Chapter 7: Modes of Cell Death

7.1. Background

Cell death can follow one of two distinct pathways, apoptosis or necrosis, and can occur in response to severe stress conditions or after exposure to toxic agents. Apoptosis is a normal physiological event taking place continuously in the development of multicellular organisms or during the immune response (Samali *et al.*, 1999). Apoptosis involves activation of the intra-cellular caspase (cysteine-aspartic proteases) enzymes. Whether cell death occurs by apoptosis or necrosis is dependent on the physiologic milieu, developmental stage, tissue type, and the nature of the cell death signal (Ziess, 2003).

Sloviter (2002) recommended replacing necrosis with the term 'passive cell death', and apoptosis and programmed cell death with the term 'active cell death'. The reason for this suggested change in terminology is found in the underlying dependence/independence on cellular energy of each of these modes to manifest cell death. Apoptosis is an ordered form of cell death which depends on the ability of dying cells to initiate well-regulated, ATP-dependent self-degradation without initiating an immune response. In contrast, the core event of necrosis is rapid loss of plasma membrane integrity associated with energy depletion and release of pro-inflammatory molecules (Lin and Yang, 2008).

Apoptosis can be initiated by one of two routes: the 'intrinsic' apoptotic pathway and the 'extrinsic' apoptotic pathway. In the 'intrinsic' apoptotic pathway the mitochondria play the central role and involves the Bcl-2 family of pro-apoptotic proteins (Bad, Bid, Bax). The Bcl-2 proteins facilitate permeabilisation of the mitochondrial membrane with subsequent release of mitochondrial proteins such as cyt C and apoptotic protease activating factor-1 (Apaf-1). Cyt C and Apaf-1, in turn, form a new complex, together with po-caspase-9, known as the apoptosome, which contains active caspase-9. The cyt C/Apaf-1/Caspase-9 apoptosome then recruits effector caspases like pro-caspase-3 that is cleaved to form active caspase-3 (Cas-3) (Harwood *et al.*, 2005). Activation of Cas-3, considered by many as a final executioner protein of the apoptotic cell death sequence, leads to rapid cleavage of a

diverse spectrum of key structural and functional proteins in the cell. These targets for proteolytic attack include, amongst others, cytoskeletal components, signal transduction molecules and DNA repair enzymes (Carambula *et al.*, 2002).

The 'extrinsic' apoptotic pathway is a receptor-mediated, ordered sequence of events starting with activation of a cell surface “death receptor” such as Fas or a receptor from the tumour necrosis factor family of receptors. Receptor occupation allows interaction between receptor death domains and death effector domain on pro-caspase-8. This results in the formation of the death-inducing signalling complex or DISC (similar to the apoptosome from the 'intrinsic' pathway), followed by subsequent cleavage and activation of caspase-8, which is then able to activate the effector Cas-3 (Harwood *et al.*, 2005).

Apoptosis that follows after activation of either the mitochondria-mediated or receptor-mediated pathways rapidly leads to characteristic morphological changes associated with apoptosis including, cytoplasmic vacuolization, cellular shrinkage, an increase in cellular density, nuclear fragmentation, membrane budding and apoptotic bodies which are removed by phagocytic cells. The absence of this set of morphological changes indicates that cell death occurs by necrosis, which is characterised by chromatin flocculation and a progressive loss of plasma membrane integrity, which allows an influx of Na^{+}, Ca^{2+} and water. This constant influx causes cellular swelling, which eventually results in cell rupture and leakage of the cytoplasmic contents into the surrounding tissue. Clinically, the main difference between these modes of cell death lies in the fact that necrosis results in localised inflammation due to spillage of cytoplasmic contents, which in turn releases pro-inflammatory factors. On the other hand, apoptotic cells are broken down to apoptotic bodies with intact membranes, which are then engulfed by nearby phagocytic cells and macrophages with no subsequent inflammatory response (Harwood *et al.*, 2005).

The experiments presented in this chapter were conducted in an attempt to elucidate the mode of cell death caused by each of the test compounds, in order to provide information regarding the mechanism of toxicity of the compounds in question.

