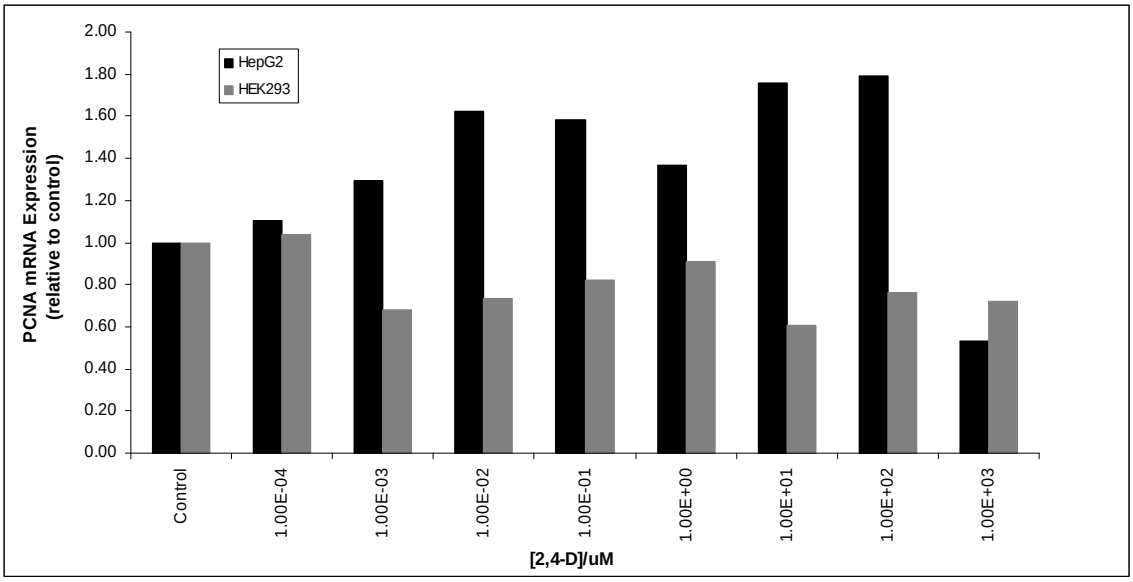
4.5 The effects of commercial 2,4-D on protein expression in cells

Western blot assays were performed to determine if exposure to environmental concentrations of 2,4-D would induce up-regulation of FTL, FTH1 and PCNA gene expression at the level of protein accumulation. The expression of Hsp72, which is involved in cellular stress response (reviewed by Aufricht, 2005), was used as an additional marker of the potential induction of intracellular oxidative stress following 2,4-D exposure. Western blot assays are the conventional method used for detection of protein expression levels in cell and molecular research.

Horse spleen ferritin was used as a positive control in western blot assays to assess the efficacy of the anti-ferritin antibody. The anti-ferritin antibody used in this study was anticipated to recognize both FTL and FTH1 polypeptide subunits. However, only one subunit band was detected; either the FTL or FTH1 subunit. Thus, the protein band that was detected was referred to as ferritin.

Target protein expression level in each sample was calculated relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

Figure 27: Comparison of the effects of commercial 2,4-D on proliferating cell nuclear antigen (PCNA) mRNA expression in exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D in complete growth media for 24 hrs. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cells were harvested and RNA extracted. Single-stranded (SS) cDNA was produced using first strand synthesis; subsequently real time polymerase chain reaction assay was performed. Target mRNA expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH).


PCNA mRNA Expression
(relative to control)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
HepG2
HEK293
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

4.5.1 The effects of commercial 2,4-D on ferritin protein expression in cells

4.5.1.1 The effects of commercial 2,4-D on ferritin protein expression in HepG2 cells

Figure 28 (A) and (B) illustrates that exposure to 2,4-D induces ferritin protein expression in HepG2 cell cultures. The effect was not concentration-dependent. The highest degree of induction (2.09 fold) of ferritin protein expression was observed in cells exposed to 10 μM.

4.5.1.2 The effects of commercial 2,4-D on ferritin protein expression in HEK293 cells

Figure 29 (A) and (B) illustrates that exposure to 2,4-D alters (< 2 fold) ferritin protein expression. A consistent pattern in protein expression was not observed. Ferritin protein expression was repressed in cells exposed to 0.1 to 10 nM, 1 μM to 1 mM. The greatest degree of repression (0.86 fold) of protein expression was observed in cells exposed to 1 mM. Exposure to 100 nM induced a 1.13 fold increase in ferritin protein expression.

4.5.1.3 Comparison of the effects of commercial 2,4-D on ferritin protein expression in HepG2 and HEK293 cells

Figure 30 illustrates that 2,4-D elicits different effects on ferritin protein expression in HepG2 and HEK293 cell cultures. Induction of ferritin protein expression was observed in HepG2 cells over the concentrations tested. In contrast, inconsistent changes in protein expression were observed in HEK293 cells exposed to 0.1 nM to 1 mM. Exposure to 2,4-D induced less than 2 fold alterations in protein expression in HEK293, compared to HepG2 cells.

4.5.2 The effects of commercial 2,4-D on PCNA protein expression in cells

4.5.2.1 The effects of commercial 2,4-D on PCNA protein expression in HepG2 cells

Figure 31 (A) and (B) illustrates that exposure to 2,4-D alters (< 2 fold) PCNA protein expression. A consistent pattern in protein expression was not observed over the

Figure 28: The effects of commercial 2,4-D on ferritin protein expression in exposed HepG2 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. Cells were harvested and protein lysates obtained. Thirty µg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Ferritin protein expression was detected using anti-ferritin antibody (rabbit polyclonal to ferritin, catalog number: ab7332; Novus Biologicals). The ferritin protein band detected may have been either FTL (19kDa) or FTH1 (21kDa). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). A representative western blot **(A)** of 3 replicates from one independent experiment is shown. Results of densitometric analysis **(B)** are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

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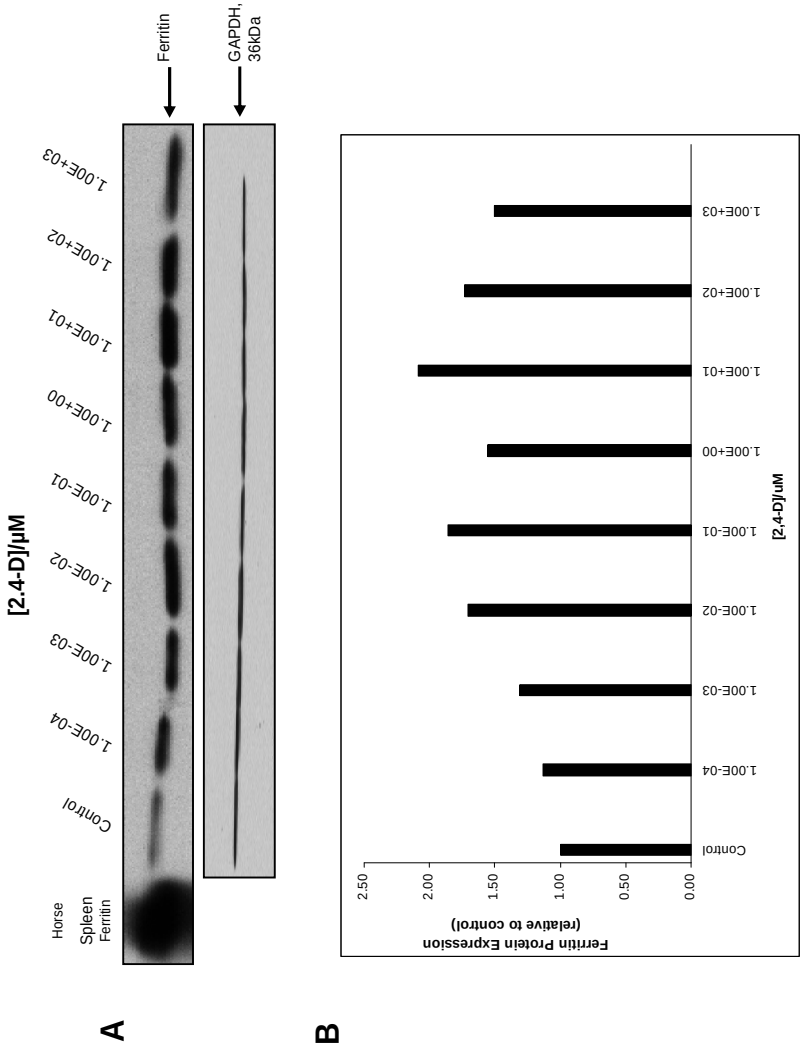

A
Ferritin
GAPDH, 36kDa
[2.4-D]/µM
Horse Spleen Ferritin
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
B
Ferritin Protein Expression (relative to control)
2.50
2.00
1.50
1.00
0.50
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2.4-D]/µM

Figure 29: The effects of commercial 2,4-D on ferritin protein expression in exposed HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. Cells were harvested and protein lysates obtained. Thirty μg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Ferritin protein expression was detected using anti-ferritin antibody (rabbit polyclonal to ferritin, catalog number: ab7332; Novus Biologicals). The ferritin protein band detected may have been either FTL (19kDa) or FTH1 (21kDa). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). A representative western blot **(A)** of 3 replicates from one independent experiment is shown. Results of densitometric analysis **(B)** are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

