Chapter 6: Mitochondrial Toxicity

6.1. Background

Mitochondria are the main source of chemical energy in a cell. They provide more than 15 times the ATP than that generated by anaerobic glycolysis (Alberts *et al.*, 2002). Mitochondria generate ATP through the action of ATP-synthase. The respiratory complexes I, III and IV of the electron transport chain are responsible for actively transporting H^+ ions into the mitochondrial intermembrane space (Figure 6.1). ATP-synthase then uses the backflow of H^+ ions from the mitochondrial intermembrane space into the mitochondrial cytosol as a driving force to synthesise ATP from adenosine diphosphate and inorganic free phosphate. Due to the constant active efflux of H^+ from the mitochondrial cytosol into the intermembrane space, a difference in H^+ concentration across the inner membrane develops, which creates an electrochemical gradient known as the mitochondrial membrane potential ($\Delta\psi_m$) (Hutteman *et al.*, 2008). In addition to H^+ re-entry into the mitochondrial matrix via ATP-synthase, all mitochondria also possess a parallel endogenous H^+ leak (Nicholls, 1977). Under normal physiological conditions the H^+ leak may serve an important purpose in limiting the $\Delta\psi_m$ to prevent dielectric breakdown of the membrane (Brand *et al.*, 1994; Rolfe and Brand, 1997).

The $\Delta\psi_m$ lies at the heart of all the major bioenergetic functions of the mitochondrion, as it provides a force that drives the influx of H^+, from the intermembrane space, by simply moving it into the mitochondria down the electrochemical potential gradient. The actions of the respiratory complexes as H^+ translocators also means that the respiratory rate is regulated by $\Delta\psi_m$ in that respiration will run faster when the membrane is depolarised as a thermodynamic consequence of the reduced energy required to move H^+ out of the intermembrane space, and will run more slowly if the membrane is hyperpolarised (Duchen, 2004).

