Chapter 5: Oxidative Stress

5.1. Background

ROS are unstable molecules, which make them highly reactive. These molecules are naturally generated in small amounts during metabolic reactions of the body. ROS can react with and damage cellular molecules such as lipids, proteins and DNA. Four chemical reactions have been reported through which reactive molecules can modify other molecules and include (Wu and Cederbaum, 2003):

- *Hydrogen abstraction:*

  A radical reacts with a hydrogen donor, to yield a stable molecule, which in turn makes the hydrogen donor reactive.
- *Addition:*

  A radical binds to an originally stable molecule converting the combined molecule into a reactive one.
- *Termination:*

  Two radicals react with each other to form a stable compound.
- *Disproportionation:*

  A radical reacts with another identical radical and donates its unpaired electron to the other, thereby yielding two stable compounds.

Under certain conditions the production of ROS is enhanced and/or the level or activity of antioxidants is reduced. The resulting state, which is characterized by a disturbance in the balance between ROS production, on the one hand and ROS removal and repair of damaged molecules on the other, is called oxidative stress (Cederbaum *et al.*, 2009).

The principal source of ROS production in mammalian cells is the mitochondria, which produces reactive species including $\bullet O_2^-$, H_2O_2 and the highly reactive $\bullet OH$. Mitochondrial ROS are produced by the electron transport chain, which consists of four electron carrier complexes, I-IV. Electrons derived from metabolic reducing equivalents are fed into the

electron transport chain through either complex I or complex II, and eventually pass to molecular O_2 to form H_2O in complex IV (Liu *et al.*, 2002). Theoretically, all of these complexes are able to transfer electrons to O_2 to form $\bullet O_2^-$, which is quickly dismutated to H_2O_2 by mitochondrial superoxide dismutase. However, studies have shown that mitochondrial ROS originate mainly from carrier complexes I and III (Turrens and Boveris, 1980; Turrens *et al.*, 1985).

Another major source of ROS, especially in the liver, is the membrane-bound microsomal monooxygenase system (MMO) (Wu and Cederbaum, 2003). This system, which catalyses the oxygenation of a wide variety of exogenous and endogenous compounds, contains CYPs as the terminal oxidases. Monooxygenation reactions typically require the input of two electrons that are transferred to P450 by the flavoprotein NADPH-P450 reductase but may also come from cytochrome b_5, a small hemoprotein, and its NADH-dependent reductase. The efficiency of electron transfer from NADPH through the electron carriers to the CYP for monooxygenation of substrate is referred to as coupling (Zangar *et al.*, 2004). Although the degree of coupling of NADPH consumption to substrate oxidation varies for different P450 species, it is usually less than 50%. An uncoupled state leads to ROS production as electrons that enter the system do not end in the oxygenation of substrate but escape their natural reaction sequence, finally affecting other molecules present through one of the four reactions mentioned at the beginning of this chapter.

Results from the CYP1A1 experiments show that the test compounds in question are able to induce CYP activity. As mentioned above, MMO is a major source of ROS, especially in the liver, and the up-regulation of CYP1A1 activity is suggestive of possible increased ROS generation and a resultant state of oxidative stress. For this reason, examining DDT/DDE/DDD-induced ROS generation could illuminate the mechanism of the cytotoxicity of the three test compounds.

5.2. Methods

5.2.1. Detection of intracellular ROS by fluorometery

Nine independent endpoint fluorometry experiments were carried out in duplicate, six with the test compounds alone (*n* = 12) and three that included a 1 h NAC pre-treatment (*n* = 6). Intracellular ROS was detected according to the method described by Zhang et al. (2009), with slight modifications. Following the 48 h seeding incubation, 40 µl of 2',7'-dichlorofluorescein diacetate DCFDA (20 µM) in PBS was added to each well and incubated for 1 h at 37°C. The loading medium was then carefully removed and cells washed with 200 µl PBS. Cells were kept hydrated by the addition of 50 µl of PBS to each well followed by the addition of 50 µl of either PBS (1% DMSO), 2',2'-azobis(2-methylpropionamidine) dihydrochloride AAPH (300 µM) or test compound (10 - 300 µM) to yield final concentrations of 0.5% DMSO, 150 µM AAPH and 5 - 150 µM of the relevant test compounds. Cells were incubated for 3 h before fluorescence was measured on a FluoStar Optima fluorescent plate reader using λ_{ex} = 492 nm and λ_{em} = 525 nm at a gain setting of 750. AAPH was used as positive oxidant control (Ximenes *et al.*, 2009).