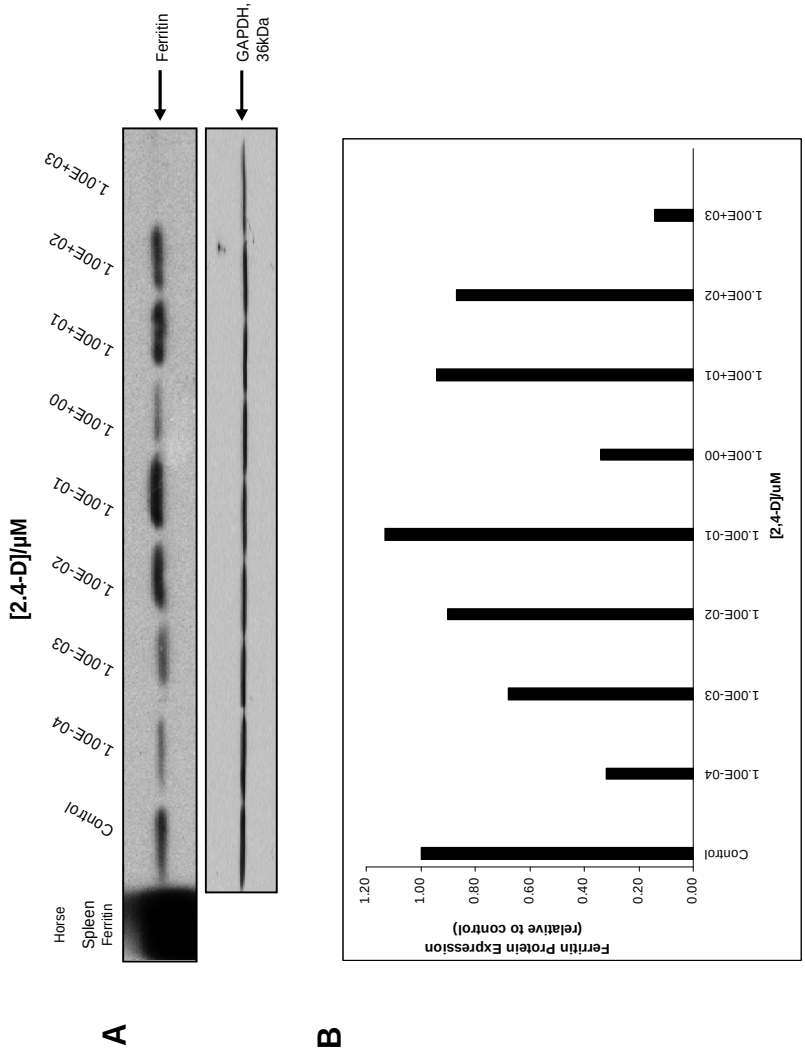

A
Ferritin
GAPDH,
36kDa
[2.4-D]/µM
Horse
Spleen
Ferritin
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
B
Ferritin Protein Expression
(relative to control)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
[2,4-D]uM

Figure 30: Comparison of the effects of commercial 2,4-D on ferritin protein expression in exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cells were harvested and protein lysates obtained. Thirty μg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Ferritin protein expression was detected using anti-ferritin antibody (rabbit polyclonal to ferritin, catalog number: ab7332; Novus Biologicals). The ferritin protein band detected may have been either FTL (19kDa) or FTH1 (21kDa). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). Results of densitometric analysis are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

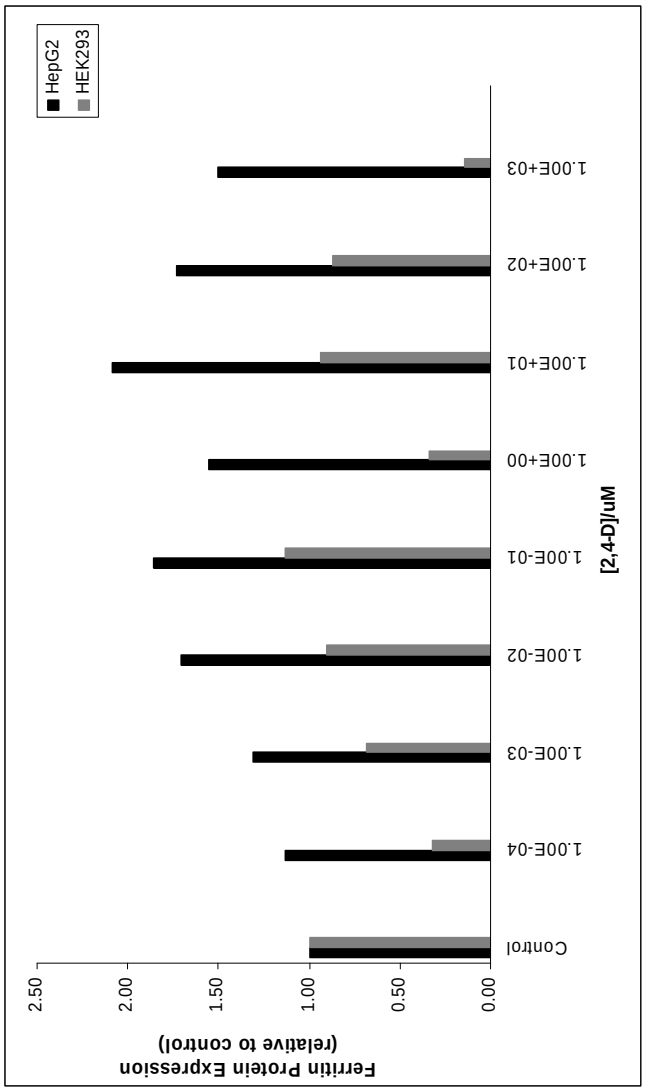
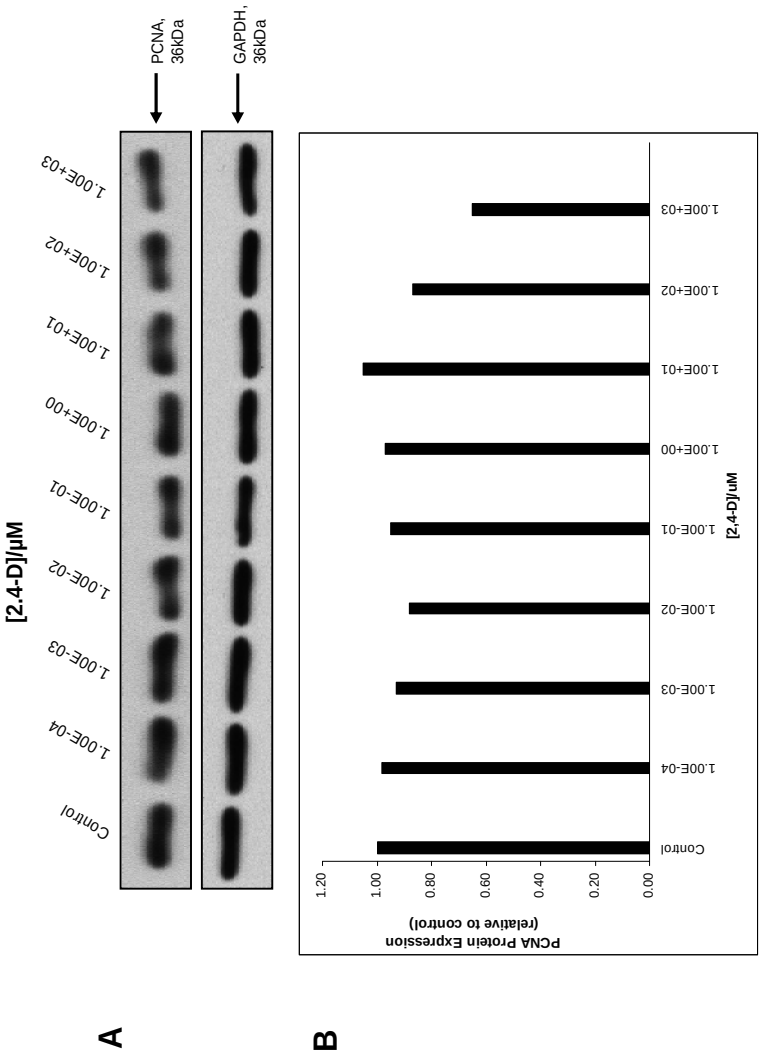

HepG2
HEK293
Ferritin Protein Expression
(relative to control)
2.50
2.00
1.50
1.00
0.50
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 31: The effects of commercial 2,4-D on proliferating cell nuclear antigen (PCNA) protein expression in exposed HepG2 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. Cells were harvested and protein lysates obtained. Ten μg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The protein expression of PCNA was detected using anti-PCNA antibody (mouse monoclonal to PCNA, catalog number: SC-56, Santa Cruz Biotechnologies, Inc.). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). A representative western blot **(A)** of 3 replicates from one independent experiment is shown. Results of densitometric analysis **(B)** are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.


A
PCNA, 36kDa
GAPDH, 36kDa
[2.4-D]/µM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
B
PCNA Protein Expression (relative to control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2.4-D]/uM

concentrations tested. The PCNA protein expression was repressed in cells exposed to 0.1 to 100 nM, 1 μM, 100 μM and 1 mM. Exposure to 1mM induced the greatest degree of repression (0.35 fold) of PCNA protein expression. Exposure to 10 μM induced (1.05 fold) protein expression.

4.5.2.2 The effects of commercial 2,4-D on PCNA protein expression in HEK293 cells

Figure 32 (A) and (B) illustrates that exposure to 2,4-D induces (< 2 fold) PCNA protein expression. Exposure to 1 mM induced the greatest degree of induction (1.90 fold) of PCNA protein expression.

