CHAPTER 3

Mechanism of cadmium (Cd^{2+}) induced cell death in *Dictyostelium discoideum*

Introduction

Cadmium (Cd^{2+}) is an abundant transition metal of worldwide concern because it accumulates in the environment as a result of its numerous industrial uses. In humans, non-occupational exposure to Cd^{2+} results predominantly from the consumption of contaminated food, from smoking as a result of Cd^{2+} uptake by tobacco plants from contaminated soil, and from inhalation of polluted air (Waalkes *et al.*, 1992). Cd^{2+} has an extremely long biological half-life that essentially makes it a cumulative toxin and, at present, there is no proven effective treatment for chronic Cd^{2+} intoxication (Goering *et al.*, 1994). Cd^{2+} has been designated a human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Occupational exposure to Cd^{2+} is associated with lung cancers in humans, while other sites, including the prostate, have not been definitively established (Metka and Filipi, 2004).

The mechanism of the carcinogenic activity of Cd^{2+} is not clearly defined. In general, the expression of antioxidant genes such as those coding for the synthesis of superoxide dismutase and catalase are repressed by Cd^{2+} (Casalino *et al.*, 1997; Hussain *et al.*, 1987; Liu *et al.*, 2002; Ognjanovic *et al.*, 1995; Shukla *et al.*, 1989) and this has been regarded as the reason for Cd^{2+}-induced lipid peroxidation, oxidative stress, and the associated toxicity. Genes encoding for metallothionein (a low molecular weight protein containing about 30% cysteine) are the most studied genes with respect to the potential of Cd^{2+} to induce gene expression. Metallothionein (MT) sequesters Cd^{2+} with high affinity resulting in decreased availability of Cd^{2+} capable of interacting with cellular targets to elicit toxicity, including carcinogenicity. Lack of expression of MT protein, under basal and Cd^{2+}-stimulated conditions, has been regarded as one of the major underlying causes of tissue susceptibility to Cd^{2+} toxicity and/or carcinogenicity (Waalkes *et al.*, 1992). It has been demonstrated that exposure to Cd^{2+} results in induction of the genes for γ-glutamylcysteine synthetase (γ-GCS), glutathione-S-transferases (GST-α and GSTπ) and the elevated synthesis of GSH so as to result in rapid and efficient detoxification of Cd^{2+} as well as the ROS generated from this metal (Chin and Templeton, 1993; Eneman *et al.*, 2000). However, either continuous exposure or exposure to toxic doses of Cd^{2+} may

overwhelm the cellular supply of GSH and the related defense system so as to result in toxicity, including carcinogenesis (Liu *et al.*, 1990).

It is clear that defective DNA repair is a contributing factor to cancer (Hoeijmakers *et al.*, 2001), and inhibition of DNA repair by Cd^{2+} is likely to play an important role in carcinogenesis (Waalkes *et al.*, 2003). Hengstler *et al.* (2003) reported a correlation between single strand breaks in DNA of mononuclear leukocytes and blood Cd^{2+} levels in factory workers co-exposed to Cd^{2+}, cobalt and lead. Repair of 8-oxoguanine DNA damage in the lymphocytes of exposed workers was inversely correlated with strand breaks, and decreased with increasing Cd^{2+} exposure (Hengstler *et al.*, 2003). At noncytotoxic concentrations, Cd^{2+} inhibited base excision repair of DNA damaged by light in HeLa cells (Hartwig *et al* 2002; Dally *et al.*, 1997). In nucleotide excision repair, Cd^{2+} inhibited the first step, which is recognition of DNA damage (Hartmann *et al.*, 1998). It has been shown that Cd^{2+} inhibits the binding to DNA of the xeroderma pigmentosum group A protein, XPA, a protein necessary for the recognition of DNA damage (Asmuss *et al.*, 2000). Cd^{2+} also inhibits binding of the p53 tumour suppressor to DNA, and in MCF7 breast cancer cells Cd^{2+} suppresses the cell cycle arrest mediated by p53 in response to DNA damage (Méplan *et al.*, 1999). Yeast exposed to low concentrations of Cd^{2+} showed decreased mismatch repair and increased hypermutability (Jin *et al.*, 2003). Furthermore, Cd^{2+} inhibits human 8-oxo-dGTPase, an enzyme that protects against the incorporation of 8-oxo-dGTP into DNA (Bialkowski *et al.*, 1999). Thus, there is ample evidence that Cd^{2+} can inhibit DNA repair at multiple levels, leading to genome instability.