5.2.2. Detection of intracellular ROS by flow cytometry

To confirm the findings obtained with fluorometry, ROS detection with DCFDA was repeated using flow cytometry. After harvesting cells by trypsinisation, 1×10^7 cells were pooled into a single centrifuge tube and incubated with 5.7 µM DCFDA in 1 ml EMEM for 1 h at 37°C. This was done to ensure that all the cells for a particular experiment were pre-loaded with the same concentration of the oxidant sensitive dye.

While the cells were being incubated to pre-load with DCFDA, 5 µl of DMSO (vehicle solvent), AAPH (30 mM) or test compound (1 - 30 mM), were added to individual flow cytometry tubes.

Following DCFDA pre-loading, cells were diluted to 2×10^5 cells/ml (same cell density as used in all other experiments) using PBS. Of this cell suspension, 1 ml was added to each of the prepared flow cytometry tubes. Cells were then incubated for 3 h at 37°C, after which the fluorescence was measured using channel FL1 (525 nm) on a Beckman Coulter FC500 flow cytometer. A total number of 5000 events were recorded per sample, from which a mean was obtained. Three individual experiments on individual batches of cells were performed.

5.2.3. Kinetic evaluation of ROS detected by fluorometry

To determine whether any ROS generation occurred over a longer exposure period, kinetic experiments were conducted. As with fluorometric detection (described in *Section 5.2.1.*), cells were loaded with 5.7 µM DCFDA for 1 h at 37°C. The solution was then carefully aspirated and the cells washed with PBS. This was followed by the addition of 50 µl PBS and an additional 50 µl of PBS containing either DMSO (1%), AAPH (300 µM) or the relevant test compound (0.2 - 200 µM) to each well. Final concentrations of DDT, DDE and DDD were 0.01, 0.1, 1, 10 and 100 µM (0.5% DMSO final concentration). The initial concentration range was broadened to allow for the detection of ROS below 5 µM of test compound in order to determine whether lower concentrations may induce any significant ROS generation. After adding the test compounds and controls, fluorescence was determined at 30 min intervals over a period of 14 h at 37°C. Fluorescence was detected on a FluoStar Optima fluorescent plate reader using λ_{ex} = 492 nm and λ_{em} = 525 nm at a gain setting of 750.

5.2.4. Statistical analyses

Nine independent endpoint fluorometry experiments were carried out in duplicate, six with the test compounds alone (***n* =** 12) and three that included a 1 h NAC pre-treatment (***n* =** 6). Outliers were detected using Grubb's test and removed, before normality of the data distributions were evaluated with the Shapiro-Francia test. Hypothesis testing was then

performed utilising either Student's ***t***-tests or Mann-Whitney tests, to determine whether any observable differences between means were statistically significant. In addition to the endpoint ROS experiments, three independent flow cytometry experiments (***n* =** 3) and three independent kinetic experiments in quadruplicate (***n* =** 12) were performed. Flow cytometry results were tested using only Mann-Whitney tests since only three observations were available for scrutiny. All results, except those from kinetics experiments, were standardized to percentage of control and are reported as Mean ± SEM. For kinetic experiments, raw data was analysed using a two-way analysis of variance with a Bonferroni *post hoc* test to compare all groups to the control groups.

The possible effects that NAC may have on test compound-induced changes in intracellular ROS were also determined. These results were standardised to percentage of control but no preliminary tests (Grubb's and Shapiro-Francia) were performed because of the small sample size (***n* =** 6). Therefore, Mann-Whitney tests were performed without removal of outliers.

5.3. Results

5.3.1. Endpoint fluorometry

Three of the six groups of data in the DDT data set contained outliers with ***p*** < 0.05 according to Grubb's test. Similarly, 3 different groups in the DDE data set contained outliers, while the DDD data set contained 4 groups with outliers (Table 5.1). A maximum of 2 outliers were removed per group. Hypothesis testing was then performed on a minimum of 10 observations. Most of the data presented with normal distributions according to the Shapiro-Francia test (Table 5.2). Only the 50 µM group of DDT and the 10 µM group of DDE were not normally distributed, accordingly these were analysed with Mann-Whitney tests.

Table 5.1. Grubb's test results for detecting outliers in the observed ROS data. Values given in the table are p-values. Instances where $p < 0.05$ (*) indicates the presence of outliers.