4.5.2.3 Comparison of the effects of commercial 2,4-D on PCNA protein expression in HepG2 and HEK293 cells

Figure 33 illustrates that exposure to 2,4-D elicits different effects on PCNA protein expression in HepG2 and HEK293 cell cultures. The magnitudes of alterations in protein expression were less than 2 fold in HepG2 and HEK293 cells. Induction of PCNA protein expression was observed in HEK293 cells over the concentrations tested. In contrast, inconsistent changes in protein expression were observed in HepG2 cells exposed to 0.1 nM to 1 mM.

4.5.3 The effects of commercial 2,4-D on Hsp72 protein expression in cells

4.5.3.1 The effects of commercial 2,4-D on Hsp72 protein expression in HepG2 cells

Figure 34 (A) and (B) illustrates exposure to 2,4-D induces (< 2 fold) Hsp72 protein expression. Exposure to 1 mM induced the greatest degree of induction (1.45 fold) of Hsp72 protein expression.

Figure 32: The effects of commercial 2,4-D on proliferating cell nuclear antigen (PCNA) protein expression in exposed HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. Cells were harvested and protein lysates obtained. Ten µg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The protein expression of PCNA was detected using anti-PCNA antibody (mouse monoclonal to PCNA, catalog number: SC-56, Santa Cruz Biotechnologies, Inc.). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). A representative western blot **(A)** of 3 replicates from one independent experiment is shown. Results of densitometric analysis **(B)** are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

A
PCNA, 36kDa
GAPDH, 36kDa
[2.4-D]/μM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
B
PCNA Protein Expression (relative to control)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 33: Comparison of the effects of commercial 2,4-D on proliferating cell nuclear antigen (PCNA) protein expression in exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cells were harvested and protein lysates obtained. Ten µg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The protein expression of PCNA was detected using anti-PCNA antibody (mouse monoclonal to PCNA, catalog number: SC-56, Santa Cruz Biotechnologies, Inc.). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). Results of densitometric analysis are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

HepG2
HEK293
PCNA Protein Expression
(relative to control)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

Figure 34: The effects of commercial 2,4-D on the inducible form of heat shock protein (Hsp72) protein expression in exposed HepG2 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. Cells were harvested and protein lysates obtained. Thirty µg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The protein expression of Hsp72 was detected using anti-Hsp72 antibody (rabbit polyclonal to Hsp70 (Hsp72), catalog number: SPA-812, Stressgen Bioreagents). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). A representative western blot **(A)** of 3 replicates from one independent experiment is shown. Results of densitometric analysis **(B)** are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

A
Hsp72, 70kDa
GAPDH, 36kDa
[2.4-D]/µM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
B
Hsp72 Protein Expression (relative to control)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

4.5.3.2 The effects of commercial 2,4-D on Hsp72 protein expression in HEK293 cells

Figure 35 (A) and (B) illustrates that 2,4-D alters (< 2 fold) Hsp72 protein expression. A consistent pattern in protein expression was not observed over the concentrations tested. The protein expression of Hsp72 was repressed in cells exposed to 0.1, 10 and 100 nM. The greatest magnitude of repression (0.06 fold) of protein expression was observed in cells exposed to 0.1 nM. Induction of Hsp72 protein expression was observed in cells exposed to 1 nM, 1 μM to 1 mM. The greatest magnitude of induction (1.09 fold) of protein expression was observed in cells exposed to 1 mM.

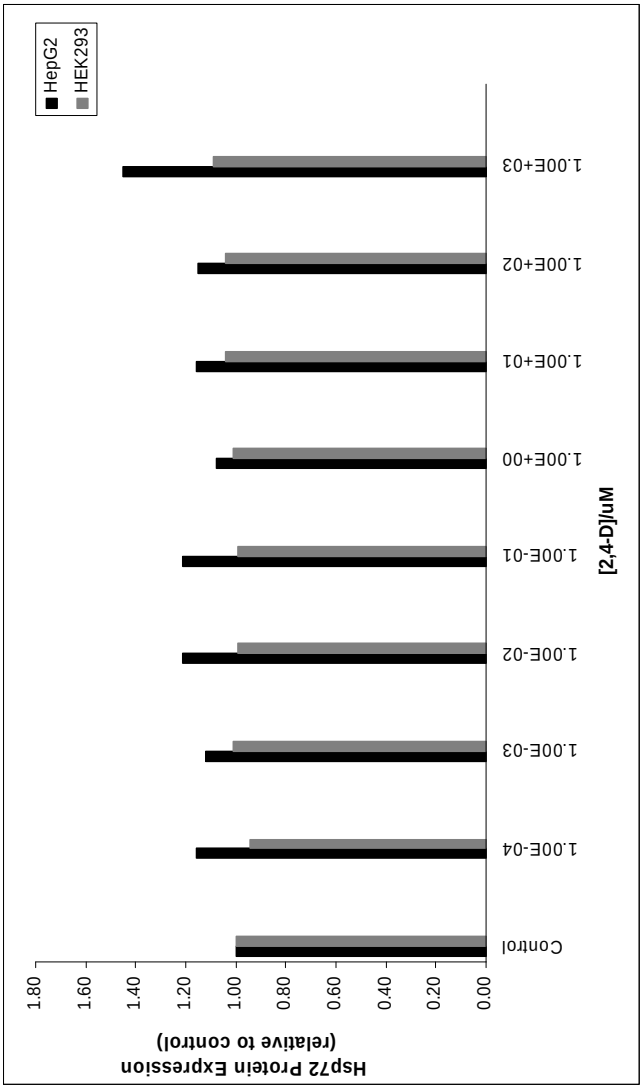
4.5.3.3 Comparison of the effects of commercial 2,4-D on Hsp72 protein expression in HepG2 and HEK293 cells

Figure 36 illustrates that 2,4-D elicits different effects on Hsp72 protein expression in HepG2 and HEK293 cell cultures. The magnitudes of alterations in protein expression were less than 2 fold in HepG2 and HEK293 cells. Induction of Hsp72 protein expression was observed in HepG2 cells over the concentrations tested. In contrast, inconsistent changes in protein expression were observed in HEK293 cells exposed to 0.1 nM to 1 mM.

Figure 35: The effects of commercial 2,4-D on the inducible form of heat shock protein (Hsp72) protein expression in exposed HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. Cells were harvested and protein lysates obtained. Thirty µg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The protein expression of Hsp72 was detected using anti-Hsp72 antibody (rabbit polyclonal to Hsp70 (Hsp72), catalog number: SPA-812, Stessgen Bioreagents). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). A representative western blot **(A)** of 3 replicates from one independent experiment is shown. Results of densitometric analysis **(B)** are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.

A
Hsp72, 70kDa
GAPDH, 36kDa
[2,4-D]/μM
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
B
Hsp72 Protein Expression (relative to control)
1.15
1.10
1.05
1.00
0.95
0.90
0.85
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/μM

Figure 36: Comparison of the effects of commercial 2,4-D on the inducible form of heat shock protein (Hsp72) protein expression in exposed HepG2 and HEK293 cells. Cells were exposed to 0 to 1mM of 2,4-D for 24 hrs. The HepG2 and HEK293 cells were not simultaneously exposed to 2,4-D. Cells were harvested and protein lysates obtained. Thirty µg of total proteins was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The protein expression of Hsp72 was detected using anti-Hsp72 antibody (rabbit polyclonal to Hsp70 (Hsp72), catalog number: SPA-812, Stressgen Bioreagents). Equal loading was ensured through detection of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) protein expression using anti-GAPDH antibody (mouse monoclonal to GAPDH, catalog number: MAB374, Chemicon International Inc.). Results of densitometric analysis are presented as the mean of triplicate assays from one independent experiment. Target protein expression levels are presented as the mean of triplicate assays from one independent experiment; and are expressed relative to the expression level in control cell cultures, normalized to the expression level of the reference gene, GAPDH.


HepG2
HEK293
Hsp72 Protein Expression
(relative to control)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
[2,4-D]/uM

5. DISCUSSION

The cytotoxic effects of low environmental concentrations (0.1 to 100 nM) of 2,4-D were investigated in the present study. The major findings from the present investigation were low environmental concentrations of 2,4-D (1) induce limited cytotoxic effects, reflected by small decreases in HepG2 and HEK293 cell viability that are independent of the duration of exposure, (2) induce cell specific viability effects (3) do not induce significant ROS production in exposed cells and (4) induce inconsistent patterns of alterations (< 2 fold increases and decreases) in FTL, FTH1 and PCNA gene expression, at the levels of mRNA and protein accumulation.

Cell viability studies revealed that low environmental concentrations (0.1 to 100 nM) induce limited, but statistically significant (P<0.05) cytotoxic effects in HepG2 (Figs. 1 and 3 (A to C)) and HEK293 cells (Figs. 6 and 8 (A to C)), as reflected by small decreases (mean decreases in cell viability < 20%) in cell viability. Cytotoxic effects were exacerbated (mean decreases in viability > 40%) at higher concentrations (5 and 10 mM). Decreases in viability were not concentration-dependent and did not change significantly when durations of exposure were increased in cells exposed to the concentration range of 0.1 to 100 nM. Statistically significant (P<0.05) increases in cell viability were observed in HepG2 cells exposed to 1mM of technical 2,4-D for 72 hrs (Fig. 1(D)) and commercial 2,4-D for 24 to 72 hrs (Fig. 3 (B) to (C)). Both forms of 2,4-D (i.e. technical or commercial) induced similar cell viability effects within individual cell types (i.e HepG2 or HEK293 cells) suggesting that toxicity was induced directly by 2,4-D and 'inert ingredients' in the commercial formulation had limited ability to act synergistically with 2,4-D and enhance toxic effects in exposed cells. An inert ingredient is a substance other than an active ingredient that is added to a herbicide formulation and plays a role in its effectiveness (i.e. better absorption, longer shelf-life) (US EPA, 2006).