The unicellular phase of cellular slime mold *Dictyostelium discoideum* has long been recognized for its higher resistance to UV, gamma, ionizing radiations and other chemical mutagens/oxidants. In this study we investigated the mechanism of Cd^{2+} induced mechanism of cell death and cellular toxicity during multicellularity in *D. discoideum* cells.

Results

3.1 Oxidative stress induction by Cd^{2+}

3.1.1 Cd^{2+} induced cell death in *Dictyostelium discoideum*

As can be seen from the Figure 3.1.1a, Cd^{2+} induces dose dependent cell death monitored by trypan blue exlusion method. Cell death was found to be 12% (LD_{12}), 25% (LD_{25}), 50% (LD_{50}) and 80% (LD_{80}) at 12 hours after treatment with 0.1mM, 0.2mM, 0.5mM and 0.7mM Cd^{2+} respectively.

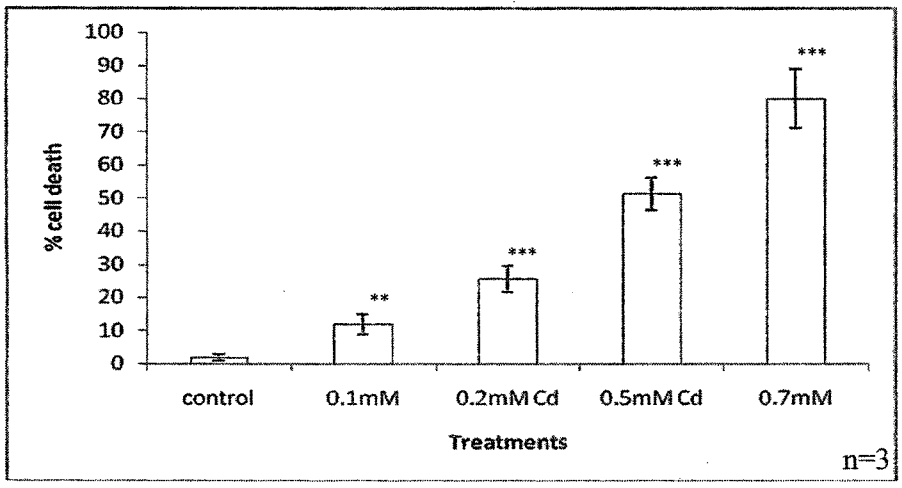


Figure 3.1.1a Dose dependent effect of Cd^{2+} induced cell death. Cd^{2+} induces dose dependent cell death monitored by trypan blue exlusion method. Data (mean ± S.E.) are from three independent experiments. ** p value <0.01 and *** p value <0.001 as compared to control.

As can be seen from the figure 3.1.1b, staining with Annexin V-FITC in conjunction with dyes such as propidium iodide (PI) allows to distinguish apoptotic cells (Annexin V positive, PI negative) from necrotic cells (Annexin V positive, PI positive). Based on PS-PI dual staining, *D. discoideum* cells treated with 0.2mM and 0.5mM Cd^{2+} showed paraptotic and necrotic cell death characteristics respectively.