Concentration	DDT	DDE	DDD
Control	0.35	0.08	0.01*
5 µM	0.05	0.00*	0.02*
10 µM	0.01*	0.00*	0.00*
50 µM	0.00*	0.06	0.00*
100 µM	0.02*	0.00*	0.08
150 µM	0.11	0.56	0.10

Table 5.2. Shapiro-Francia test normality results of the observed ROS data after removal of outliers detected with Grubb's test. Values given in the table are p-values. Instances where $p < 0.05$ are significantly non-normal. * indicates $p < 0.05$.

Concentration	DDT	DDE	DDD
Control	0.98	0.18	0.13
5 µM	0.23	0.27	0.91
10 µM	1.00	0.03*	0.29
50 µM	0.01*	0.19	0.61
100 µM	0.27	0.36	0.26
150 µM	0.19	0.45	0.64

The positive control (AAPH) yielded the large expected increase in ROS generation when compared to vehicle only treated controls (Table 5.3 and Figure 5.1). Fluorometric evaluation showed no ROS generation in HepG2 cells exposed to DDT for 3 h. Rather, results showed a trend of decreasing ROS with an increase in DDT concentration (Figure 5.3A). A

significant ($p < 0.05$) deviation from the vehicle control was only detected at the highest concentration of DDT (150 µM) where a 30% decrease in intracellular ROS levels occurred.

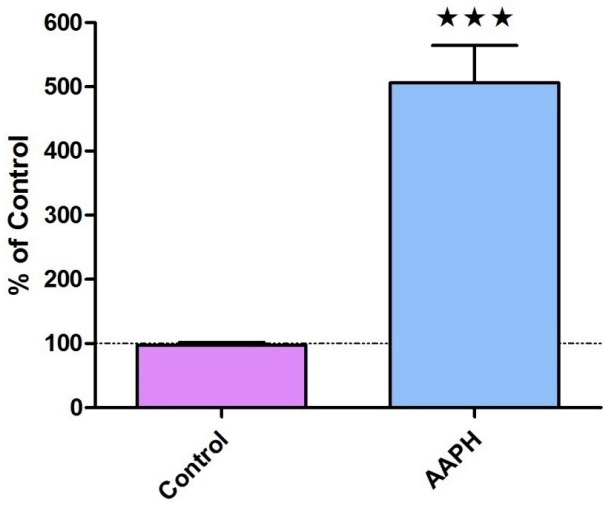

Figure 5.1. Generation of H_2O_2 in HepG2 cells following 3 h exposure to vehicle control *vs.* AAPH (150 µM) using DCFDA as ROS probe (mean ±SEM) as detected by fluorometry. *** = $p < 0.001$.

DDE exposure also decreased the amount of intracellular ROS following a dose-dependent trend. This was more pronounced than in cells exposed to DDT at all of the tested concentrations, except 50 µM, causing a significant decrease in the detected ROS of approximately 40% ($p < 0.01$), compared to controls. Similar to the other tested compounds, DDD did not induce ROS generation. DDD also produced a decrease in ROS with significant reductions at concentrations of 50 and 100 µM DDD with $p < 0.01$ and $p < 0.05$, respectively.

Table 5.3. ROS generation in HepG2 cells following 3 h exposure to DDT, DDE, DDD and AAPH (positive control). Results (% of Control) are presented as mean ±SEM. * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as determined by Mann-Whitney tests.

Concentration	DDT	DDE	DDD	AAPH (150 µM)
Control	100 ± 8	100 ± 10	92 ± 4	
5 µM	97 ± 12	62 ± 7**	73 ± 10	
10 µM	84 ± 10	59 ± 9**	87 ± 12	506 ± 58***
50 µM	87 ± 9	75 ± 12	64 ± 7**	
100 µM	81 ± 11	53 ± 10**	62 ± 10*	
150 µM	69 ± 11*	61 ± 9**	76 ± 11	

Table 5.4. ROS generation in HepG2 cells due to DDT, DDE, DDD, with or without 1 h pre-treatment with NAC. There were no statistically significant differences between cells pre-treated with NAC and those that were exposed to test compounds only.