Compared to HepG2, HEK293 cells appeared to be more susceptible to the toxic effects of either form of 2,4-D, reflected by a more pronounced reduction in cell

viability (Figs. 11 and 12 (A to D)). At all exposure times, mean decreases in HEK293 cell viability were 4 to 26% greater in magnitude, as compared to HepG2 cell viability over the range of 2,4-D concentrations used (0.1 nM to 10 mM). The differences between HepG2 and HEK293 cell viability effects at all concentrations and durations of treatment were statistically significant ($P<0.05$).

Results from ROS studies revealed that low environmental concentrations of 2,4-D do not induce significant ROS production in exposed cells. Statistically significant ($P<0.05$) ROS production was observed at concentrations greater than 10 μM and 100 nM in HepG2 (Fig. 16) and HEK293 (Fig. 17) cells, respectively. ROS production was concentration-dependent in HepG2 and HEK293 cells. ROS production increased with longer durations of exposure. Figure 18 illustrates that ROS production was consistently 50 to 70% less in HEK293, compared to HepG2 cells, at all concentrations and times.

Preliminary results from the present study reveal that exposure to low environmental concentrations of 2,4-D induce alterations (> 2 fold increases and decreases) in FTL (Figs. 19 and 20), FTH1 (Figs. 22 and 23) and PCNA (Figs. 25 and 26) mRNA expression in exposed HepG2 and HEK293 cells. A concentration-dependent increase or decrease in mRNA accumulation was not observed in cells exposed to the range of environmentally realistic concentrations of 2,4-D.

Preliminary studies of ferritin and PCNA protein expression reveal that exposure to low environmental concentrations of 2,4-D induce alterations (< 2 fold increases and decreases) in ferritin (Figs. 28 and 29) and PCNA (Figs. 31 and 32) protein expression in HepG2 and HEK293 cells. A concentration-dependent increase or decrease in protein accumulation was not observed in cells exposed to the range of environmentally realistic concentrations of 2,4-D.

The present study is the first to report that low environmental concentrations of 2,4-D decrease HepG2 and HEK293 cell viability. Previous *in vivo* and *in vitro* 2,4-D toxicological studies have utilized high concentrations of 2,4-D (see section 2.1.6.1 to 2.1.6.9) and have not directly tested concentrations found in the environment. Bharadwaj et al. (2005) assessed the cytotoxic effects of 2,4-D in HepG2 cells using the same low concentrations and commercial form of 2,4-D that was used in the present study. However it was illustrated that exposure to low concentrations of 2,4-D for 24

hrs did not induce statistically significant decreases in HepG2 cell viability. Compared to the present study, the decreases in cell viability that were observed by Bharadwaj et al. (2005) were approximately 20% greater in magnitude. The differences in the reported significance of results in the studies performed by Bharadwaj et al. (2005) and the present investigation may have been due to the different statistical multiple comparison tests that were used to determine significant differences in treatment effects between control and treated groups in the two studies. Bharadwaj et al. (2005) used ANOVA followed by a student Neuman-Keul's post-hoc test and the present study used ANOVA followed by a two-sided Dunnett's t-test. In contrast to a student Neuman-Keul's post-hoc test, Dunnett's t-test is a t-statistic test that is considered to be less conservative indicator for the detection of differences between control and treated groups (Lane, 1997). The power of a statistical test is the probability that the test will reject the null hypothesis (High, 2000).

Decreases in HepG2 and HEK293 cell viability were not dependent on duration of exposure to 0.1 to 100 nM. In humans, 2,4-D has an average $t_{½}$ of 17.7 hrs (Sauerhoff *et al*., 1977). Sauerhoff et al. (1977) reported that following oral exposure, approximately 82.3% of 2,4-D was eliminated unchanged and 12.8% was excreted as an unidentified conjugate in urine. Therefore, it is possible that in the present study, more pronounced decreases in HepG2 and HEK293 cell viability were not observed following longer durations of exposure due to the rapid intracellular metabolism of 2,4-D to non-toxic 2,4-D conjugates and elimination from cells that may have occurred, *in vitro*, prior to the exposure time points of 24, 48 and 72 hrs.

The significant increases in HepG2 cell viability observed following exposure to 1mM of either form of 2,4-D was a unique observation in the present study that has not been observed in previous studies investigating the effects of 2,4-D on cell viability, *in vitro*. For instance, using the neutral red assay, Bharadwaj et al. (2005) illustrated that 24 hrs of exposure to 1 mM of commercial 2,4-D (the same commercial form used in the present study) induced a significant decrease (70%) in HepG2 cell viability. Kaioumova et al. (2001b) reported that approximately 45 and 55% of Jurkat T cells underwent apoptosis following exposure to 1 mM of 2,4-D DMA for 24 hrs, as determined by the nicoletti and annexin-V assays, respectively. The significant increases in HepG2 cell

viability observed following exposure to 1 mM of 2,4-D may be due to the reasons outlined below.

The Resazurin assay is a relatively non-toxic (O'Brien *et al*., 2000) reductase-based assay that is easy to use and inexpensive (McMillian *et al*., 2002). The Resazurin assay is the most sensitive assay, compared to other assays (i.e. MTT, neutral red assay) for determination of cytotoxicity (Davoren *et al*., 2007), *in vitro*. The basis of this fluorometric assay is the enzymatic reduction of Resazurin (blue and non-fluorescent) to resorufin (pink and highly-fluorescent) (O'Brien *et al*., 2000). Compared to dead or dying cells, viable cells are able to reduce Resazurin to resofurin (McWilliams *et al*., 2002; Miret *et al*., 2006; O'Brien *et al*., 2000). Thus, fluorescence is more pronounced in viable cells, compared to dead or dying cells. However, O'Brien et al. (2000) explained that viable cells may further reduce resorufin (pink and highly-fluorescent) to hydroresorufin (colourless and non-fluorescent). According to O'Brien et al. (2000), if viable cells further reduce resorufin to hydroresorufin then fluorescence will be more pronounced in dead or dying cells, compared to viable cells. Thus, in the present study, if viable cells reduced resorufin to hydroresorufin, then fluorescence may have been more pronounced in dead or dying cells, compared to viable cells. Therefore, in the present study, the increases in fluorescence that were observed in cells exposed to the toxic concentrations of 1 mM of 2,4-D may have been a reflection of decreased cell viability and not increased cell viability, relative to untreated control cell cultures as interpreted. Compared to untreated control cell cultures, more pronounced fluorescence was not observed at concentrations below 1 mM. However, in the present study, the theory that viable cells may have further reduced resorufin to hydroresorufin is challenged by the less pronounced fluorescence that was observed following exposure to toxic concentrations of 5 and 10 mM, compared to 1 mM of either form of 2,4-D for all durations of exposure. In the present study, if viable cells were able to further reduce resorufin to hydroresorufin, then, theoretically, increased cell death at toxic concentrations of 5 and 10 mM should have been represented by more pronounced fluorescence, compared to concentrations equal to and lower than 1 mM.

An increase in HepG2 cell proliferation could be the second reason for the increased fluorescence observed at 1 mM. An increase in cell proliferation may have

been induced by exposure to 1 mM. O'Brien et al. (2000) illustrated that there was a good correlation between HepG2 cell number and Resazurin reduction. According to the authors, increased fluorescence indicates increased reduction of Resazurin to resofurin, which in turn reflects an increased cell number. 2,4-D is considered to be a peroxisome proliferator (Ge *et al*., 2002) and has been shown to induce proliferation of rat hepatic peroxisomes (Vainio *et al*., 1983) and human peripheral blood lymphocytes (Figgs *et al*., 2000; Holland *et al*., 2002). Significant proliferation of lymphocytes was observed at a 2,4-D exposure concentration of 5 µM (Holland *et al*., 2002). Thus, in the present study, 1 mM may have induced proliferation of HepG2 cells, which would have resulted in increased fluorescence due to increased reduction of Resazurin to resorufin. However, Bharadwaj et al. (2005) illustrated that exposure to 1mM of commercial 2,4-D (the same commercial form of 2,4-D used in the present study) induced a 50% decrease in HepG2 cell proliferation. Furthermore, the decreased fluorescence observed following exposure to 5 and 10 mM, compared to 1 mM for 6 to 72 hrs is in disagreement with the theory that increased Resazurin reduction to resofurin reflects increased cell proliferation. The reason(s) for the apparent increase in cell viability in HepG2 cells exposed to 1 mM of 2,4-D presently remains unclear, as the increases in fluorescence in cells at this exposure concentration, relative to untreated control cell cultures may be due to the biochemical characteristic (i.e. further reduction of resorufin to hydroresorufin by viable cells) of the assay or increased cell proliferation.