7.2. Methods

7.2.1. Assessment of cell death by apoptosis

Staurosporine, the potent inhibitor of serine/threonine kinases known to induce apoptosis in hepatocytes (Giuliano *et al.*, 2004), was used as positive control. To induce apoptosis in positive control samples, 100 μl of staurosporine working solution was added to 100 μl of cell suspesion, giving exposure to a final concentration of 11 μM for 6 h at 37°C. To quantify Cas-3 activity, the method accompanying the CASP3F Cas-3 fluorometric detection kit from Sigma-Aldrich was followed with slight modification. (The CASP3F kit was not used, just the protocol and Ac-DEVD-AMC)

Following 6 h exposure to either positive control or test compounds, plates were put on ice for 20 - 30 min to cool. As soon as the plates were not warm to the touch, medium was aspirated and replaced with 25 μl of ice-cold lysis buffer and incubated on ice for a further 20 min. After this 100 μl of assay buffer was added and the plates incubated overnight at 37°C. The following day, the cleaved 7-amino-4-methylcoumarin (AMC) was quantitated using a FluoStar Optima using λ_{ex} = 360 nm and λ_{em} = 460 nm.

7.2.2. Assessment of cell death by necrosis

The detergent Triton X-100, which is reported to disrupt most cell membranes (Tate *et al.*, 1983), was used as positive control for PI staining. To disrupt plasma membranes of the positive control sample, a 100 μl of Triton X-100 (working solution) was added to 100 μl of cells to give a final concentration of 0.5% (v/v). Cells were treated with Triton X-100 for 30 min at 37°C. Plasma membrane integrity was assessed by a modified version of PI fluorometry described by Nieminen *et al.* (1992). Prior to use, the PI stock solution was

diluted to 75 µM in PBS. Following exposure to the test compounds, a 50 µl of PI working solution was added to 200 µl of cells and stained with a final concentration of 15 µM PI for 15 min at 37°C. After staining, medium was discarded and cells washed with 200 µl of PBS, followed by the addition of 100 µl PBS before detecting the fluorescence using a FluoStar Optima at λ_{ex} = 544 nm and λ_{em} = 595 nm.

7.2.3. Statistical analyses

Six independent fluorometry experiments were carried out in duplicate (n=12). Relevant blank values were deducted from all experimental values before observed values were standardised to percentage of controls. 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 are presented as Mean ± SEM.

Three additional experiments were carried out in duplicate to assess the possible effects that NAC may have on the pesticide-induced mode of cell death. 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.

7.3. Results

7.3.1. Assessment of cell death by apoptosis

Analysis confirmed the presence of outliers in the 5 µM groups of both the DDE and DDD data sets, with ***p*** < 0.05 (Table 7.1). Subsequently, these were identified and removed. This

was followed by normality testing, which revealed normal distributions for most of the data except the 5, 10 and 100 µM groups of the DDE data set (p < 0.05) (Table 7.2.). Non-normal data was analysed using Mann-Whitney tests.

Table 7.1. Grubb's test results for detecting outliers in Cas-3 data. Values given in the table are *p*-values. Instances where p < 0.05 (*) indicates the presence of outliers.

Concentration	DDT	DDE	DDD
Control	0.40	0.78	0.49
5 µM	0.14	0.02*	0.00*
10 µM	0.51	0.46	0.31
50 µM	0.54	0.61	0.20
100 µM	0.44	0.77	0.08
150 µM	0.47	0.27	0.11

Table 7.2. Shapiro-Francia test normality results of the observed Cas-3 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	0.74	0.38	0.42
5 µM	0.84	0.05*	0.95
10 µM	0.82	0.02*	0.92
50 µM	0.47	0.04*	0.09
100 µM	0.41	0.18	0.22
150 µM	0.22	0.23	0.57

The positive control (11 µM staurosporine) significantly (p < 0.001) induced Cas-3 activity when compared to controls with a mean increase of 97% (Figure 7.1). All the tested concentrations of DDT (5 - 150 µM) were found to significantly (p < 0.001) increase Cas-3

activity in a dose-dependent manner, ranging from a 21% increase (5 µM) to a 63% increase (150 µM). DDT-induced Cas-3 activity followed a dose-dependent trend (Figure 7.2 and Table 7.3).