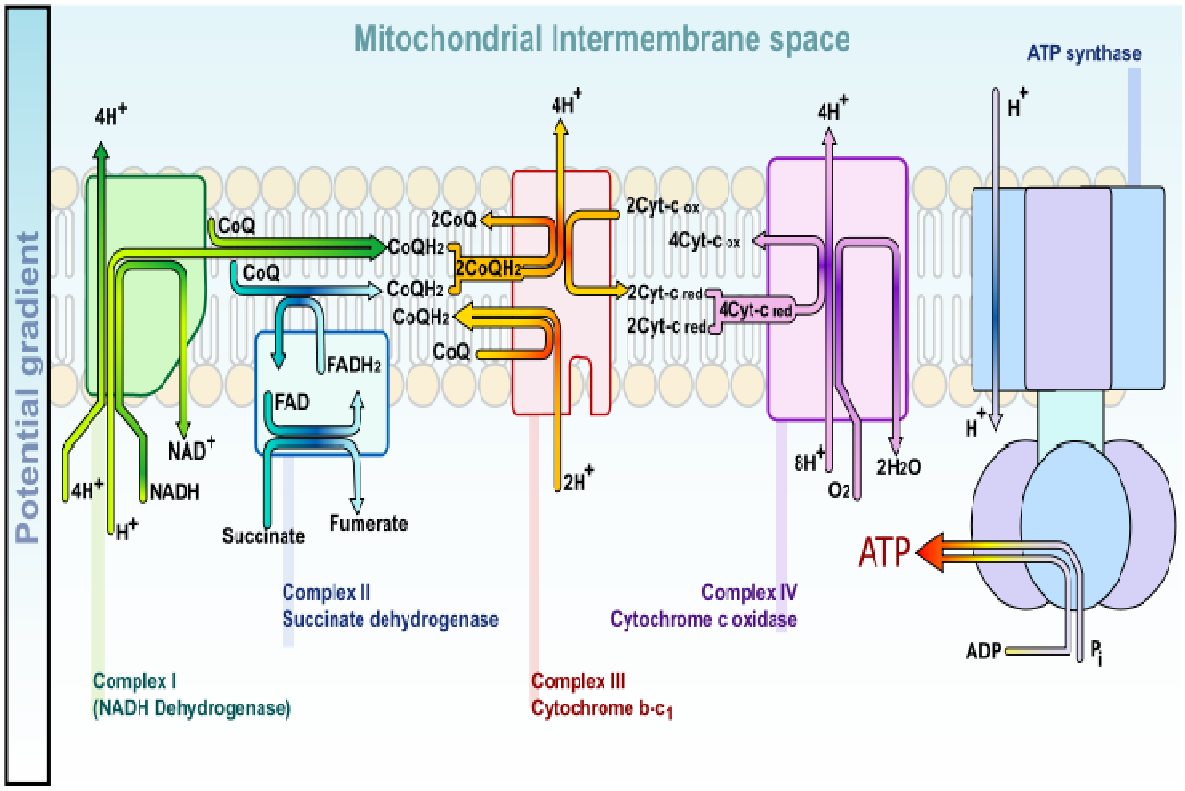

Figure 6.1. **Illustration of the electron transport chain and ATP-synthase embedded in the inner mitochondrial membrane. Electrons enter the system via reduced nicotinamide adenosine dinucleotide (NADH) and reduced flavin adenosine dinucleotide ($FADH_2$). As electrons are transferred from one respiratory complex to the next, H+ ions are driven from the mitochondrial cytosol into the intermembrane space. O_2 is the final electron receptor, which is reduced to H_2O. ATP-synthase is coupled to this system by the backflow of H+ through the proton channel of ATP-synthase, an ATPase that works in backwards, forming ATP from ADP and inorganic phosphate (Duchen, 2004) (Figure adapted from Wikipedia, 2011).**

$\Delta\psi_m$ typically ranges from -150 mV to -180 mV under normal physiological conditions. This value is negative since it is the electrochemical potential relative to the cell cytoplasm (Perry *et al.*, 2011). Cationic dyes, such as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), have been used to evaluate $\Delta\psi_m$ in HepG2 cells (Nerurkar *et al.*, 2004; Perry *et al.*, 2011). As a positively charged molecule, this dye accumulates in mitochondria in inverse proportion to the $\Delta\psi_m$. JC-1 is unique in its ability to exist in two states, each with its own excitation-emission spectrum. When the dye is present in high concentrations within mitochondria, due to a highly negative $\Delta\psi_m$, the dye

forms J-aggregates with λ_{em} = 590 nm and when present at low concentrations, due to low $\Delta\psi_m$, the dye exists as J-monomers with λ_{em} = 520 nm (Gravance *et al.*, 2000).

Many of the cytotoxicity assays used by authors in previous attempts to detect hepatotoxicity are non-specific and fail to detect specific types of toxicity, such as mitochondrial toxicity, particularly when cell viability is not affected (Farkas & Tannenbaum, 2005). A good example of this is troglitazone, an antidiabetic drug, which was commercialised after pre-clinical *in vitro*, *in vivo* as well as clinical testing but was eventually withdrawn because it proved to be hepatotoxic due to mitochondrial toxicity that was not detected during early drug development (Labbe *et al.*, 2008). Troglitazone toxicity highlighted the importance of evaluating the mitochondrial status of cells following exposure to a certain compound. As mitochondrial effects can have an important role in cell toxicity and cell death it is prudent to assess the mitochondrial status during cytotoxicity testing as was done in the present study.

6.2. Methods

6.2.1. Evaluation of $\Delta\psi_m$ using JC-1

$\Delta\psi_m$ was evaluated using the method of Nuydens *et al.* (1999), with slight modifications. Following the 48 h seeding incubation, a 100 µl of JC-1 (20 µM) in EMEM was added to each well and cells loaded with the dye for 30 min at 37°C. Loading medium was then discarded and cells washed with 200 µl PBS. To keep cells hydrated, 50 µl of PBS was added to each well. This was followed by the addition of 50 µl of either PBS (1% DMSO), Tamoxifen (300 µM) or test compound (10 - 300 µM) to yield final concentrations of 0.5% DMSO, 150 µM Tamoxifen and 5 - 150 µM of the relevant test compounds, all diluted in PBS. Cells were then exposed for 1 h before fluorescence was measured on a FluoStar Optima at both λ_{ex} = 492 nm and λ_{em} = 525 nm (monomeric JC-1 form) and λ_{ex} = 545 nm and λ_{em} = 595 nm (aggregate JC-1 form) at a gain setting of 1000.