Figures 11 and 12 (A to D) illustrate that compared to HepG2, HEK293 cells appeared to be more susceptible to the toxic effects of either form of 2,4-D, reflected by more pronounced decreases in cell viability. The first reason that may account for the more pronounced decreases observed in HEK293 may be due to differences in the biotransformation system of HepG2 and HEK293 cells. Eaton and Klaassen. (2001) explained that one of the main reasons why chemicals exert different degrees of toxicity between organs is due to the differences in the biotransforming ability of various organs. The biotransforming ability ultimately determines the differences in the accumulation of the ultimate toxicant in organs (Eaton and Klaassen, 2001). Xenobiotic biotransformation is the principal mechanism that is responsible for maintaining homeostasis during exposure to xenobiotics (Parkinson, 2001). In humans, xenobiotics

undergo biotransformation, which generally changes the physical properties of a chemical to favor increased hydrophilicity and subsequent excretion in the urine or feces (Parkinson, 2001). Almost all tissues in the human body are able to carry out biotransformation reactions (Riddick, 1998). The liver is described as the most important organ of biotransformation (Riddick, 1998).

There are two phases of biotransformation (phase I and II) (Parkinson, 2001; Riddick, 1998). Phase I reactions induce small increases the hydrophilicity of a xenobiotic (Parkinson, 2001), by introducing or exposing a functional group via oxidation, reduction or hydrolysis (Riddick, 1998). The reactions increase, maintain or decrease the activity of the compound (Riddick, 1998). Phase II reactions consists of synthetic or conjugation processes (Parkinson, 2001). In phase II reactions, an endogenous compound (i.e. glutathione, glycine, taurine) combines with the functional group (originally present in the xenobiotic or introduced or unmasked in the phase I reaction (Parkinson, 2001)) of the xenobiotic (Parkinson, 2001). Xenobiotics may undergo Phase I and II reactions sequentially; however there are exceptions to that order (Parkinson, 2001; Riddick, 1998). Some xenobiotics may only undergo either a phase I or II reaction followed by elimination from the body (Riddick, 1998). Some xenobiotics are eliminated unchanged, without prior biotransformation.

The chemical, 2,4-D acid, having a partition coefficient (K_{ow}) of 2.81 (reviewed by EXTOXNET, 1996) is a hydrophobic compound and theoretically should be biotransformed in humans, to enhance its hydrophilicity and subsequent elimination. There is controversy regarding whether or not 2,4-D is biotransformed in humans. Gregus et al. (1999) and Van Ravenzwaay et al. (2003) explained that 2,4-D does not require biotransformation prior to its elimination in humans and following oral exposure, 82.3% of 2,4-D is eliminated unchanged in the urine of humans (Sauerhauff *et al*., 1977). Also, it has been reported that 12.8% of 2,4-D is excreted in the urine as an unidentified conjugate (Sauerhauff *et al*., 1977). It has been illustrated that dietary exposure to 2,4-D acid induces the activity of epoxide hydrolases (phase II biotransformation enzymes) in the livers of mice (Lundgren *et al*., 1987). Taurine and glycine conjugates of 2,4-D have been identified in the bile of rats and mice (Griffin *et al*., 1997) exposed to the chemical via intragastric injection. *In vitro* studies performed

by Li et al. (2003) illustrated that 2,4-D may form a 2,4-D-CoA reactive metabolite. Li et al. (2003) illustrated that synthetic 2,4-D CoA forms a glutathione conjugate or alternatively, it covalently binding to rat hepatic proteins, *in vitro*. The results of current literature suggest that 2,4-D may undergo both phase I and II reactions, or only phase II reactions. So how is this all relevant to the present study?

HepG2 and HEK293 cells are both metabolically active cells. HepG2 cells have retained high levels of biotransforming enzymes (Mersch-Sundermann *et al*., 2004). Parknison. (2001) explained that biotransformation enzymes are present in large amounts in the liver, and to a lesser degree in other organs including the lung, eye, pancreas and kidney. The promoter activity of several isoforms of the uridine diphosphate – glucuronsyltransferase (UGT) genes was recently found to be decreased in HEK293, compared to HepG2 cells (Gardner-Stephen and Mackenzie, 2007). UGT is a biotransformation enzyme responsible for Phase II glucuronidation reactions (Parkinson, 2001; Riddick, 1998). Therefore there are enormous differences between tissues in their ability to biotransform xenobiotics (Parkinson, 2001). These differences may subsequently contribute to the different degrees or patterns of toxic effects induced by xenobiotics in different tissues/organs. Thus, in the present study, the biotransformation enzyme(s) responsible for the detoxification of 2,4-D may have been absent or reduced in HEK293, compared to HepG2 cells. The above may explain the enhanced susceptibility of HEK293 cells to 2,4-D toxicity, compared to HepG2 cells.

The enhanced active transportation of 2,4-D into HEK293 cells may also explain the more pronounced decreases that were observed in HEK293, relative to HepG2 cell viability. *In vivo* and *in vitro* experimental studies performed by Charles et al. (2001) and González et al. (2005) suggest that in humans, various forms of 2,4-D (i..e 2,4-D DMA, 2,4-D ester) undergo rapid metabolic conversion to 2,4-D acid. The 2,4-D acid is ionized at physiologic pH, and hence does not diffuse into cells (Garabrant and Philbert, 2002). The 2,4-D anion is actively transported into cells, including those of the liver and kidney by the organic anion transporter system (Pritchard *et al*., 1982). This transport system may facilitate the rapid elimination of xenobiotics, and could favour the increased intracellular accumulation of foreign molecules (i.e 2,4-D) and subsequently increase toxic effects in cells that possess the transport system (Pritchard *et al*., 1982).

Results from experimental studies have identified several organic anion transporters that are specific to the kidney, and are responsible for the uptake of 2,4-D in rats and mice (Hasegwawa *et al*., 2003; Imaoka *et al*., 2004; Younghood and Sweet, 2004). These organic anion transporters were not found to be expressed in other tissues, including the liver (Imaoka *et al*., 2004; Younghood and Sweet, 2004). Therefore, in the present study, the increased toxicity observed in HEK293 cells may have been due in part to increased uptake and subsequent accumulation of 2,4-D in HEK293, compared to HepG2 cells.

The mechanisms of 2,4-D toxicity have yet to be fully elucidated. Results from previous experimental studies suggest that ROS and associated oxidative stress may be one potential mechanism involved in 2,4-D cytotoxicity. Recently it has been shown that 2,4-D alters the activity of antioxidant enzymes and glutathione levels in human erythrocytes (Bukowska, 2003) (see section 2.1.7.5). It has also been illustrated that 2,4-D induces lipid peroxidation in isolated rat hepatocytes (Palmeira *et al*., 1995) and directly induced ROS (hydroxyl radicals) production and associated oxidative stress in yeast (Teixeira *et al*., 2004) (see section 2.1.7.5). In this present study, the potential role of ROS in 2,4-D – induced cytotoxicity was investigated. For the very first time, it was shown that high concentrations of 2,4-D directly induces significant intracellular ROS production in human cell cultures (HepG2 and HEK293). Figure 18 illustrates that reduced amounts of ROS were produced in HEK293, compared to HepG2 cells, at all concentrations and durations of exposure tested. The difference in ROS production that was observed between HepG2 and HEK293 cells may have been due to differences in the antioxidant defense systems between the two cells. All eukaryotic aerobic cells have tremendous antioxidant systems, such as vitamins (C and E) and enzymes (SOD, catalase, GSH-Px) (reviewed by Kehrer, 1993; Kohen and Nyska, 2002) that are responsible for the detoxification of ROS. However, there may be variations in antioxidant levels or responses between cell types. Szymonik-Lesiuk et al. (2003) illustrated that there are variations in antioxidant levels and responses between rat tissues (brain, liver, kidney and heart) following exposure to carbon tetrachloride (an inducer of oxidative stress). Thus, in the present study, antioxidant levels or responses

may have differed between HepG2 and HEK293 cells. Therefore, greater amounts of ROS may have been detoxified in HEK293, compared to HepG2 cells.

However, cell viability studies demonstrated that HEK293 cells were more susceptible to 2,4-D toxicity, compared to HepG2 cells. So why did ROS studies indicate that less ROS was produced in HEK293, compared to HepG2 cells? The answer may be due to the biochemical nature of the ROS assay. In contrast to $O_2^{\cdot -}$ and $OH^{\cdot}$, H_2O_2 is considered to be the primary ROS responsible for the oxidation of the non-fluorescent 2',7'-DCFH molecule to the fluorescent 2'7'-DCF molecule (Bass *et al*., 1983). It has recently been shown that $O_2^{\cdot -}$ oxidizes the 2',7'-DCFH molecule, but to a lesser degree than H_2O_2 (Hempel *et al*., 1999). Therefore, compared to HepG2, more $O_2^{\cdot -}$ and $OH^{\cdot}$ species may have been produced in HEK293 cells resulting in less oxidation of 2',7'-DCFH to 2',7-DCF and less fluorescence. Nevertheless, the observation that 2,4-D directly induces ROS production in HepG2 and HEK293 cells is an intriguing one, that suggest a potential role for ROS and associated oxidative stress in 2,4-D – induced cytotoxicity.