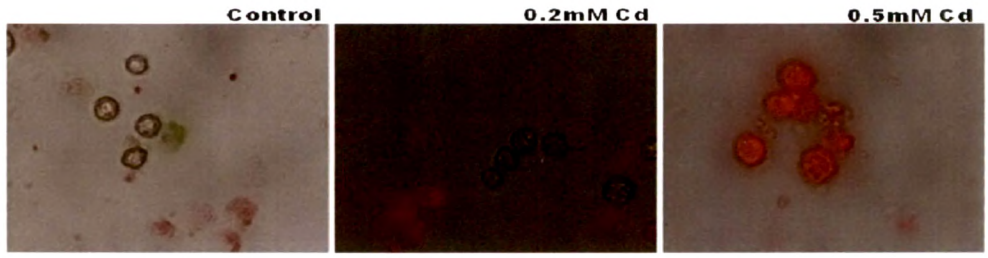


Figure 3.1.1b Cd^{2+} induced cell death by PS-PI dual staining. A (Control) PS and PI negative could be seen in control cells. **B** (0.2mM Cd^{2+}) PS exposure is seen at 5 hours while PI staining at 12 hours with 0.2 mM Cd^{2+}. **C** (0.5mM Cd^{2+}) 0.5 mM Cd^{2+} was found to be necrotic as both AnnexinV-FITC and PI staining were observed at 3 hours. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

It would be of interest to see whether Cd^{2+} induces cell death in *D. discoideum* via ROS generation. Hence we have monitored ROS by DCFDA method.

3.1.2 Cd^{2+} induced intracellular Reactive Oxygen Species (ROS) production

As can be seen fig. 3.1.2, ROS production increases as the dose of Cd^{2+} increases in *D. discoideum* cells.

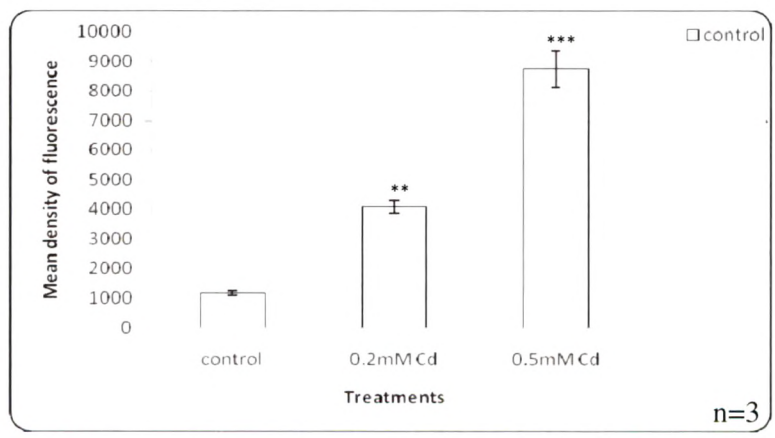


Figure 3.1.2a Cd^{2+} induced intracellular Reactive Oxygen Species (ROS) production (5 min post treatment) detected by fluorometry. Cd^{2+} led to dose dependent increase in ROS production. Data (mean ± S.E.) are from three independent experiments.** p value <0.01; *** p value <0.001 as compared to control.

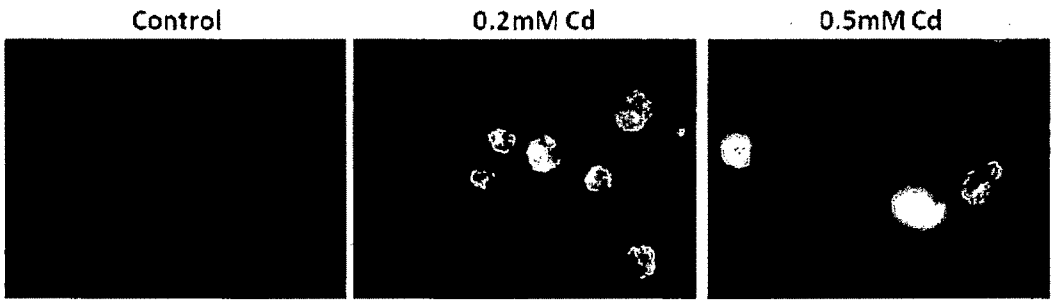


Figure 3.1.2b Effect of Cd^{2+} on ROS production detected by fluorescence microscopy. Photographs were taken with 60X objective.