	DDT		DDE		DDD	
	--	NAC	--	NAC	--	NAC
5 µM	97 ± 12	93 ± 12	62 ± 7	72 ± 8	73 ± 10	94 ± 12
10 µM	84 ± 10	87 ± 14	59 ± 9	68 ± 6	87 ± 12	87 ± 10
50 µM	87 ± 9	89 ± 12	75 ± 12	81 ± 15	64 ± 7	97 ± 24
100 µM	81 ± 12	93 ± 12	53 ± 10	73 ± 7	62 ± 10	89 ± 11
150 µM	69 ± 10	84 ± 10	61 ± 9	75 ± 6	76 ± 11	82 ± 11

NAC pre-treatment had no significant effect on test compound-induced changes in intracellular ROS levels. There is a trend in the results that NAC pre-treatment appeared to inhibit the reductions in ROS levels induced by the test compounds (Table 5.4 and Figure 5.5).

5.3.2. Flow cytometry

The positive control, AAPH, yielded expected results by significantly ($p < 0.001$) inducing ROS. AAPH produced a 2-fold increase in ROS generation when compared to the vehicle controls (Figure 5.2). Flow cytometry results support the fluorometric findings in that no significant increase in ROS generation was detected after DDT exposure. None of the flow cytometry results were significantly different from controls. A slight increase in ROS occurred in the 5 µM of DDT group but this was not a statistically significant elevation (Figure 5.3D). Flow cytometry indicated no ROS generation after DDE exposure. Similar to cells exposed to DDT, there may be a trend of decreased ROS with an increase in DDE concentration (Figure 5.3E). None of the flow cytometry results showed any significant deviation from control values. No significant DDD-induced increase in ROS generation was detected by flow cytometry but, as with DDT, results do show a slight increase in ROS generation in cells exposed to 5 µM of DDD (Figure 5.3F). A similar trend to the other test

compounds was seen, with reduced ROS production associated with increases in DDD concentration. However, none of these observations were significant (Figure 5.3F).

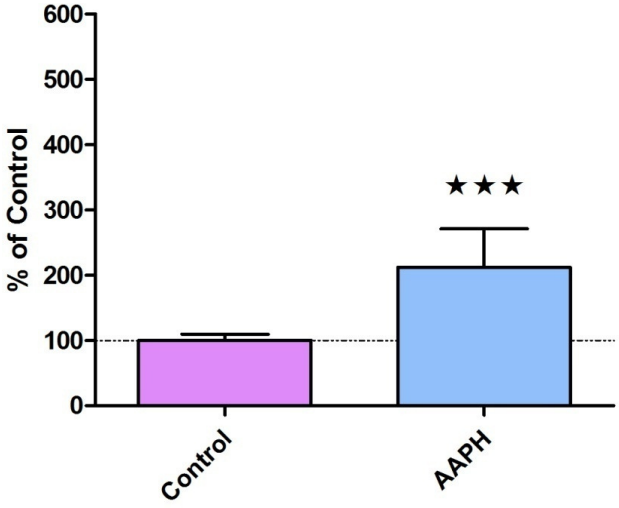

Figure 5.2. Generation of H_2O_2 in HepG2 cells following 3 h exposure to vehicle control *vs.* AAPH (150 μM) using DCFDA as ROS probe (mean ±SEM) as detected by flow cytometry. *** = $p < 0.001$.

5.3.3. Kinetic fluorometry

Kinetic evaluation of AAPH-induced ROS generation showed a sharp increase in ROS generation up to ≈ 6 h exposure, after which the rate of ROS generation decreased, reaching a plateau. AAPH significantly ($p < 0.01$) elevated intracellular ROS from 2 h of exposure onwards, compared to vehicle controls (Figure 5.4). As evident from Figure 5.4, no increase or decrease in ROS generation was observed when cells were exposed to DDT, DDE or DDD over a period of up to 14 h.