6.2.2. Statistical analyses

Six independent fluorometry experiments were carried out in duplicate (***n*** = 12). Relevant blank values were deducted from all values before the ratio of 595 nm/525 nm fluorescence intensities was calculated, which is used as an indication of $\Delta\psi_m$ - the more intense the fluorescence at 595 nm, the more J-aggregates are present indicating a high $\Delta\psi_m$, and *vice versa* (Nuydens *et al.*, 1999; Nerurkar *et al.*, 2004; Perry *et al.*, 2011). Data 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 (normal) or Mann-Whitney tests (non-normal), to determine whether any observable differences between means were statistically significant. Results were standardized to percentage of control and are presented as Mean ± SEM.

Three additional experiments were carried out in duplicate to assess the possible effects that NAC may have on pesticide-induced changes in $\Delta\psi_m$. These results were also standardised to percentage of control but no preliminary tests (Grubb's and Shapiro-Francia) were performed. Mann-Whitney tests were performed due to the small sample size (***n*** = 6), no outliers were removed and normality of the data could not be established.

6.3. Results

One group out of each of the data sets contained outliers (Table 6.1). After removal, data distributions were tested for normality, which revealed that the data was normally distributed except for one group from both the DDT (5 µM) and DDD (100 µM) data sets (Table 6.2). Accordingly, these data groups were analysed using Mann-Whitney tests, while the rest of the data were analysed with Student's ***t***-tests to determine whether any deviations from the control mean were significant.

Table 6.1. Outliers in $\Delta\psi_m$ data, detected by Grubb's test. Values given in the table are ***p***-values. Instances where $p < 0.05$ (*) indicates the presence of outliers.

Concentration	DDT	DDE	DDD
Control	0.49	0.69	0.02*
5 µM	0.00*	0.10	0.19
10 µM	0.31	0.02*	0.09
50 µM	0.20	0.05	0.24
100 µM	0.08	0.18	0.96
150 µM	0.11	0.09	0.82

Table 6.2. Shapiro-Francia test normality results of the observed $\Delta\psi_m$ 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	0.24	0.56	0.46
5 µM	0.02*	0.34	0.06
10 µM	0.34	0.15	0.58
50 µM	1.00	0.41	0.71
100 µM	0.29	0.72	0.04*
150 µM	0.89	0.93	0.08

The positive control, Tamoxifen, yielded expected results as it hyperpolarised the membrane as has been previously reported for HepG2 cells (Donato *et al.*, 2009). Tamoxifen caused an approximate 7-fold increase in $\Delta\psi_m$ compared to vehicle controls (Table 6.3 and Figure 6.2). Considerable variation was seen in Tamoxifen results but hyperpolarisation was sufficiently high enough for the results to be highly significant ($p < 0.001$) (Table 6.3).

Table 6.3. Changes in $\Delta\psi_m$ in HepG2 cells following 1 h exposure to DDT, DDE, DDD and Tamoxifen (positive control). Results (% of Control) are presented as mean ±SEM. *** = $p < 0.001$ as determined by Student's *t*-tests and Mann-Whitney tests.

Concentration	DDT	DDE	DDD	Tamoxifen (150 µM)
Control	100 ± 9	100 ± 10	100 ± 4	
5 µM	86 ± 6	89 ± 5	97 ± 5	
10 µM	86 ± 6	87 ± 6	90 ± 6	688± 199***
50 µM	101 ± 7	103 ± 6	146 ± 7***	
100 µM	145 ± 13***	164 ± 15***	301 ± 20***	
150 µM	162 ± 17***	194 ± 18***	340 ± 22***	