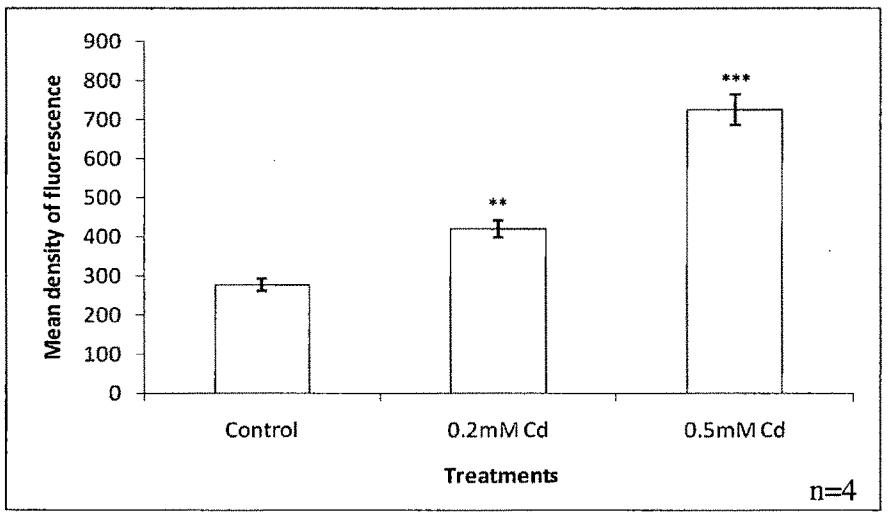


Figure. 3.1.2c Densitometric analysis: Cd^{2+} induced ROS production. ROS were measured at 5 min post Cd^{2+} stress. Data (mean ± S.E.) are from four independent experiments. *** p value <0.001 as compared to control.

3.1.3 Effect of GSH on Cd^{2+} induced cell death

To further confirm that Cd^{2+} induced cell death in *D. discoideum* via ROS generation, we have studied the effect of GSH on Cd^{2+} induced cell death. GSH pre treatment could rescue Cd^{2+} induced ROS and cell death. As can be seen in fig. 3.1.3 GSH pre treatment could intercept Cd^{2+} induced cell death in *D. discoideum.* Cells were pre treated with 1mM GSH for 2 hrs in fresh sterile HL_5 medium prior to Cd^{2+} exposure. Paraptotic cell death was found to be reduced from 25% to 10% and necrotic cell death rescued from 50% to 25% in cells pretreated with 1mM GSH.

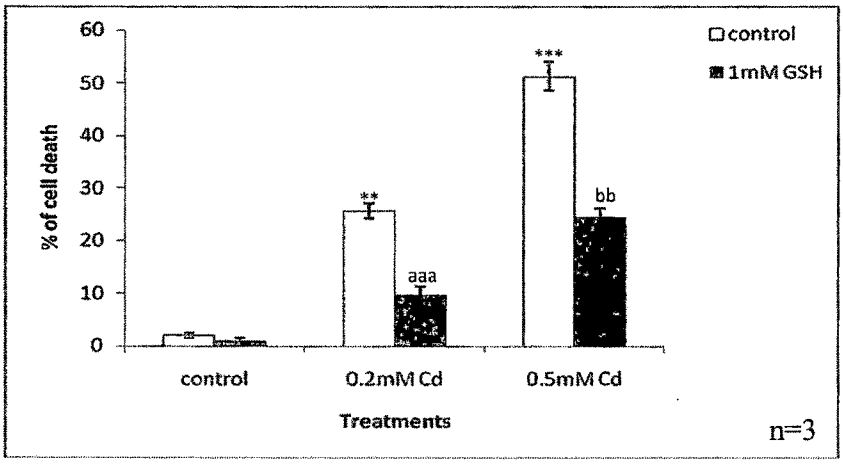


Figure 3.1.3 Effect of GSH on Cd^{2+} induced cell death. Cell death was partially rescued with 1 mM GSH at both paraptotic and necrotic doses. Data (mean ± S.E.) are from three independent experiments. *** p value <0.001 as compared to control; aaa p value <0.001; bb p value <0.01 compared to 0.2 and 0.5 mM Cd^{2+} respectively.

3.1.4 Effect of GSH on Cd^{2+} induced intracellular Reactive Oxygen Species (ROS) production

Effect of GSH pretreatment on ROS production was monitored. As can be seen from the fig. 3.1.4, GSH pre treatment prevented ROS production significantly as compared to control under Cd^{2+} induced cell death.