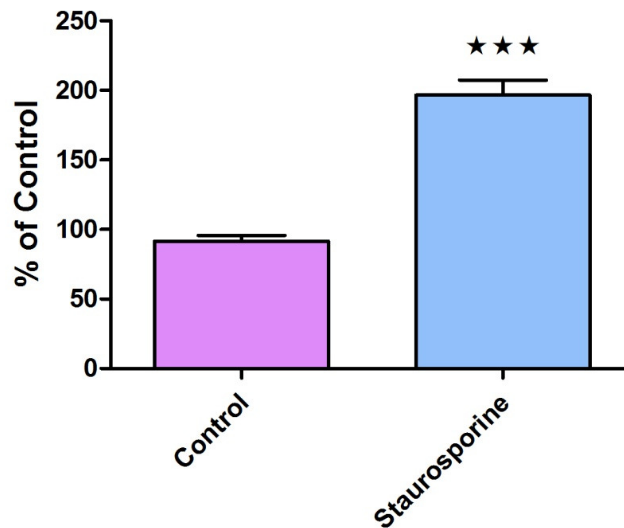

Figure 7.1. Active caspase-3 in HepG2 cells following 6 h exposure to Control *vs.* Staurosporine (11 µM) (mean ±SEM). Staurosporine significantly induced caspase-3 activity with $p < 0.001$ (***).

Table 7.3. Relative Cas-3 activity in HepG2 cells following 6 h exposure to DDT, DDE, DDD and Staurosporine (positive control). Results (% of Control) are presented as mean ±SEM. ** indicates $p < 0.01$ and *** $p < 0.001$ as determined by Mann-Whitney and Student's *t*-tests.

Concentration	DDT	DDE	DDD	Staurosporine (11 µM)
Control	100± 2	100± 2	100± 2	
5 µM	121± 4***	104± 3	119± 4***	
10 µM	128± 4***	116± 7	119± 5**	197± 11***
50 µM	143± 5***	133± 8***	141± 8***	
100 µM	149± 5***	148± 7***	182± 9***	
150 µM	163± 5***	165± 9***	239± 6***	

DDE affected Cas-3 activity in a dose-dependent manner and was also found to significantly ($p < 0.001$) increase Cas-3 activity. However, DDE was not as potent an inducer as DDT, yielding insignificant increases at the lower concentration range tested (5 - 10 µM). DDE

significantly increased Cas-3 activity at higher concentrations by 34% (50 μM), 49% (100 μM) and 66% (150 μM) (Figure 7.2 and Table 7.3).

At concentrations of 5 - 50 μM, DDD yielded results similar to that of DDT, causing significant (***p*** < 0.01) increases in Cas-3 activity of between 20% and 40%. However, at concentrations of 100 and 150 μM, DDD caused highly significantly (***p*** < 0.001) elevated Cas-3 activity when compared not only to the controls, but also DDT and DDE. The effect of DDD on Cas-3 activity followed a dose-dependent trend (Figure 7.2 and Table 7.3).