The results from RT-PCR assays revealed that 24 hrs of exposure to low environmental concentrations (0.1 to 100 nM) of commercial 2,4-D induces alterations (< 2 fold increases and decreases) in FTL and FTH1 mRNA expression in HepG2 and HEK293 cells. A concentration-dependent increase or decrease in mRNA expression was not observed over the concentration range of 0.1 nM to 1 mM. The regulation of mammalian ferritin gene expression is complex and poorly understood (Torti and Torti, 2002). Previously, studies have illustrated that ferritin expression was mainly regulated at the translational level in response to intracellular levels of iron (Arosio and Levi, 2002; Leibold and Guo, 1992). However, ferritin expression may also be regulated at the transcriptional level in response to increased iron levels (Tacchini *et al*., 1997; White and Munro, 1988). Several studies illustrate that in addition to iron, several other regulatory factors (i.e. tumor necrosis factor (TNF), oxidative stress, and ROS ($O_2^{\cdot -}$ and H_2O_2)) may also be directly involved in the regulation of ferritin gene expression at the transcriptional or translational levels (for review see Torti and Torti, 2002). Several authors have suggested that increases in FTL and FTH1 mRNA and protein expression may be responsive to oxidative stress (Balla *et al*., 1992; Cairo *et al*., 1995; Epsztejn *et*

al., 1999; Lin and Girotti, 1997; Orino *et al*., 2001; Regan *et al*., 2002; Tsuji *et al*., 2000). Tsuji et al. (2000) explain that the increase in transcription of cytoprotective genes in response to oxidative challenge is termed the antioxidant response. Currently, there is an absence of relevant and supportive scientific literature to explain the repression of FTL and FTH1 mRNA expression that was observed in HepG2 (FTL mRNA: 0.1 nM to 1 μM; FTH1 mRNA: 1 nM) and HEK293 (FTL mRNA: 1 nM, 100 nM and 10 μM; FTH1 mRNA: 0.1 nM to 100 μM) cells. However, in the present study, the observed increases in FTL and FTH1 mRNA expression may have been a cytoprotective response to ROS production and associated intracellular oxidative stress in HepG2 (FTL mRNA: concentrations > 1 μM, FTH1 mRNA: concentrations > 1 nM) and HEK293 (FTL mRNA: 0.1 nM, 1 μM, 100 μM and 1 mM; FTH1 mRNA: 1 mM) cells.

The more pronounced magnitude of increases in FTL mRNA expression in HEK293 may be due to the action of $O_2^{\cdot -}$ on ferritin protein. Biemond et al. (1984) illustrates that $O_2^{\cdot -}$ derived from polymorphonuclear leukocytes mobilizes iron from human ferritin. Subsequent studies (Thomas et al. (1985) and Agrawal et al. (2001)) illustrated that $O_2^{\cdot -}$ induces the release of iron from ferritin protein, *in vitro*. The release of iron being mediated by the reduction of Fe^{3+} to Fe^{2+} by $O_2^{\cdot -}$ and subsequent release from the ferritin molecule (Agrawal *et al*., 2001). Biemond et al. (1986) and Thomas and Aust. (1985) illustrated that $O_2^{\cdot -}$ produced by xanthine oxidase releases iron from ferritin during the conversion of xanthine to uric acid. Furthermore, Biemond et al. (1986) illustrated that, compared to H_2O_2 or $OH^{\cdot}$, $O_2^{\cdot -}$ is specifically responsible for release of iron from ferritin and the release was inhibited by superoxide dismutase, an enzyme that is specifically involved in the detoxification of $O_2^{\cdot -}$ (Gregus and Klaassen, 2001). Therefore, in the present study, the possible increased presence of $O_2^{\cdot -}$ in HEK293 cells may have served as a fuel for increased ROS production and associated oxidative stress via release of iron from ferritin. The increased concentration of released iron may have been subsequently involved in the generation of noxious ROS via its participation in the Haber-Weiss reaction. The effect may have induced more pronounced increase in FTL mRNA expression in HEK293, compared to HepG2 cells as an antioxidant response.

Results from western blot and densitometric analyses illustrated that low environmental concentrations of 2,4-D induces alterations (< 2 fold increases and decreases) in ferritin protein expression in HepG2 (Fig. 28 (A) and (B)) and HEK293 (Fig. 29 (A) and (B)). The induction of ferritin protein expression in HepG2 and HEK293 cells may have been a protective response to increased ROS production and possible oxidative stress conditions. The induction of ferritin protein expression serves as a protective mechanism against oxidative stress via sequestration of excess free iron. Excess free iron participates in Haber-Weiss reaction to produce noxious ROS and associated oxidative stress conditions (Biemond *et al*., 1988; Chiancone *et al*., 2004; Harrison and Arosio, 1996; McCord, 1998; Papanikolaou and Pantopoulos, 2005). Ferritin expression is known to be regulated at the translational level in response to intracellular levels of iron (Arosio and Levi, 2002; Leibold and Guo, 1992). Several studies have shown that ferritin protein expression may also be regulated at the translational level in response to increased intracellular ROS production and associated oxidative stress (Balla *et al*., 1992; Epsztejn *et al*., 1999; Lin and Girotti, 1997; Orino *et al*., 2001; Regan *et al*., 2002; Tacchini *et al*., 1997; Tsuji *et al*., 2000) (see section 2.2.3). In the present study, the increased expression of ferritin protein in exposed cells may have been a protective response to increased ROS production and possible oxidative stress conditions.

The degradation of oxidized ferritin protein may have been a possible reason for the above observed repression of ferritin protein expression that was observed in HEK293 cells (0.1 nM to 10 nM, 1 µM to 1 mM). The ROS can cause oxidative damage to proteins (for review see Kohen and Nyska, 2002). Oxidized proteins are then targeted for proteolytic degradation (Davies *et al*., 2001). Rudeck et al. (2000) have shown that exposure to oxidants including H_2O_2, induce ferritin protein degradation in human erythrocytes. In the present study it was shown that 2,4-D exposure induced ROS production in both HepG2 and HEK293 cells. The production of ROS may also have induced some oxidative damage to ferritin protein in HepG2 cells exposed to concentrations of 100 µM and 1 mM, as the magnitude of increases in ferritin protein expression in HepG2 cells decreased over the concentration range of 10 µM to 1 mM.

Similar to FTL and FTH1, the induction of PCNA mRNA expression in HepG2 (0.1 nM to 100 μM) and HEK293 (0.1 nM) cells may have been a response to oxidative DNA damage. Repression of PCNA mRNA expression was also observed in HepG2 (1 mM) and HEK293 (concentrations < 0.1 nM) cells. However the reasons for the repression of PCNA mRNA expression in HepG2 and HEK293 cells in the present study remains unclear due to a lack of relevant and supportive scientific literature. The protein, PCNA, an acidic nuclear protein (Mathews *et al*., 1984) plays an integral role in nucleic acid metabolism (Kelman, 1997), including DNA replication, repair and cell cycle regulation (Kelman, 1997; Schurtenberger *et al*., 1998). Several *in vivo* and *in vitro* experimental studies utilized PCNA protein expression as a potential marker of oxidative DNA damage (Balajee *et al*., 1999; Holmes *et al*., 2002; Savio *et al*., 1998) (see section 2.3.3).

Repression (< 2 fold) of PCNA protein expression was observed in HepG2 cells exposed to 0.1 nM to 1 μM, 100 μM and 1 mM. Induction (< 2 fold) of PCNA protein expression was observed in HepG2 (10 μM) and HEK293 (0.1 nM to 1 mM) cells. Increased PCNA protein expression has been observed in response to oxidative stress (Balajee et al. (1999), Holmes et al. (2002) and Savio et al. (1998)) (see section 2.3.3). The authors suggest that due to its involvement in DNA repair, the induction of PCNA protein expression may be indicative of oxidative damaged DNA. Therefore, in the present study it may have been possible that the increased ROS production in HEK293 cells resulted in oxidative damage to DNA, which subsequently induced PCNA protein expression to aid in the repair of damaged DNA. If this is true, then why did 2,4-D repress PCNA protein expression in HepG2, compared to HEK293 cells? In the present investigation, ROS studies illustrated that 2,4-D induced ROS production in both cell types, with more pronounced production in HepG2, compared to HEK293 cells. A possible reason for the intracellular repression of PCNA protein expression may be due to the increased expression of the protein, p21. As a protective mechanism during oxidative stress, there is activation of mechanisms that arrest the progression of the cell cycle at specific checkpoints (Russo *et al*., 1995). The arrest in cell cycle progression allows time for the repair of damaged DNA (Russo *et al*., 1995). The results from several *in vitro* studies have shown that oxidative stress induces G1 arrest in human lung

epithelial (Gehen *et al*., 2007; Rancourt *et al*., 2001) and human colon carcinoma cells (Helt *et al*., 2001). Tuschl and Schwab. (2003) and (2004) reported that exposure to 4, 8 and 16 mM of 2,4-D acid for 24 hrs induced G1 arrest in HepG2 cell cycle. The activation of G1 arrest during oxidative stress is mediated through p53 tumor suppressor protein (Russo *et al*., 1995). The p53 transcription factor subsequently binds to the promoter regions of several genes, with activation of gene transcription (Russo *et al*., 1995). Russo et al. (1995) explains that the gene, WAF1/CIP1 is amongst the genes whose transcription is activated by p53. The WAF1/CIP1 gene encodes a 21kDa protein (p21) that is required for progression from G1 into S phase of the cell cycle (Russo *et al*., 1995). The enhanced expression of p21 protein during oxidative stress inhibits PCNA-dependent DNA synthesis (Gehen *et al*., 2007). Bendjennat et al. (2003) illustrated that p21 inhibited PCNA-dependent DNA synthesis by inhibiting PCNA loading onto DNA. However, Gehen et al. (2007) illustrated that during oxidative stress conditions, in addition to inhibiting PCNA function, p21 directly controlled the abundance of PCNA protein. Thus, in the present study, enhanced intracellular ROS production and associated oxidative stress may have induced more pronounced increases in p21 protein expression and reduction of PCNA protein abundance in HepG2, compared to HEK293 cells.