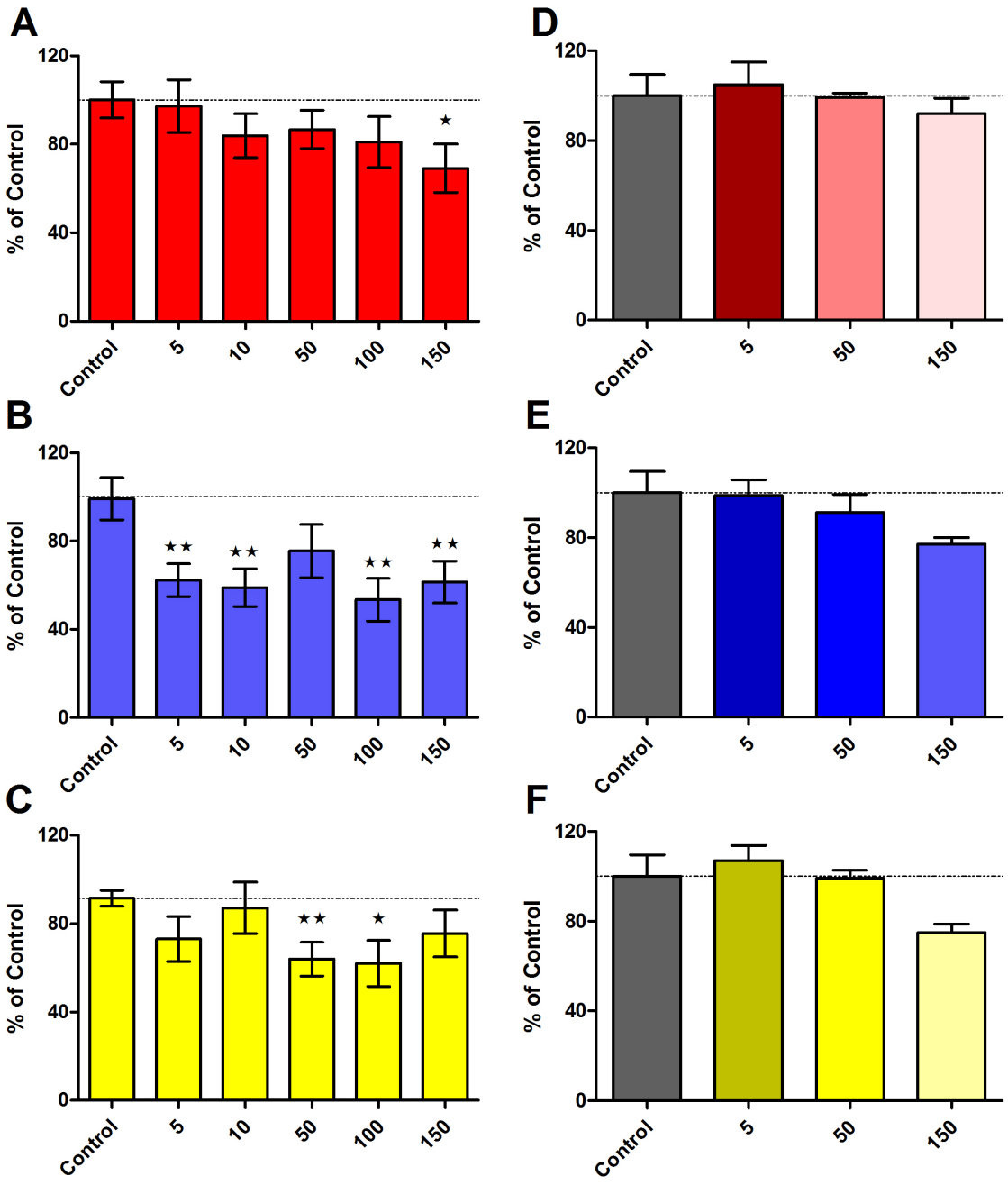

Figure 5.3. Fluorometric detection (endpoint) of H_2O_2 in HepG2 cells following 3 h exposure to DDT (A), DDE (B) and DDD (C) (mean ±SEM). * indicates $p < 0.05$ and ** $p < 0.01$ as determined by Mann-Whitney tests. Graphs (D), (E) and (F) illustrate the corresponding flow cytometry results of DDT, DDE and DDD, respectively. Dashed horizontal lines represent control values.