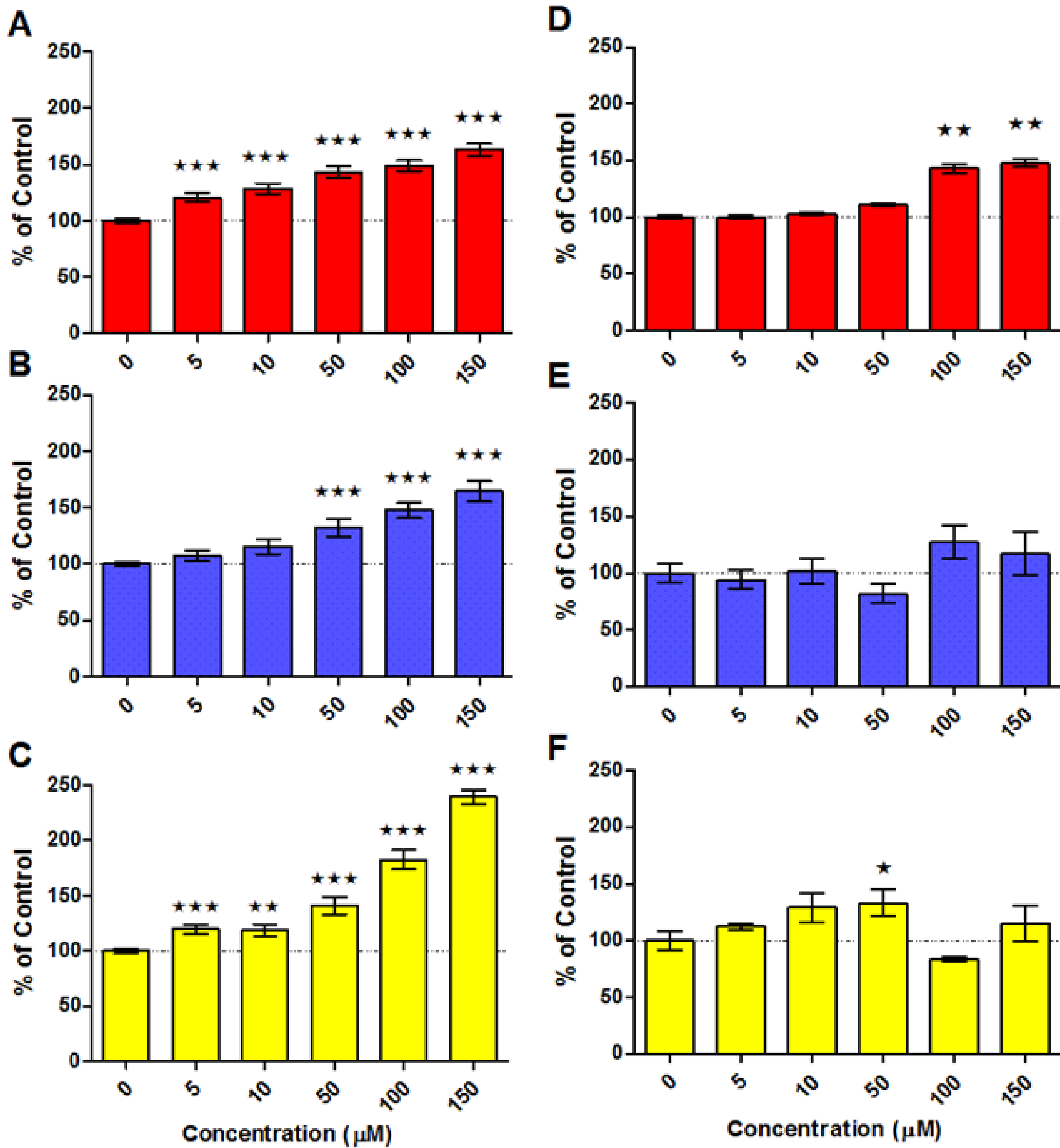

Figure 7.2. Caspase-3 activity in HepG2 cells following 6 h exposure to DDT (A), DDE (B) and DDD (C) (mean ±SEM). Caspase-3 activity was used as a measure of cell death by apoptosis. Graphs (D), (E) and (F) represent the PI staining of cells exposed to DDT, DDE and DDD, respectively. Propidium iodide was used as a measure of membrane integrity and cell death by necrosis. Dashed horizontal lines represent untreated control values. Results are given as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** $p < 0.001$ as determined by Mann-Whitney and Student's *t*-tests, where applicable.

Table 7.4. Relative Cas-3 activity in HepG2 cells after exposure to DDT, DDE, DDD, with or without 1 h pre-treatment with NAC. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 as determined by Mann-Whitney and Student's *t*-tests.

	DDT		DDE		DDD	
	--	NAC	--	NAC	--	NAC
5 µM	121± 4	125 ± 9	104± 3	106 ± 5	119± 4	107 ± 3
10 µM	128± 4	115 ± 6	116± 7	102 ± 4	119± 5	126 ± 17
50 µM	143± 5	121 ± 12*	133± 8	100 ± 5**	141± 8	134 ± 16
100 µM	149± 5	118 ± 12*	148± 7	110 ± 7**	182± 9	166 ± 27
150 µM	163± 5	127 ± 17*	165± 9	118 ± 7**	239± 6	139 ± 16***

Results demonstrate a trend in which NAC pre-treatment significantly reduced the degree of apoptosis induction resulting from exposure to the three test compounds. This is most prominent in DDE results, where NAC pre-treatment reduced Cas-3 activity at concentrations between 50 - 150 µM (p < 0.01) (Table 7.4).

7.3.2. Assessment of cell death by necrosis

According to Grubb's test 5 groups out of the 3 data sets included outliers with p < 0.05 (5 µM DDT, 10 and 50 µM DDE and 5 and 100 µM DDD) (Table 7.5). Outliers were removed and followed by normality testing, which revealed that all of the data, except the 100 µM group from the DDE data set, was normally distributed. Subsequently all of the data were analysed with Student's ***t***-tests except 100 µM DDE, which was compared to controls using the Mann-Whitney test.