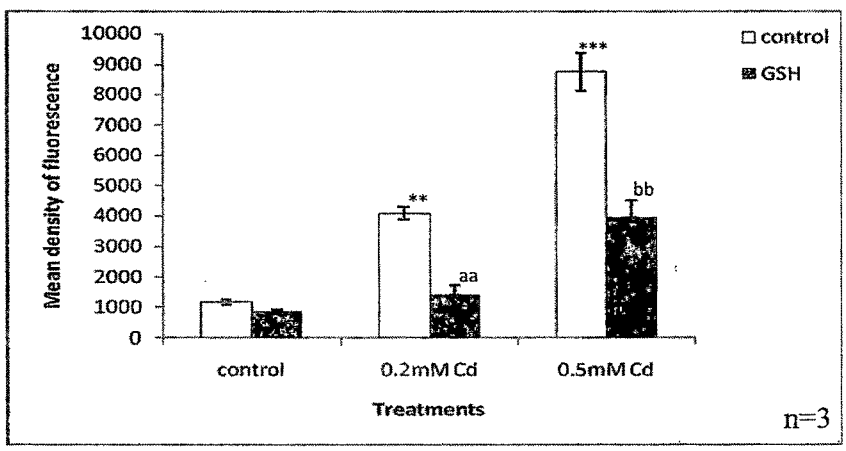


Figure 3.1.4 Effect of GSH on Cd^{2+} induced ROS production. GSH pretreatment prevent the Cd^{2+} induced ROS production within 5 min. Data (mean ± S.E.) are from three independent experiments. ** p value <0.01; *** p value <0.001 as compared to control; aa & bb p values <0.01 compared to 0.2 and 0.5 mM Cd^{2+} respectively.

3.1.5 Induction of DNA damage by Cd^{2+} exposure

ROS generated by Cd^{2+} treatment are known to cause DNA lesions; the most abundant being base modification and phosphorylation of gamma H2AX protein (Minami *et al.*, 2005). Also there is evidence that Cd^{2+} interferes with several DNA repair mechanisms including the repair of oxidative DNA damage (Filipi and Hei, 2004). To explore the effect of Cd^{2+} on *D. discoideum* DNA cells were treated with Cd^{2+} and oxidative DNA damage was assessed using anti-H2AX antibody. Immunofluorescence microscopy results revealed that treatment with increasing doses of Cd^{2+} resulted in increased H2AX phosphorylation within 5 minutes (Fig. 3.1.5a, b), suggesting an early DNA damage induction post Cd^{2+} stress induction.