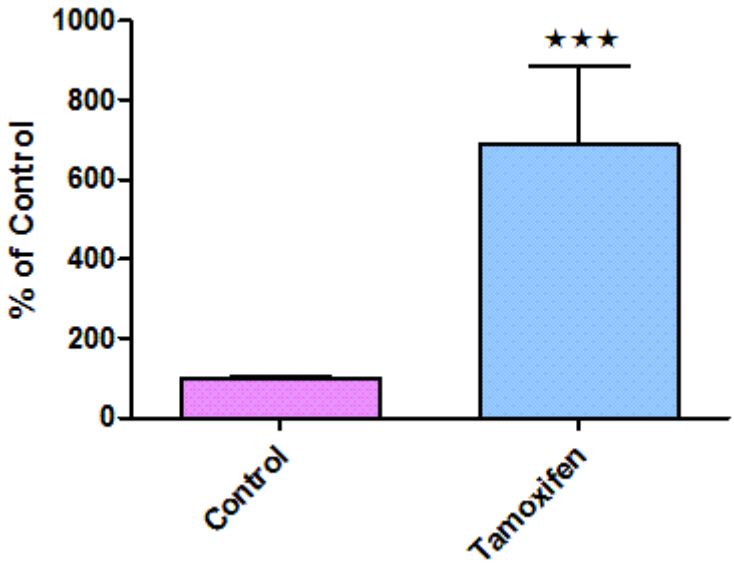

Figure 6.2. Changes in $\Delta\psi_m$ detected by JC-1 in HepG2 cells following 1 h exposure to vehicle control *vs.* Tamoxifen (150 µM) (mean ±SEM). Tamoxifen caused significant hyperpolarisation of the membrane potential with $p < 0.001$ (Student's *t*-test).

At the lower concentration range tested (5 - 10 µM), DDT reduced $\Delta\psi_m$ by 14%, although not statistically significant. At 50 µM, DDT did not affect $\Delta\psi_m$, with only a 1% difference from controls. However, highly significant increases in $\Delta\psi_m$ were seen at the higher concentration range tested, from 100 - 150 µM ($p < 0.001$), producing 45% and 62%

increases, respectively. DDT demonstrated a dose-dependent increase in $\Delta\psi_m$ from 10 - 150 µM (Table 6.3 and Figure 6.3).

A similar trend was seen with DDE. From 5 - 10 µM, there was a non-significant decrease in $\Delta\psi_m$ of approximately 12%. At 50 µM, DDE caused only a 3% increase in $\Delta\psi_m$, while 100 and 150 µM concentrations resulted in very large increases in $\Delta\psi_m$ of 64% and 94%, respectively. These increases were greater than those induced by equivalent dosages of DDT and were statistically highly significant ($p < 0.001$) (Table 6.3 and Figure 6.3).

Low concentrations of DDD (5 and 10 µM) also decreased $\Delta\psi_m$, non-significantly. However, DDD (≥ 50 µM) induced highly significant ($p < 0.001$) increases in $\Delta\psi_m$. At 50 µM, DDD caused an increase equivalent to that produced by 100 µM of DDT (46% *vs.* 45%). A 100 and 150 µM concentrations yielded 201% and 240% increases in $\Delta\psi_m$ compared to untreated controls ($p < 0.001$) (Table 6.3 and Figure 6.3).

Pre-treating cells with NAC stabilised $\Delta\psi_m$, especially at higher concentrations of the test compounds, where NAC pre-treatment significantly reduced the hyperpolarisation induced by the test compounds at concentrations of 150 µM (Table 6.4 and Figure 6.4).

Table 6.4. Changes in $\Delta\psi_m$ in HepG2 cells due to DDT, DDE, DDD, with or without 1 h pre-treatment with NAC. * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as determined by Mann-Whitney tests.

	DDT		DDE		DDD	
	--	NAC	--	NAC	--	NAC
5 µM	95 ± 11	109 ± 8*	89 ± 5	108 ± 5*	97 ± 5	99 ± 6
10 µM	86 ± 6	111 ± 8	93 ± 8	105 ± 6	90 ± 6	114 ± 16
50 µM	101 ± 7	112 ± 4	103 ± 6	132 ± 8*	146 ± 7	117 ± 5*
100 µM	145 ± 13	123 ± 6	164 ± 15	122 ± 10	301 ± 21	153 ± 5***
150 µM	162 ± 17	110 ± 11*	194 ± 18	116 ± 9**	340 ± 22	138 ± 14***