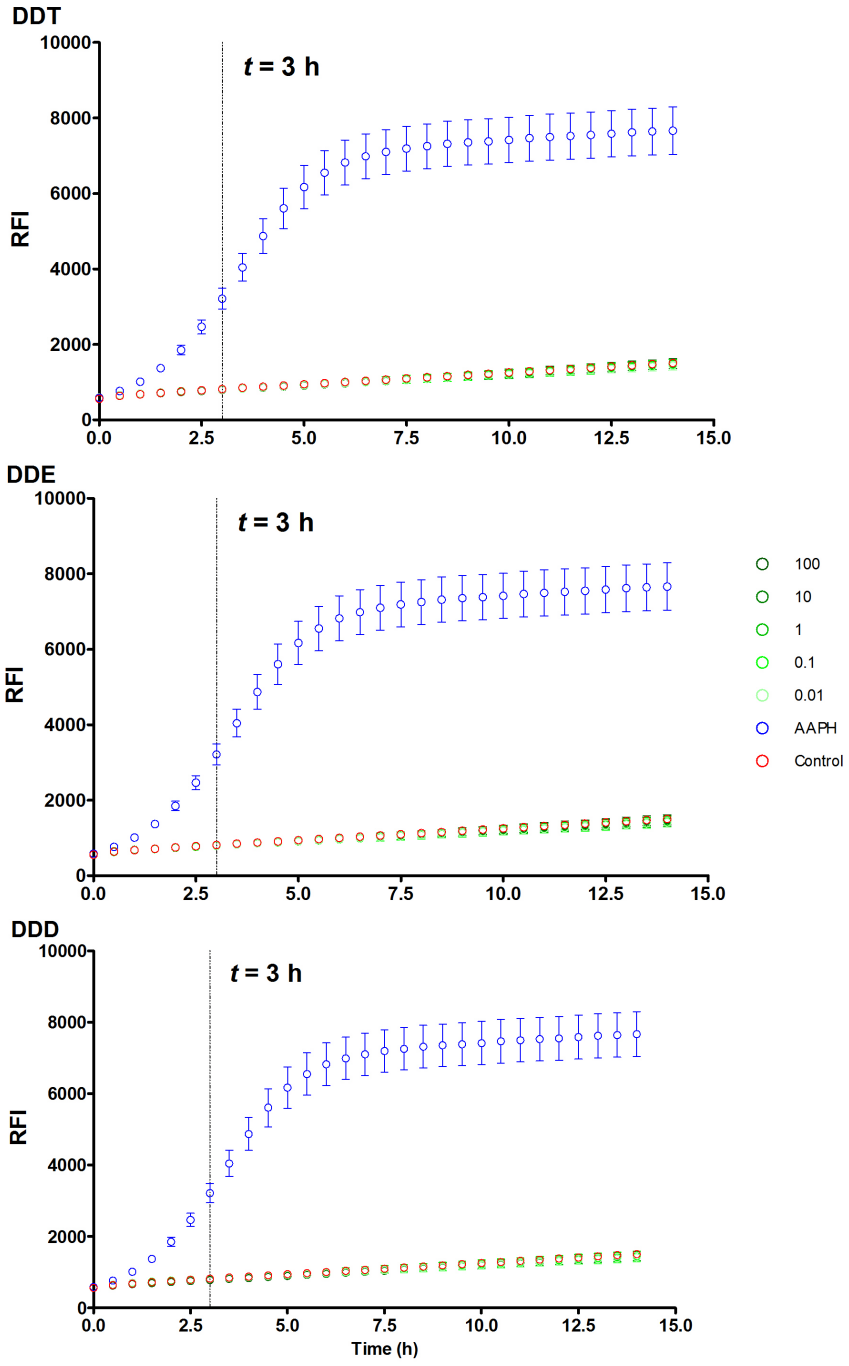

Figure 5.4. Raw data (no data manipulation) from three independent experiments showing H_2O_2 generation in HepG2 cells following exposure to various concentrations of DDT, DDE and DDD over a 14 h incubation period (mean ±SEM). Dashed vertical lines represent X = 3 h, which is the incubation period used in all other experiments. RFI = relative fluorescence intensity. AAPH (150 μM) alone induced significant ($p < 0.001$) ROS generation from 2 h onwards. All test compounds at all tested concentrations showed no significant difference from the negative control values for the same time.