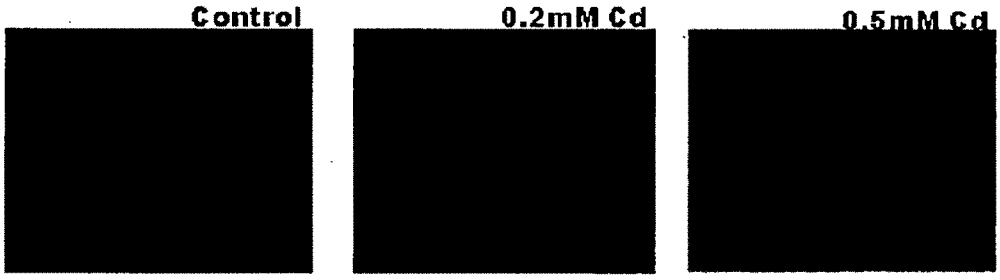


Figure 3.1.5a. Cd^{2+} induced DNA damage assayed using anti-H2AX

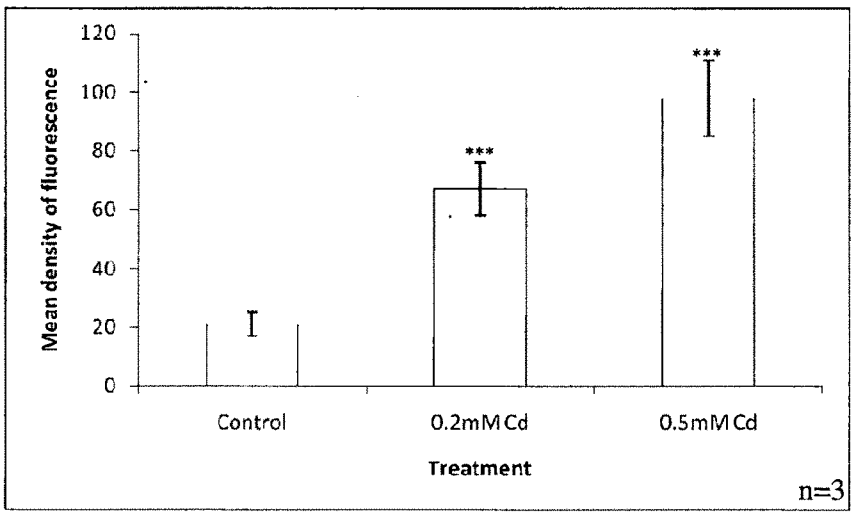


Figure 3.1.5b Densitometric analysis: Cd^{2+} induced DNA damage. Cd^{2+} led to significant oxidative DNA damage within 10 min at post exposure with Cd^{2+} as assessed in terms of presence of phosphorylated histone (H_2AX). Data (mean ± S.E.) are from three independent experiments. *** p value <0.001 as compared to control.

3.1.6 Cd^{2+} induced PARP activation

DNA damage is necessary and sufficient to induce activation of PARP. As oxidative DNA damage was observed in Cd^{2+} treated cells PARP activation was assayed at 5 min after Cd^{2+} treatment. PARP activity increased initially and peaked at 10 minutes post exposure to paraptotic and necrotic doses of Cd^{2+} (Fig. 3.1.6a, b).

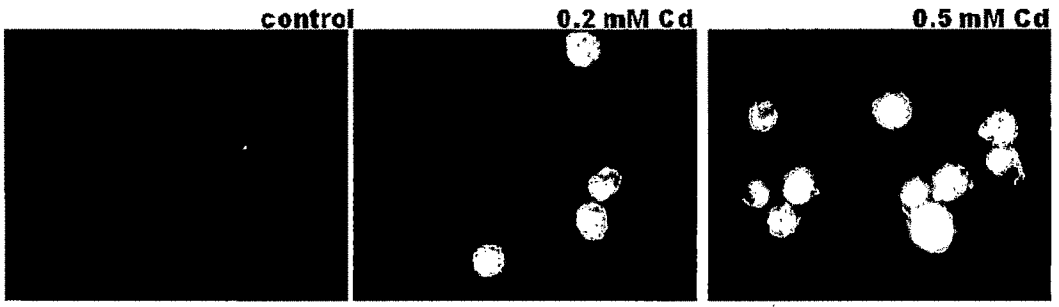


Fig. 3.1.6a Effect of Cd^{2+} on PARP activation in *D. discoideum*. Photographs were taken with 60X objective.