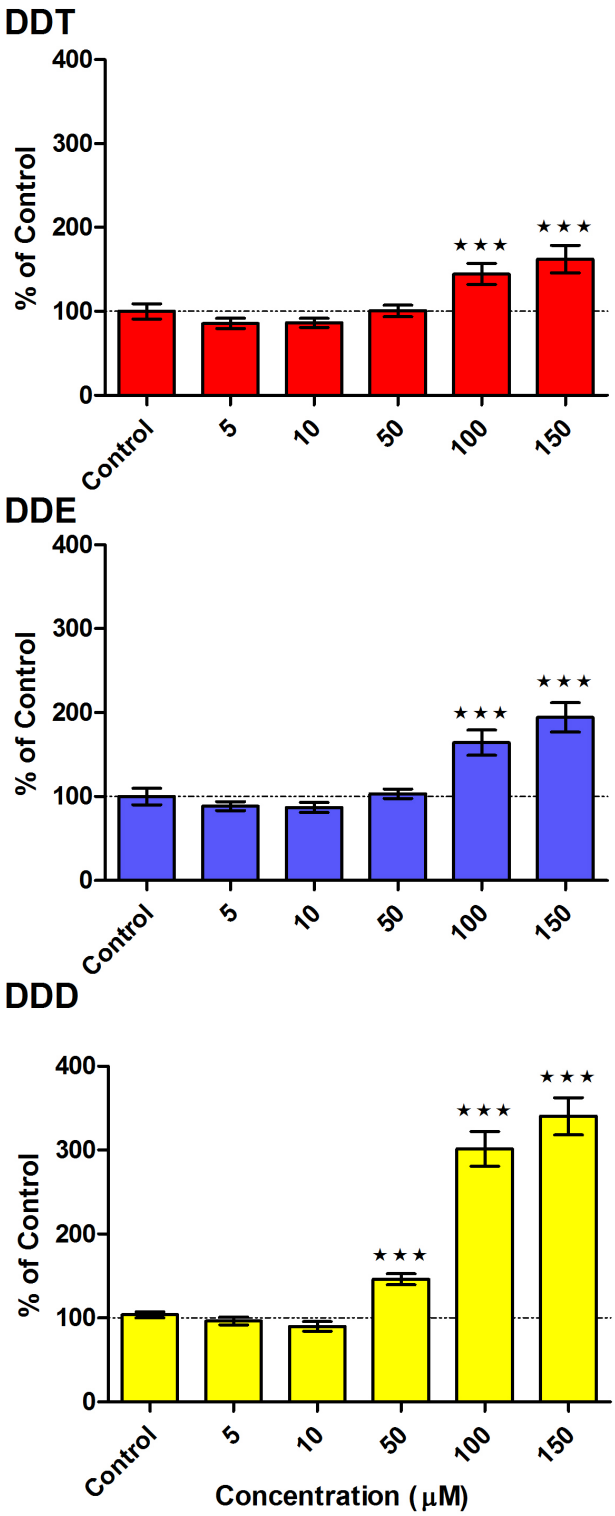

Figure 6.3. Changes in $\Delta\psi_m$ in HepG2 cells following a 1 h exposure to various concentrations of DDT, DDE and DDD (mean ± SEM) relative to untreated controls. *** = $p < 0.001$ as determined by Students *t*-tests and Mann-Whitney tests. Dashed horizontal lines represent Y = 100%.