Table 7.5. **Grubb's test results for detecting outliers in the data from PI staining. Values given in the table are *p*-values. Instances where *p* < 0.05 (*) indicates the presence of outliers.**

Concentration	DDT	DDE	DDD
Control	0.19	0.24	0.32
5 µM	0.01*	0.26	0.03*
10 µM	0.70	0.01*	0.07
50 µM	0.33	0.02*	0.69
100 µM	0.33	0.64	0.00*
150 µM	0.13	0.35	0.05

Table 7.6. **Shapiro-Francia test normality results of the observed PI 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	0.68	0.11	0.06
5 µM	0.89	0.78	0.06
10 µM	0.30	0.08	0.88
50 µM	0.54	0.05	0.59
100 µM	0.43	0.05*	0.47
150 µM	0.82	0.15	0.33

Positive controls yielded an expected increase in membrane damage, causing significantly (***p*** < 0.001) higher PI staining (755%) than the untreated controls (Figure 7.3). DDT demonstrated significant loss of membrane integrity at higher concentrations (100 and 150 µM), which is indicated by the significantly higher PI staining (***p*** < 0.01). This effect followed a dose-dependent trend (Table 7.7).

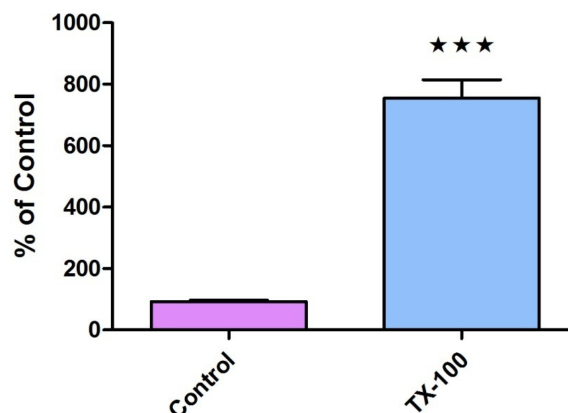

Figure 7.3. Propidium iodide staining as a measure of membrane integrity in HepG2 cells following treatment with 0.5% (v/v) Triton X-100 (TX-100) (mean ±SEM). *** = $p < 0.001$.

DDE did not have any statistically significant effect on PI staining when compared to untreated controls but higher concentrations of 100 and 150 µM did cause a slight increase in PI staining (Table 7.7).

DDD had the largest effect on membrane integrity at the lower concentration range tested increasing PI staining by 13%, 29% and 33% at concentrations of 5, 10 and 50 µM, respectively. However, only the 33% increase proved to be statistically significant. No significant influence was found at 100 and 150 µM of DDD (Table 7.7). Neither DDE nor DDD results followed any dose-response trend.

Table 7.7. PI staining in HepG2 cells following 24 h exposure to DDT, DDE, DDD and Triton X-100 (positive control). Results (% of Control) are presented as mean ±SEM. * indicates $p < 0.05$ and ** $p < 0.01$ as determined by Mann-Whitney tests.

Concentration	DDT	DDE	DDD	Triton X-100 (0.5%)
Control	100± 5	100± 8	100± 8	
5 µM	100± 4	94± 8	113± 9	
10 µM	103± 5	102± 11	129± 12	755± 60***
50 µM	111± 4	82± 8	133± 11*	
100 µM	143± 13**	127± 15	84± 7	
150 µM	148± 13**	117± 19	115± 16	

Table 7.8. PI staining in HepG2 cells due to DDT, DDE, DDD, with or without 1 h pre-treatment with NAC. * indicates $p < 0.05$ as determined by Mann-Whitney tests.

	DDT		DDE		DDD	
	--	NAC	--	NAC	--	NAC
5 µM	100± 4	94 ± 9	94± 8	96 ± 4	113± 9	98 ± 12
10 µM	103± 5	87 ± 9	102± 11	96 ± 10	129± 12	90 ± 10*
50 µM	111± 4	184 ± 41*	82± 8	136 ± 35	133± 11	115 ± 20
100 µM	143± 13	160 ± 16	127± 15	153 ± 48	84± 7	147 ± 28*
150 µM	148± 13	171 ± 23	117± 19	157 ± 51	115± 16	148 ± 14

A general trend can be observed from results where NAC pre-treatment appeared to elevate the number of cells in necrotic cell death exposed to higher concentrations of the test compounds (50 - 150 µM) (Table 7.8 and Figure 7.4).