Results from western blot and densitometric analyses from the present study illustrated that low environmental concentrations of 2,4-D induce alterations (< 2 fold) in Hsp72 protein expression in HepG2 (Fig. 34 (A) and (B)) and HEK293 (Fig. 35 (A) and (B)). Induction of Hsp72 protein expression was observed in HEK293 exposed to 1 nM. Induction of Hsp72 protein expression in HepG2 and HEK293 cells may have been a protective response to increased ROS production and possible oxidative stress conditions. Hsp72 belongs to the family of heat shock protein 70 (Hsp70) (for review see Morimoto *et al*., 1992; Pelham *et al*., 1986). The protein family is involved in several intracellular processes, including signal transduction, cell cycle regulation, cell differentiation and programmed cell death (Mayer and Bukau, 2005). There is a diversity of physiological and chemical conditions that induce Hsp biosynthesis. The conditions that result in Hsp70 gene expression are classified into three categories; (1) environmental stress (i.e. heat shock, amino acid analogues, transition heavy metals), (2)

pathophysiological state (i.e. fever, inflammation, ischemia, viral and bacterial infection and (3) non-stressful conditions (i.e. normal cell growth and differentiation) (for review see Morimoto *et al*., 1992). Morimoto et al. (1992) have explained that Hsp72 protein expression may be induced by oxidative stress.

In humans, Hsp72 gene expression is regulated at the transcriptional level in response to binding of the heat shock transcriptional factor 1 (HSF1) to the heat shock element (HSE) in the promoter region of the Hsp72 gene (Baler *et al.,* 1992). Briefly, under normal, non-stressed conditions HSF is maintained in a non-DNA binding form through interactions with Hsp70 proteins. In contrast, in response to stress, the accumulation of misfolded or abnormal proteins competes with HSF for binding to Hsp70 proteins. The released HSF undergoes oligomerization to its DNA binding form. The HSF subsequently binds to HSE, with increased transcriptional activity of the Hsp70 gene. The return to normal un-stressed conditions results in the dissociation of HSF from DNA and the conversion of HSF to its non-DNA binding form (reviewed by Morimoto *et al*., 1992).

In the present study, exposure to 2,4-D elicited ROS production in HepG2 and HEK293 cells. Several studies have reported that increased Hsp72 protein expression may be a protective response to oxidative stress (Ferrante *et al*., 2006; Gorman *et al*., 1999; Oh *et al*., 2006; Ohkawara *et al*., 2006; Seo *et al*., 2005). Ochratoxin (fungal metabolite) – induced lipid peroxidation induced Hsp72 protein expression in peritoneal macrophages of mice (Ferrante *et al*., 2006). Increased Hsp72 protein expression inhibited morphological alterations, growth reduction and cell death in human colon cells (CaCo2) following exposure to the oxidant, H_2O_2 (Ohkawara *et al*., 2006). Additionally, Gorman et al. (1999), Oh et al. (2005) and Seo et al. (2005) have shown that ROS may be directly involved in the induction of Hsp72 protein expression. Gorman et al. (1999) illustrated that in the presence of either pyrrolidine dithiocarbamate, 1,10-phenanthroline or *N*-acetyl-L-cysteine (NAC) antioxidant molecules; Hsp72 protein expression was repressed in heat-shocked human myelocytic cells (HL-60). Seo et al. (2005) exposed cultured rat glioma cells (C6 cells) to sodium salicylate (an anti-inflammatory agent). The protein expression of Hsp72 was induced in cells, following recovery from sodium salicylate exposure. Pre-treatment with NAC,

abolished Hsp72 protein expression, and inhibited HSF1 DNA binding. Thus, suggesting that ROS played an integral role in the induction of Hsp72 protein expression (Seo *et al*., 2005). Oh et al. (2005) illustrated that methyl jasmonate (a plant hormone) induced intracellular O_2^{-} and H_2O_2 and mitochondrial ROS production in human C6 glioma cell cultures. Increased intracellular and mitochondrial Hsp72 protein expression was observed following recovery from exposure to methyl jasmonate. There was repression of Hsp72 protein expression and HSF1 DNA binding following pre-treatment with NAC (non-specific antioxidant), catalase (antioxidant specific for detoxification of H_2O_2), and sodium formate (antioxidant specific for detoxification of OH^{-}).

In the present study, the magnitude of induction of Hsp72 protein expression was less pronounced in HEK293 cells, compared to HepG2 cells (Fig. 36). The differences in the effects could be due to the probable constitutive expression of Hsp72 in HEK293 cells, compared to HepG2 cells. Western blot analyses of untreated HEK293 control cells illustrated that there appeared to be a high constitutive expression of Hsp72 in HEK293 (Fig. 35(A)). Yaglom et al. (2007) explained that Hsp72 protein may be constitutively expressed in many tumor cell lines. The HEK293 cells have been transformed with a human adenovirus, with elaboration of a virus-specific tumor antigen and are capable of inducing tumors in mice (Graham and Nairn, 1977). Therefore, HEK293 cells appear to a tumor cell line which constitutively expresses Hsp72 protein. Baler et al. (1996) suggested that Hsp70 may act as a negative regulator of HSF1, and illustrated that over expression of Hsp72 inhibited induced Hsp72 gene expression in heat stressed monkey cells (COS). The authors explained that over expression of Hsp72 may have inhibited HSF1 activation. Baler et al. (1996) postulated that the inhibition of HSF1 activation by over expression of Hsp72 protein may have been due to the stabilization of HSF1-Hsp72 complexes formed in the presence of excess Hsp72. Thus, in the present study, the high constitutive expression of Hp72 protein expression in HEK293 cells may have resulted in the increased presence of stable HSF1-Hsp72 protein complexes. The effect may have inhibited large increases in Hsp72 protein expression in HEK293 cells at concentrations equal to and greater than 1µM. The above may also explain the repression of Hsp72 protein expression in HEK293 cells exposed to lower 2,4-D concentrations. The induction of Hsp72 protein expression at

concentrations equal to greater than 1 μM may have been due to the possibility that the inhibitory effect was partially removed following exposure to higher concentrations, due to more pronounced stressed conditions, presence of abnormal proteins and enhanced competition with HSF1 for binding Hsp72. Released HSF1 would have subsequently undergone oligomerization and triggered transcription of Hsp72 gene to increase mRNA and protein accumulation levels.

The preceding paragraphs discussed the many interesting findings that were revealed in the present study. However, as with all experimental research, several strengths and weakness could be identified in the present investigation. In the study, a concentration range of 0 to 10 mM (cell viability analyses) or 0 to 1 mM (ROS, RT-PCR and western blot analyses) was used. The concentrations that were used reflected low environmental and toxic concentrations of 2,4-D. However, the main purpose of the present study was to assess the toxic effects of low environmental concentrations of 2,4-D. Thus, a smaller range of concentrations (i.e. 0, 0.1 nM, 1 nM, 10 nM, 100 nM and 1 mM) could be assessed in future. One purpose of the present study was to determine if more pronounced toxic effects would be observed with longer durations of exposure to low environmental concentrations of 2,4-D. However, the present study was truly an acute toxicity study and thus the design was unable to efficiently assess the chronic effects of low concentrations of 2,4-D. Acute toxicity effects refer to the immediate effects that are observed 0 to 7 days after a single exposure of a chemical (PAN Pesticides Database, 2000). Humans who use groundwater as a source of drinking water are typically exposed to daily concentrations of 2,4-D from groundwater. Thus, ideally, a repeated daily exposure sub-acute, sub-chronic or chronic toxicity study could be performed in future. However, the use of cell models, such as HepG2 cells, limits the ability to perform sub-acute, sub-chronic or chronic toxicity studies, *in vitro*. The metabolic capacity of immortalized cells is reduced as cells differentiate over long durations of times, thus compromising their accurate correlation with toxicity (Fabre *et al*., 2003).

In the present study, HepG2 cells were considered an ideal cell model to use for *in vitro* assessment of 2,4-D – induced toxicity. However, Fabre et al. (2003) recently made a novel discovery that revealed that HepG2 cells actually contain lower levels of

phase I and II biotransformation enzymes, compared to primary hepatocytes or the new human hepatoma cell line, HBG BC2. Thus, in future studies, HepG2 and other cell models (i.e HBG BC2) could be used to assess 2,4-D toxicity, *in vitro*. Data from the present study revealed that more pronounced decreases in HepG2 and HEK293 cell viability were observed following 6 hrs, compared to 24 to 72 hrs of exposure to either form of 2,4-D. Thus, alterations in gene expression could be assessed following exposure to 2,4-D for 6 hrs, in contrast to 24 hrs. More pronounced alterations in FTL, FTH1 and PCNA gene expression, both at the level of mRNA and protein accumulation may be observed at 6 hrs, compared to 24 hrs of exposure.

The present study reported preliminary data for mRNA and protein expression. The results illustrate that 2,4-D induced inconsistent patterns (i.e. increases and decreases) in mRNA and protein expression. Target mRNA and protein expression were expressed as the mean of triplicate assays from one experiment, however, there were variations between individual RT-PCR and western blot assays. Thus, mRNA and protein analyses should have been performed on RNA and protein extracts obtained from at least 3 separate 2,4-D exposure experiments to determine reproducibility and significance of data.

As described above, several strengths and weakness were identified in the present study. The identification of the above aids in the design of future studies that would expand on results obtained from cell viability, ROS, RT-PCR and western analyses in the present study. In the present study, HepG2 and HEK293 cells were used as cell models to assess the cytotoxic effects of 2,4-D. Their use allowed for the determination of the cell specificity of 2,4-D – induced toxicity. However, in future studies, HepG2 cells would be used to evaluate the cytotoxic effects of 2,4-D, *in vitro*. HepG2 cells have been used in a vast array of *in vitro* toxicological studies. Additionally, compared to other immortalized human cells, HepG2 cells retain high levels of biotransforming enzymes (Mersch-Sundermann *et al*., 2004). HepG2 cells have been described as the most desirable *in vitro* model capable of portraying a more realistic reflection of xenobiotic metabolism in humans (Mersch-Sundermann *et al*., 2004).