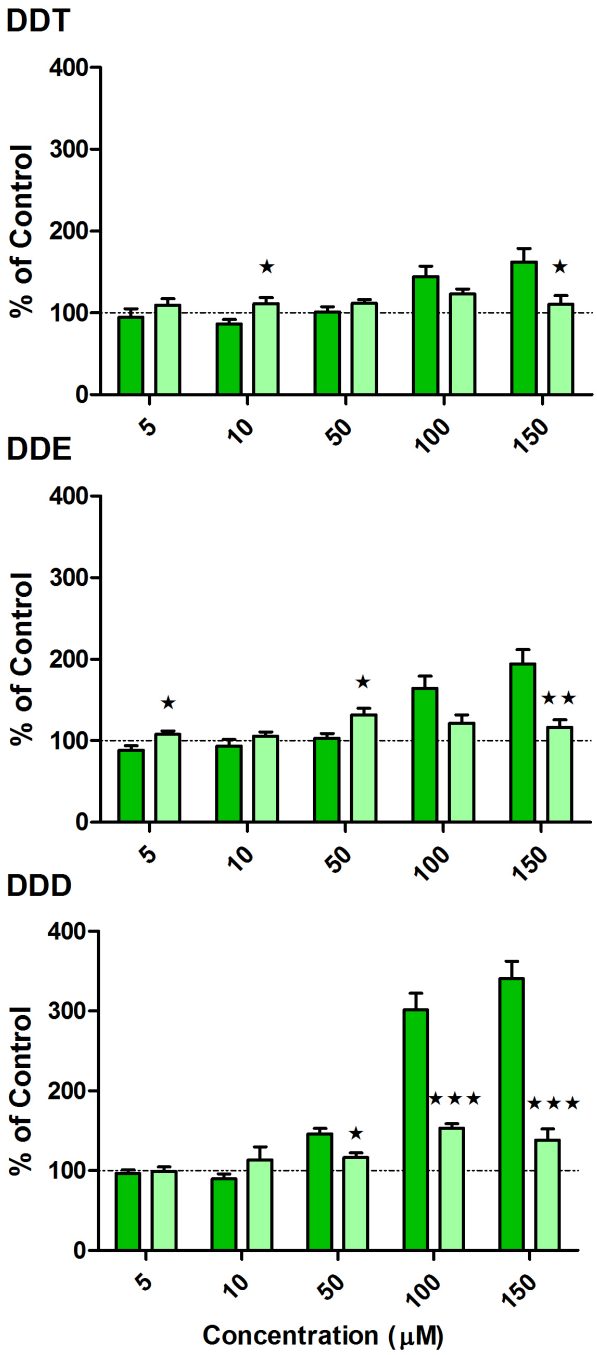

Figure 6.4. Changes in $\Delta\psi_m$ in HepG2 cells following a 1 h exposure to various concentrations of DDT, DDE and DDD relative to untreated controls. Dark green bars represent cells exposed to test compounds alone and light green bars those with a 1 h pre-treatment with NAC (mean ± SEM). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ as determined by Mann-Whitney tests. Dashed horizontal lines represent Y = 100%.

6.4. Discussion

Following the removal of outliers with the Grubb's test (Table 6.1), only 2 of the 18 data sets presented non-normal distributions (Table 6.2), indicating good reproducibility.

Exposure to the test compounds for 1 h produced similar trends across the tested concentration range with slight decreases in $\Delta\psi_m$ at 5 - 10 µM and significant increases from 100 - 150 µM. DDD was the most potent inducer of $\Delta\psi_m$ of the three test compounds.

No literature was found regarding the effects of the three test compounds on intact cells. To our knowledge this is the first report of the effects of DDT, DDE and DDD on the $\Delta\psi_m$ of intact, cultured hepatocytes. Due to the lack of literature for comparison, this discussion will focus on the possible reasons the observed effects may have occurred drawing on previous studies, which assessed the function of individual components of the respiratory chain and isolated mitochondria.

Elevations of $\Delta\psi_m$ are indicative of an increase in the slope of the electrochemical gradient formed by the difference in concentration of H^+ across the inner mitochondrial membrane. Results from this study suggest that all three compounds significantly increase the H^+ concentration in the intermembrane space. If the elicited response is the result of direct action of the compounds on the respiratory complexes involved in cellular respiration, this can occur in one of three ways, when considering oxidative phosphorylation:

1. by stimulating the electron transport chain, which in turn will drive more H^+ into the intermembrane space

2. by inhibiting the action of ATP-synthase, which would slow the backflow of H^+ ions into the mitochondrial cytosol

3. by inhibiting the H^+ leak that normally prevents mitochondrial hyperpolarisation under physiological conditions.

Mitochondrial hyperpolarisation observed in this study is not likely to be the result of stimulation of the electron transport chain as previous studies have shown that DDT is capable of inhibiting certain complexes of the respiratory chain including complex II (Nishihara and Utsumi, 1985) and complex III (Nishihara and Utsumi, 1985; Morena and Madeira, 1991). The same has been reported for DDE by Mota *et al.* (2011) who described inhibition of the same respiratory complexes in mitochondria isolated from rat liver, after exposure to DDE.

Observed increases in $\Delta\psi_m$ may have occurred due to decreased H^+ re-entry into the mitochondrial cytosol via ATP-synthase. Several studies in literature have described an inhibitory effect on mitochondrial ATP-synthase by DDT (Chefurka, 1983; Nishihara and Utsumi, 1985; Morena and Madeira, 1991; Donato *et al.*, 1997; Younis *et al.*, 2002). A similar ATP-synthase inhibitory action has also been described for DDE (Donato *et al.*, 1997; Mota *et al.*, 2011). Younis *et al.* (2002) demonstrated that DDT is capable of binding directly to the mitochondrial ATP-synthase of DDT-susceptible insects, inhibiting its action. The binding site, a 23 kDa protein segment, was not present in insects that were DDT-resistant and for this reason they suggested that ATP-synthase inhibition by DDT is the mechanism of action of DDT toxicity in these organisms. Furthermore, these authors also suggested that the 23 kDa protein segment was not present in mammalian mitochondria. In contradiction to this, Mota *et al.* (2011) reported inhibition of ATP-synthase by DDE in isolated rat liver mitochondria. It is not clear whether this inhibition in a mammalian species was due to the 23 kDa protein segment being present or some other mechanism that is independent of this protein segment. The aforementioned uncertainty leaves room for the argument that the inhibition of ATP-synthase may explain the mitochondrial hyperpolarisation observed in the present study.