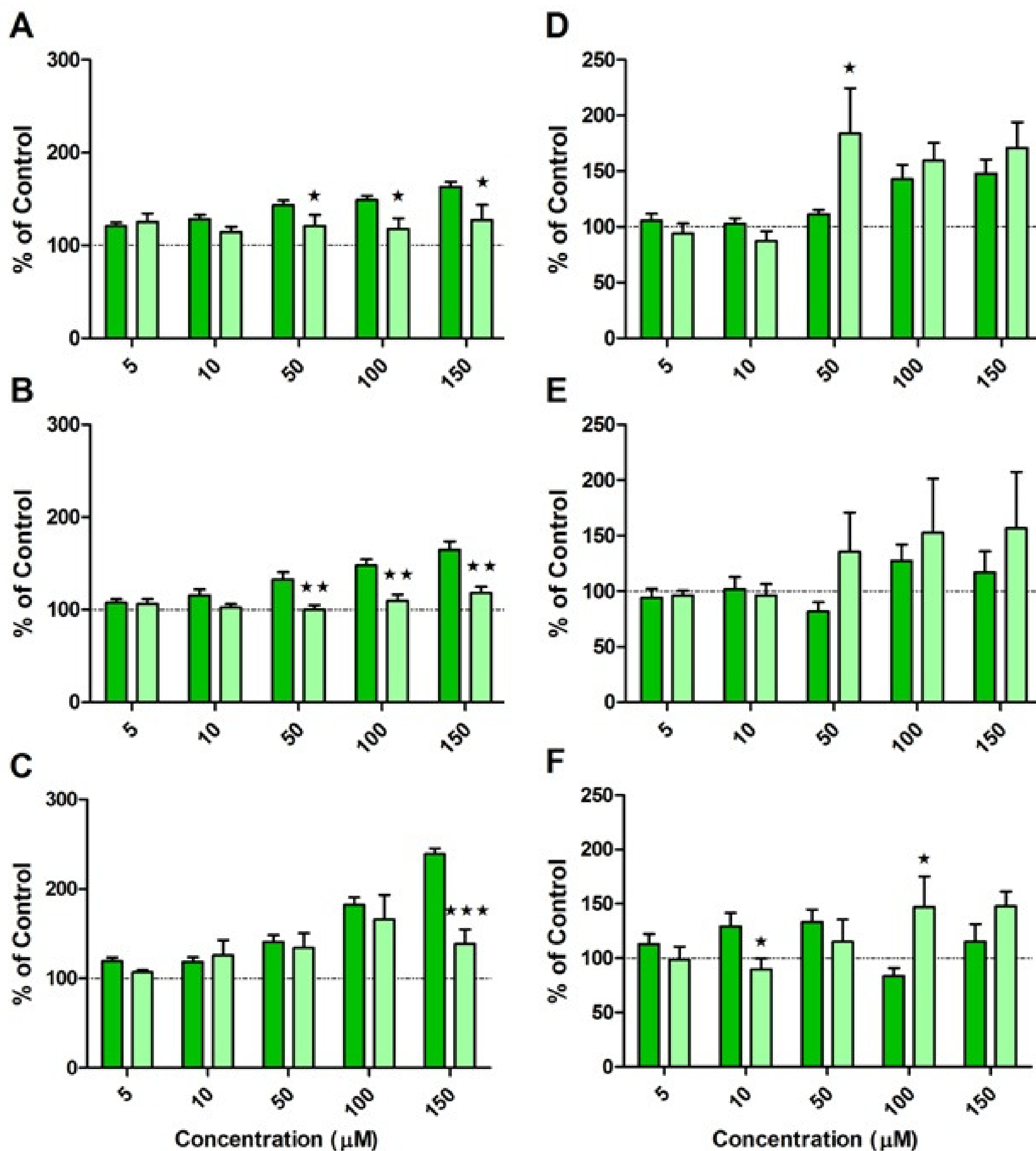

Figure 7.4. Caspase-3 activity in HepG2 cells following 6 h exposure to DDT (A), DDE (B) and DDD (C)(mean ±SEM). Graphs (D), (E) and (F) represent the propidium iodide staining of cells exposed to DDT, DDE and DDD, respectively. Caspase-3 activity and propidium iodide were used as a measure of cell death by apoptosis and necrosis, respectively. Dashed horizontal lines represent Control values. Results are given as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** $p < 0.001$ as determined by Mann-Whitney and Student's *t*-tests, where applicable. Light green bars represent 1 h pre-treatment with NAC as opposed to dark green bars, which received no pre-treatment.