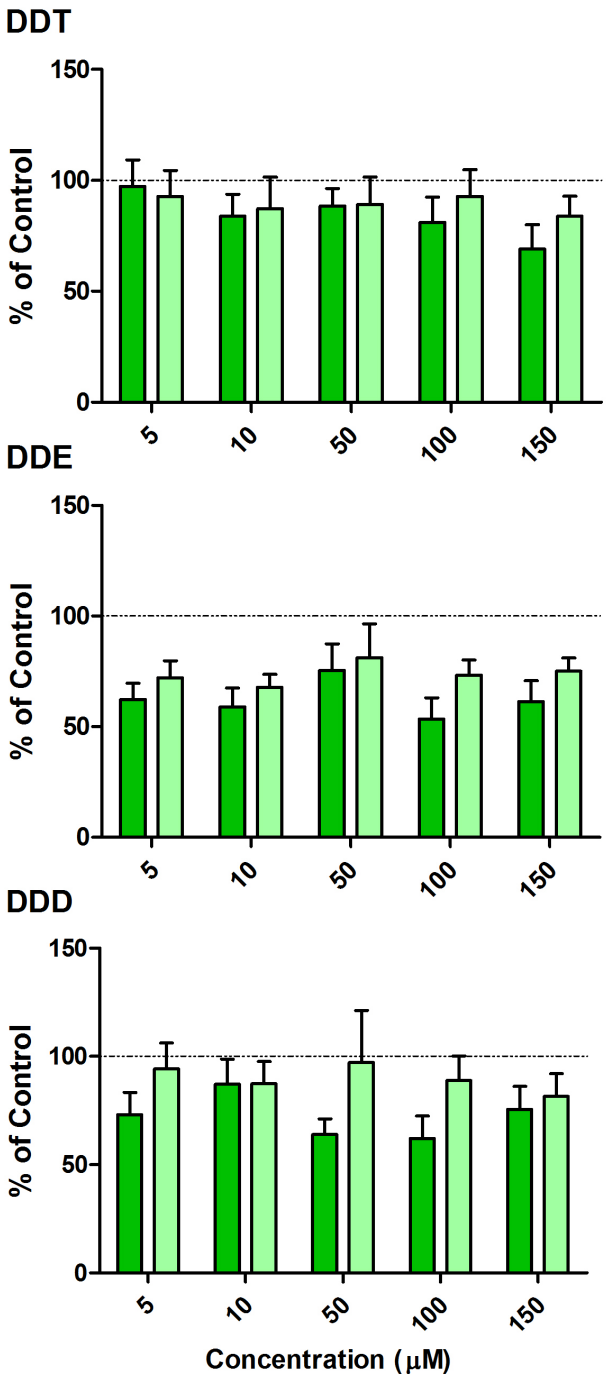

Figure 5.5. Generation of H_2O_2 in HepG2 cells following 3 h exposure to DDT, DDE and DDD (mean ±SEM), without (dark green bars) or with (light green bars) 1 h NAC pre-treatment. No significant differences were detected.

5.4. Discussion

Following the removal of outliers with the Grubb's test (Table 5.1), only 2 of the 18 data sets presented non-normal distributions (Table 5.2), indicating good reproducibility.

When comparing the results of the fluorometric and flow cytometric methods of detection of AAPH-induced ROS generation using DCFDA, fluorometry detected a 5-fold increase in fluorescence compared to a 2-fold increase observed with flow cytometry (Figure 5.1 and Figure 5.2). This would suggest that the fluorometry method is a more sensitive method than flow cytometry. However, fluorometric evaluation demonstrates a different inhibitory trend to that seen with flow cytometry (Figure 5.3).

Regarding the effect of the three test compounds on ROS levels, previous *in vitro* studies using DCFDA as probe have demonstrated DDT-induced ROS generation in various types of cells. Using fluorometry, researchers have reported DDT-induced ROS in skin tumour (Ruiz-Leal and George, 2004) and U937 monocyte lymphoma cells (Sciullo *et al.*, 2010). Studies reporting DDT-induced ROS in hepatocytes (Filipak Neto *et al.*, 2008; Shi *et al.*, 2010a) have used flow cytometry. The study by Filipak Neto *et al.* (2008) used primary hepatocytes derived from a fish species. In the human-derived L-02 hepatocyte cell line, a significant increase in intracellular ROS was observed after 24 h exposure to 10 nM and 100 nM of DDT (Shi *et al.*, 2010a). The effects of DDE and DDD on ROS generation in hepatocytes have not been reported previously. However, they have been shown to induce ROS generation in peripheral blood mononuclear cells (Perez-Maldonado *et al.*, 2004) and primary rat Sertoli cells (Song *et al.*, 2008).