Whether the results of the present study are due to the inhibition of the H^+ leak is also not clear. Inhibition of the H^+ leak could cause congestion by preventing H^+ from re-entering the mitochondrial cytosol, in this way increasing the electrochemical gradient and $\Delta\psi_m$. Mota *et al.* (2011) reported increases in the H^+ leak in rat liver mitochondria exposed to nanomolar concentrations of DDE. This may discredit the aforementioned hypothesis as an explanation of the results. However, those authors used DDE at concentrations in the nanomolar range. In the present study concentrations of DDE ≤ 10 µM also resulted in a decrease in $\Delta\psi_m$, which is in agreement with the report of Mota *et al.* (2011). Exposure to concentrations of 50 µM and higher of DDE elicited mitochondrial hyperpolarisation in the present study. Had Mota *et al.* (2011) tested higher concentrations of DDE (≥ 50 µM), they may have found inhibition of the H^+ leak due to DDE exposure.

Furthermore, an inhibitory action on the electron transport chain has some implications for ROS generation. The electron transport chain is the primary source of mitochondrial ROS, mainly from respiratory complexes I and III (Rhoads *et al.*, 2006). Stimulation of the electron transport chain may result in elevated ROS by free electron transfer to O_2, producing $\bullet O_2^-$, and H_2O_2 (Turrens and Boveris, 1980; Turrens *et al.*, 1985) (*Chapters 1* and *5*). DDT and DDE have been previously reported to inhibit the electron transport chain. For this reason it is unlikely to detect ROS generation by mitochondrial exposure to these compounds. This provides further support for the decrease in intracellular ROS observed in the present study.

With regards to the known hepatoprotectant, NAC, pre-treatment reversed the mitochondrial hyperpolarisation induced by high concentrations of each of the test compounds and stabilised $\Delta\psi_m$ (Figure 6.4). The ability of NAC to reverse changes in and stabilise $\Delta\psi_m$, has been reported before in A549 lung epithelial cells exposed to another pesticide, paraquat (Mitsopoulis and Suntres, 2011). The authors did not elaborate or speculate on how this may happen. A number of authors have reported a regulatory role of GSH in $\Delta\psi_m$ (Constantini *et al.*, 1996; Perl *et al.*, 2002; Nagy *et al.*, 2007). It is well known that NAC is a GSH precursor (Kurebayashi and Ohno, 2006) and that NAC treatment replenishes cellular GSH, which is why NAC is used as rescue medication in acute

acetaminophen toxicity (Ruffmann and Wendel, 1991). The fact that NAC pre-treatment was able to reverse the changes in $\Delta\psi_m$ indicates that NAC may, to some degree, protect against the toxicity induced by the test compounds. Furthermore, this suggests that the mitochondrial hyperpolarisation observed in the present study may be due to GSH depletion induced by the three test compounds. However, whether GSH depletion played a role in the observed mitochondrial hyperpolarisation is disputable, as it was observed that none of the test compounds increased intracellular ROS levels in the short time required to elevate $\Delta\psi_m$ (1 h exposure). A noteworthy observation was the fact that NAC pre-treatment elevated $\Delta\psi_m$ at low test compound concentrations but reduced excessive mitochondrial hyperpolarisation due to exposure to high concentrations of the test compounds. This trend correlates well with observations made regarding cell viability following exposure to the test compounds, where, at low concentrations of the test compounds, NAC pre-treatment exacerbated toxicity, but alleviated toxicity at higher concentrations of the test compounds.

In summary, all three of the tested compounds produced significant elevation of $\Delta\psi_m$ compared to controls, of which DDD was the most potent. This may be due to inhibition of ATP-synthase.