7.4. Discussion

Following the removal of outliers in the Cas-3 data sets with the Grubb's test (Table 7.1), only 3 of the 18 data sets presented non-normal distributions (Table 7.2), indicating good reproducibility for the Cas-3 assay. Regarding the membrane integrity assay, only 1 of the 18 data sets presented with a non-normal distribution (Table 7.6) after removal of the relevant outliers (Table 7.5), showing good assay reproducibility.

All three tested compounds affected HepG2 cells leading to dose-dependent activation of Cas-3, implying activation of the apoptotic pathway. DDT and DDD were more potent than DDE by highly significantly ($\boldsymbol{p} < 0.001$) raising Cas-3 activity beyond baseline (controls) at concentrations as low as 5 µM. Of the three tested compounds, DDD proved to be the most potent, causing significantly more Cas-3 activity than DDT and DDE at concentrations of 100 µM (≈ +30%) and 150 µM (≈ +70%). In terms of potency the following was observed: DDD > DDT > DDE. These results correlate very well with the IC_{50} values determined during the cytotoxicity assay where a similar trend was noted.

Shi *et al.* (2010a) examined apoptosis as a possible mode of cell death of cultured hepatocytes exposed to concentrations of 10 - 100 nM of DDT. During that study the authors did not observe any DDT-induced apoptosis, probably due to a lack of cell death (94% viability). Even if that study did report some apoptotic cell death, with cell viability that high it could have been normal cell attrition and not necessarily DDT-induced. Contrarily, another study did report apoptosis in HepG2 cells exposed to 50 nM of DDT (Filipak Neto *et al.*, 2008). The discrepancy between the results from these two studies could be ascribed to the length of exposure, 24 h (Shi *et al.,* 2010a) versus 96 h (Filipak Neto *et al.*, 2008). In the present study concentrations of 5 - 10 µM were not found to induce toxicity after 24 h exposure, but significantly raised Cas-3 activity. It could be hypothesised that lower concentrations of DDT (that are not necessarily toxic) may cause significant activation of

Cas-3 following longer incubation periods such as those used in the study by Filipak Neta *et al.* (2008)

The fact that DDT is able to induce apoptosis is not confined to the liver alone. It has also been reported to induce apoptosis in human peripheral blood mononuclear cells (Perez-Maldonado *et al.*, 2004), rat thymocytes (Tebourbi *et al.*, 1998) and murine embryos (Greenlee *et al.*, 1999). DDE has also been reported to induce apoptosis in various types of tissues such as peripheral blood mononuclear cells (Perez-Maldonado *et al.*, 2004), rat Sertoli cells (Shi *et al.*, 2009) and cells from the testes of rats exposed *in vivo* (Shi *et al.*, 2010b). DDD-induced apoptosis has only been reported in peripheral blood mononuclear cells (Perez-Maldonado *et al.*, 2004). No literature is available regarding DDE/DDD-induced apoptosis in cultured hepatocytes. From their study on rat Sertoli cells, Shi *et al.* (2009) concluded that exposure to 30 and 50 μM DDE induced apoptosis via the 'extrinsic' apoptotic pathway as indicated by the up-regulation of caspase-8 activated by the Fas receptor. The authors suggested that DDE may have activated the receptor-mediated pathway by activating nuclear factor-κβ, which can exert both pro- and anti-apoptotic effect, depending on the specific cell type as well as the type of inducer. It is therefore possible that the increase in Cas-3 activity observed in the present study could also be due to upstream caspase-8 activation. However, in the liver, nuclear factor-κβ prevents apoptosis and has a positive role in regeneration (Taub, 1998). Also, observations with regard to the mitochondrial membrane potential changes after exposure to the test compounds is suggestive of Cas-3 activation via the 'intrinsic'/mitochondrial-mediated pathway in that all three test compounds (DDT, DDE and DDD) were able to significantly raise $\Delta\psi_m$, which would lead to the release of cyt C from the mitochondria, with subsequent formation of the apoptosome and Cas-3 activation. Further investigations would be necessary to confirm which of the two apoptotic pathways are responsible for the elevated Cas-3 activity after exposure to DDT, DDE and DDD.