In the present study neither fluorometric, nor flow cytometric methods detected any significant ROS generation caused by DDT, DDE or DDD exposure (5 - 150 µM), which may be considered contradictory to reported literature. However, flow cytometry did reveal a small increase in ROS generation at the lowest tested concentration of DDT and DDD (5 µM), which could suggest that concentrations of DDT and DDD lower than 5 µM may induce more ROS generation (Figure 5.3A). To test this hypothesis, a very wide concentration range

(0.01 - 100 µM) of all three test compounds were tested in three independent kinetic-type fluorometry experiments, each spanning 14 h of exposure, in an attempt to reproduce the reported ROS generation. The chosen concentration range included concentrations of 10 and 100 nM, which has previously been reported to induce ROS generation in cultured hepatocytes (Shi *et al.*, 2010a). Together with flow cytometry results, these experiments using the extended concentration range supported the initial endpoint fluorometry results in that no ROS generation was detected in cells exposed to any of the test compounds (Figure 5.3).

Contradictory to their first article (Filipak Neto *et al.*, 2008), a second publication reported that 50 nM of DDT caused a significant decrease in intracellular ROS in primary hepatocytes (Bussolaro *et al.*, 2010). Although the same methodology was applied, hepatocytes originating from a different fish species were employed, which could indicate a species-specific response to DDT in terms of intracellular ROS generation. This may explain why ROS generation was not seen with HepG2 cells in the present study whereas ROS generation was seen in L-02 cells (Shi *et al.*, 2010a). Although both cell lines are of human origin, the one is cancerous (HepG2) and the other not (L-02) (Guo *et al.*, 2007) so they are likely to present with different genotypic and phenotypic features and could therefore respond differently.

Another explanation for the apparent discrepancy could be the duration of exposure. In the studies conducted by Filipak Neto *et al.* (2008) and Shi *et al.* (2010a), the authors exposed hepatocytes to DDT for periods of 4 days and 24 h, respectively. Morel *et al.* (1999), who specifically studied CYP1A1-generated ROS in HepG2 cells, exposed cultures for 30 h to benzo(a)pyrene, a well-known CYP1A1 inducer, in order to detect ROS generation. In the present study cells were only exposed for 3 h, which failed to yield any elevated intracellular ROS. For this reason, the kinetics experiments were conducted over a 14 h period, but still no ROS was detected. This may indicate that the DDT-induced ROS generation seen in previous studies had its origin from the MMO system rather than mitochondria. It was shown in this study that DDT induces CYP1A1 activity, which forms part of the MMO system of enzymes. Considerable time is required for CYP1A1 up-regulation to manifest intracellularly as the relevant receptors need to be activated and translocated to the nucleus, after which transcription needs to occur, followed by translation and finally protein

synthesis. If DDT-induced ROS generation is the result of high MMO activity, all of the afore-mentioned processes need to take place before any detectable increase in ROS will present itself. If DDT-induced ROS generation was due to mitochondrial uncoupling, this would manifest much faster and would probably be detectable within 14 h of exposure as was seen with AAPH-induced ROS, which reached a maximum plateau after approximately 6 h of exposure.

CYP1A1 and intracellular ROS are also related to each other in terms of a negative-feedback autoregulatory loop, in which CYP1A1, that generates ROS during its catalytic cycle, is regulated by cytosolic ROS levels, specifically H_2O_2 (Morel and Barouki, 1998; Morel *et al.*, 1999; Barouki and Morel, 2001). ROS regulation of CYP1A1 expression occurs at a transcriptional level. The aryl hydrocarbon receptor (AhR), AhR nuclear translocator (Arnt) and Nuclear Factor I (NFI) are all required for activation and transcription of the *cyp1a1* gene and studies have shown that synergy between AhR-Arnt complex and NFI as well as NFI integrity is diminished in the presence of H_2O_2 (Morel *et al.*, 1999). The fact that CYP1A1 up-regulation was observed only after 24 h exposure to the test compounds (*Chapter 4*) provides further support for the fact that DDT does not induce ROS through a rapidly activated pathway, as observed in the present study.

NAC pre-treatment for 1 h had no significant influence on the test compound changes in intracellular ROS (Table 5.4 and Figure 5.4), which is not unexpected as initial results indicated that none of the test compounds induced ROS in the first place. This provides further support in concluding that none of the tested compounds induce ROS generation in HepG2 cells following a short 3 h exposure.

Using fluorometric detection, no elevated intracellular ROS levels were observed for any of the test compounds at any of the tested concentrations after 3 h exposure. This was confirmed by flow cytometry. After extending both the exposure period to 14 h and widening the concentration range from 0.01 – 100 µM, still no elevations in ROS levels were observed. These findings suggest that the test compounds do not induce ROS generation in these cells by a rapidly inducible reaction pathway.