Performing *in vitro* toxicology studies using primary cell cultures of human hepatocytes would be the most favourable *in vitro* model. Primary cell cultures of human hepatocytes obtained from organ donors have been used extensively in both academic and industrial laboratories for evaluating the hepatic metabolism of xenobiotics (LeCluyse, 2001). Thus, they would be the ideal model for extrapolation of the cytotoxic effects of 2,4-D, *in vitro* to humans. Additionally, Zhang et al. (2006) illustrated that there are marked differences between responses in HepG2 cells and primary human hepatocytes cell cultures in response to xenobiotic exposure. The authors reported that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in a more exacerbated adverse response in HepG2 cells, compared to primary human hepatocytes cell cultures. Zhang et al. (2006) explained that the observation warrants the need for caution to be taken when extrapolating and interpreting the results obtained from cell based models. However, although, the use of primary cell cultures of human hepatocytes would be ideal for conducting future *in vitro* toxicology studies, they are less convenient to obtain and work with, compared to HepG2 cells. Other disadvantages of primary cell cultures include their ability to; (1) rapidly lose their xenobiotic metabolizing capacity (Riddick, 1998), (2) undergo limited number of cell divisions (Knasmüller *et al*., 1998; Mersch-Sundermann *et al*., 2004) and (3) contain different cell types (Mersch-Sundermann *et al*., 2004). Toxicity can be cell specific and thus, cytotoxic effects can differ between cell types (Eaton and Klaassen, 2001) of the same organ.

Two forms of 2,4-D were used in the present study. In future studies only the commercial form of 2,4-D should be employed, since this is the form that humans are more likely to be exposed to from the environment. In future studies, cell viability results should be supported with other commonly used cell viability assays (i.e MTT, neutral red, or trypan blue exclusion assay).

In the present study, it was shown for the first time that 2,4-D induces ROS production in human cell cultures. However, the present study did not illustrate that exposure to 2,4-D induces oxidative stress. Previously discussed, ROS may mediate oxidative stress through lipid, protein or DNA oxidation (for review see Kohen and Nyska, 2002). Therefore, in future studies, the induction of oxidative stress in cells

exposed to 2,4-D would be investigated. Measurement of oxidative stress in the present thesis would have provided additional evidence for the association between oxidative stress and the induction of FTL, FTH1 and PCNA mRNA or protein expression. The most commonly used techniques for determination of oxidative stress are those that measure the end-products of lipid peroxidation (Dotan *et al*., 2004). Thus, in future studies, the traditional TBA assay would be performed. The TBA assay measures malonialdehyde end-product of lipid peroxidation (Halliwell and Chirico, 1993) and has been employed in many toxicological studies.

Depending on the results of cell viability studies, in future, the alterations in PCNA gene expression patterns at the time point that induced the most pronounced decreases in cell viability would be assessed. Balajee et al. (1999), Holmes et al. (2002), Savio et al. (1998) and Xu et al. (1999) provided evidence to suggest that up-regulation of PCNA mRNA or protein expression is a cytoprotective response to DNA damage. Thus, unlike FTL or FTH1, the choice of studying PCNA would help focus on one pathway involved in 2,4-D – induced cytotoxicity, which may involve ROS mediated oxidative DNA damage. Three concentrations (no treatment (untreated control cell cultures), the concentration where there is the smallest degree of induction of mRNA expression and the concentration where there is the greatest degree of induction of mRNA expression) would be used to assess alterations in PCNA gene expression patterns in future studies. The design would help to determine concentration-dependent effects. If the results of RT-PCR and western blot analyses are consistent, then the underlying cell and molecular pathways that may be involved in the up-regulation of PCNA would be investigated. To begin this investigation, nuclear transcription assays would be performed to determine if increased PCNA mRNA accumulation is due to increased transcriptional rate of the PCNA gene. The determination of the mechanism(s) of 2,4-D – induced activation of PCNA gene transcription would then be of interest, and thus examination of the promoter region of the PCNA gene would follow. Theoretical knowledge of the transcription factors that are involved in inducing PCNA gene transcription during DNA repair and the DNA sequences of the promoter region of the PCNA gene that these factors bind would then be obtained. Next, one transcription factor and its DNA binding sequence would initially be chosen for further

investigation. A plasmid construct containing the DNA sequence of interest would then be transfected into HepG2 cells. Cells would be exposed to the respective concentrations of 2,4-D. Following 2,4-D exposure, cells would be harvested and luciferase assays would be performed to determine gene induction. This would be followed by performing electrophoretic mobility shift assay (EMSA) to determine specific DNA-protein interactions in the promoter sequence, of interest. The above experimental design would enable for identification of a sequential molecular pathway involved in oxidative DNA damage during 2,4-D – induced cytotoxicity.

In addition to the above, in future studies, the comet assay would also be employed. The comet assay is a sensitive and rapid method for the detection of DNA strand break (Fairbairn *et al*., 1995). DNA strand break may be induced by ROS (for review see Kohen and Nyska, 2002). The use of the comet assay will also provide additional evidence for 2,4-D – induced intracellular oxidative stress conditions. Compared to lipids, DNA are more significant targets of oxidative damage (Halliwell and Chirico, 1993). Additionally, the authors explained that lipid peroxidation often tend to occur late in the injury process. Thus, the TBA assay may not reflect the true extent of oxidative injury induced following exposure to 2,4-D.

The current study was performed using *in vitro* cell models. Zhang et al. (2006) explained that caution must be taken when extrapolating and interpreting the results obtained from cell based models. So what are the implications of the data obtained from the present study? How do the findings of the present *in vitro* toxicology study relate to the real world, where humans may be exposed to environmental concentrations of 2,4-D? Cell viability data suggest that low environmental concentrations of 2,4-D may induce limited cytotoxic effects. The present data suggest that the effects may not be concentration-dependent and that more pronounced adverse cytotoxic effects are not likely to be observed following longer durations of exposure (up to 72 hrs) to low environmental concentrations of 2,4-D. The observation that similar patterns of cytotoxicity were induced following exposure to low environmental concentrations of either technical or commercial 2,4-D, *in vitro*, suggests that toxicity is induced directly by 2,4-D and that 'inert ingredients' in commercial preparations of 2,4-D have limited ability to synergistically act with 2,4-D to enhance toxicity. 2,4-D appeared to induce

cell specific effects; toxic effects were enhanced in HEK293, compared to HepG2 cells, *in vitro*. Thus, the results suggest that in humans, 2,4-D may induce cell or tissue specific toxic effects. The results also suggest that in humans, the kidney may be a more significant target organ of 2,4-D – induced toxicity, compared to the liver. Kidney failure is a symptom of 2,4-D poisoning in humans (PAN Pesticide Database, 2004).

The inability of low environmental concentrations of 2,4-D to induce significant ROS production, *in vitro* implies that low concentrations of 2,4-D encountered by humans in the environment may be insufficient to induce significant amounts of cellular and/or tissue stress or damage. Nevertheless, the results from ROS assays are intriguing and suggest a potential role for ROS and associated oxidative stress 2,4-D – induced toxicity.

Preliminary results from mRNA and protein studies suggest that low environmental concentrations of 2,4-D may induce subtle alterations at the cell and molecular levels. The results also serve to highlight a potential mechanism of 2,4-D – induced toxicity, which may involve ROS and associated oxidative stress. Thus, the results of the present study imply that low environmental concentrations of 2,4-D may only have minimal impact on the health of exposed humans.

Several interesting conclusions can be drawn from results of the present study. Firstly, it can be concluded that that low environmental concentrations (0.1 to 100nM) of 2,4-D induces limited, but significant cytotoxic effects that does not become more pronounced with increasing concentrations or times. However, it should be mentioned that although the present study illustrates the low environmental 2,4-D concentrations induces statistically significant cytotoxic effects; the effects may not be biologically significant. Exposure to 2,4-D induced cell specific effects, that were reflected by the more pronounced decreases that were observed in HEK293, compared to HepG2 cell viability, *in vitro*. Exposure to either technical or commercial 2,4-D induced similar cytotoxic effects. Concluding that toxicity is induced directly by 2,4-D and that the presence of 'inert ingredients' in commercial 2,4-D had limited ability to act synergistically with 2,4-D and enhance the magnitude of cytotoxic effects. Compared to higher concentrations, exposure to low environmental concentrations of 2,4-D does not induce significant intracellular ROS production. The results also highlight a role for

ROS and associated oxidative stress in 2,4-D – induced cytotoxicity. Preliminary results revealed that low environmental concentrations of 2,4-D are sufficient to induce subtle alterations (< 2 fold) in FTL, FTH1 and PCNA gene expression patterns at the levels of mRNA and protein accumulation; an interesting revelation in the present study that also serve to highlight underlying cell and molecular pathways of 2,4-D – induced cytotoxicity that involves ROS and associated oxidative stress. The results also provide direction for future studies aimed at elucidating a molecular pathway involved in 2,4-D – induced cytotoxicity. Thus, taken all together, the results reported in this thesis suggest that exposure to low environmental concentrations of the herbicide, 2,4-D in groundwater may have a minimal impact on the health of exposed humans.

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