No literature could be found with regards to necrotic effects as a result of DDT, DDE or DDD exposure *in vitro*. It would be erroneous to conclude that DDT/DDE/DDD induces cell death

through apoptosis because of a lack of *in vitro* evidence that proves otherwise. A number of previous *in vivo* studies have reported DDT-induced necrosis. DDT, DDE and DDD exposure in chicken embryo neurons *in ovo*, revealed definitive plasma and nuclear membrane damage as visualised using scanning and transmission electron microscopy, from which the authors concluded cell death by necrosis (Bornman *et al.*, 2007). Histological examination of the livers of female rats exposed to 75 and 150 ppm dietary DDT for 36 weeks revealed typical of organ necrosis (Jonsson *et al.,* 1981). In rats exposed to DDT (5 - 500 ppm) for 5 days, histological evaluation of the livers showed cytoplasmic vacuolisation, typical signs of necrosis and no DNA fragmentation, which indicates cell death by necrosis (Kostka *et al.*, 1996; Kostka *et al.*, 1999). Another study reported necrotic cell death and cellular infiltration in livers from rats that were orally exposed to two doses of 150 mg/kg body weight of DDT (Mikhail *et al.,* 1979). These authors also reported mononuclear leukocyte infiltration in the livers of these animals. They suggested that this was indicative of an inflammatory response. Inflammation should not be prevalent in the case of apoptosis because the cytoplasmic contents are contained within apoptotic bodies.

In the present study, treating HepG2 cells with NAC, prior to test compound exposure, significantly decreased the activation of Cas-3 for all three test compounds, indicating a decrease in apoptosis. These findings correlate with literature where NAC is reported to be a broad inhibitor of apoptotic death induced by various *in vitro* stressors (De Flora *et al.*, 2001). However, NAC pre-treatment did not alleviate the cytotoxic effects of any of the test compounds. Therefore, this decrease in Cas-3 activity is expected to translate to increased cell death by necrosis. Results supporting this hypothesis is visualised in Figure 7.4, where significant decreases in Cas-3 activity can be seen for DDT (Figures 7.4.A), DDE (Figure 7.4.B) and DDD (Figure 7.4.C), which are accompanied by corresponding increases in PI fluorescence representing necrotic death for DDT (Figure 7.4.D), DDE (Figure 7.4.E) and DDD (Figure 7.4.F). The decrease in test compound-induced Cas-3 activity due to NAC treatment may be explained by the stabilising effects that NAC pre-treatment had on the $\Delta\psi_m$, as transient mitochondrial hyperpolarisation is an early event preceding Cas-3 activation and membrane phosphatidylserine externalization (Nagy *et al.*, 2007).

Literature describing apoptotic death due to DDT exposure tends to originate from *in vitro* studies, whereas necrotic effects have been reported from *in vivo* studies. A possible explanation as to why DDT-induced apoptotic death has not been described *in vivo* may be the fact that it is difficult to detect apoptosis *in vivo*. The difficulty in detecting apoptosis *in vivo* has been attributed to the fast endogenous clearance of apoptotic cells from an organism (Zhang *et al.*, 2000; Nyati *et al.*, 2006). In the present study the *in vitro* tests were able to detect necrosis due to DDT exposure but not when cells were exposed to DDE and DDD. DDE and DDD results were inconsistent, not presenting any predictable dose-response trends. This is likely to be the result of experimental procedures like wash steps, aimed at reducing background fluorescence. During these wash steps necrotic cells may have been lost when aspirating the supernatant, even though particular care was taken to try and avoid this.

In summary, there seems to be no clear distinction between which mode of cell death leads to the loss of viability due to either DDT, DDE or DDD exposure since evidence exists that supports both pathways. The inflammatory response seen *in vivo* appears the most convincing, which is indicative of necrosis.

It has been reported that a switch can take place from apoptosis to necrosis due to inactivation of caspases by ROS (Samali *et al.*, 1999; Prabhakaran *et al.*, 2004). In the present study Cas-3 activity was detected after 6 h and membrane integrity was assessed after 24 h. It is therefore possible (but unlikely) that DDT may induce activation of Cas-3 but that cell death eventually occurs due to necrosis after 24 h because of Cas-3 inactivation by CYP1A1-generated ROS. The fact that DDT may inhibit ATP-synthase provides further support for this theory as apoptosis is an energy-dependent mode of death and a loss of cellular ATP would preferentially lead to cell death by necrosis, which is the hepatocyte mode of death reported *in vivo*. However, this is disputable as no test compound-induced ROS generation was observed in the present study.