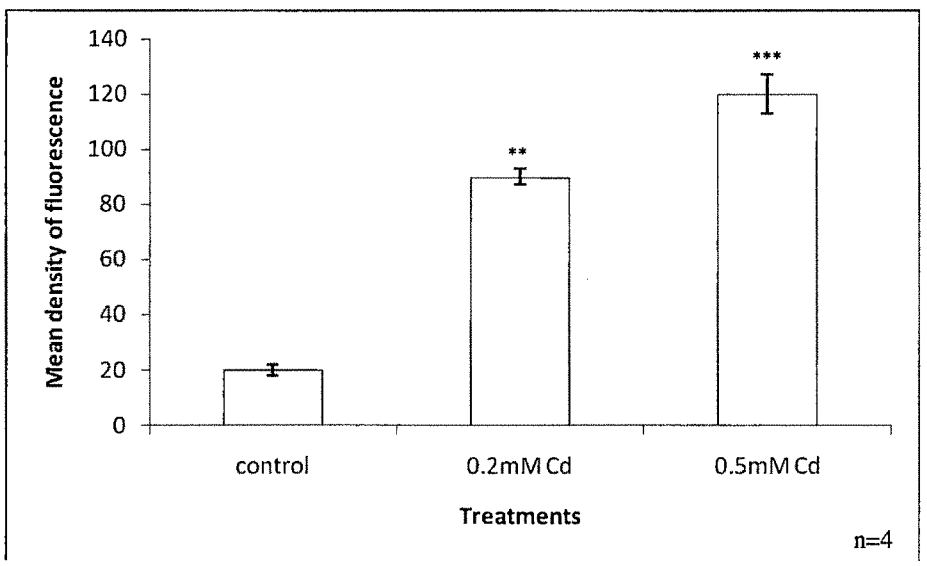


Figure 3.1.6b Densitometric analysis: Cd^{2+} induced PARP activation. Cd^{2+} led to increase in PARP activation within 10 minutes of exposure. Data (mean ± S.E.) are from four independent experiments. ** p value<0.01; *** p value <0.001 as compared to control.

3.1.7 PARP activation leads to NAD^+ depletion

PARP activation was also confirmed by monitoring cellular NAD^+ levels in control and Cd^{2+} treated cells and the results are shown in Fig. 3.1.7. Our results showed 40% and 82% reduction in NAD^+ levels in paraptotic and necrotic doses of Cd^{2+} at 1 hour. This result supports the early activation of PARP.

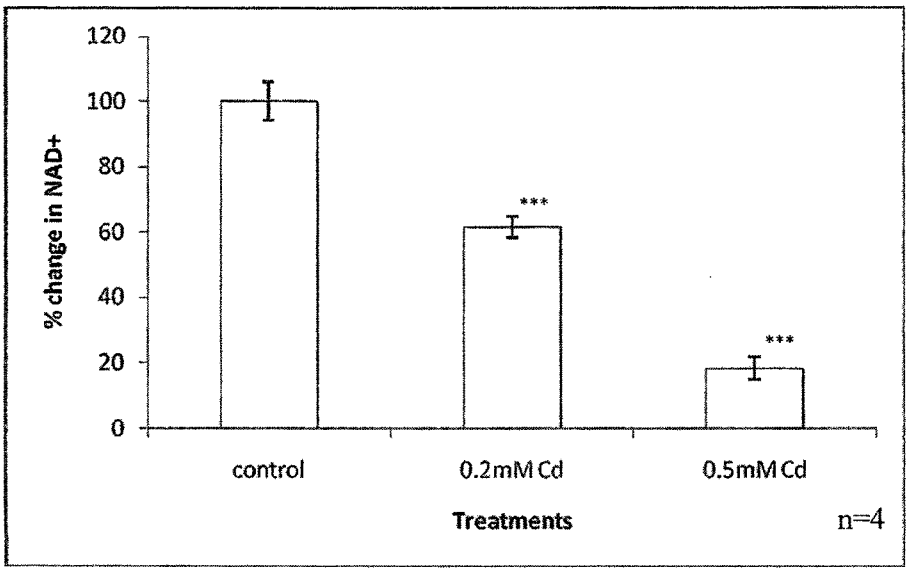


Figure 3.1.7 Effect of Cd^{2+} on cellular NAD^+ levels. Cd^{2+} led to dose dependent decrease in NAD^+ levels. Data (mean ± S.E.) are from four independent experiments. *** p value <0.001 as compared to control.

3.1.8 NAD^+ depletion leads to MMP changes

Change in mitochondrial membrane potential is one of the key events during cell death. PARP dependent cell death was accompanied by changes in mitochondrial membrane potential. In order to determine the involvement of mitochondria in cell death we examined the changes in MMP using the membrane potential sensitive dye $DiOC_6$ (Petit *et al.*, 1995) with modifications (Rottenberg and Wu, 1998; Kuhnel *et al.*, 1997 and Rajawat, 2010). As can be seen in Fig. 3.1.7 ~~3.7.1a~~ dose dependent reduction in MMP in Cd^{2+} treated *D. discoideum* cells was observed fluorometrically (Fig. 3.1.8a) and by fluorescence microscopy (Fig. 3.1.8b).

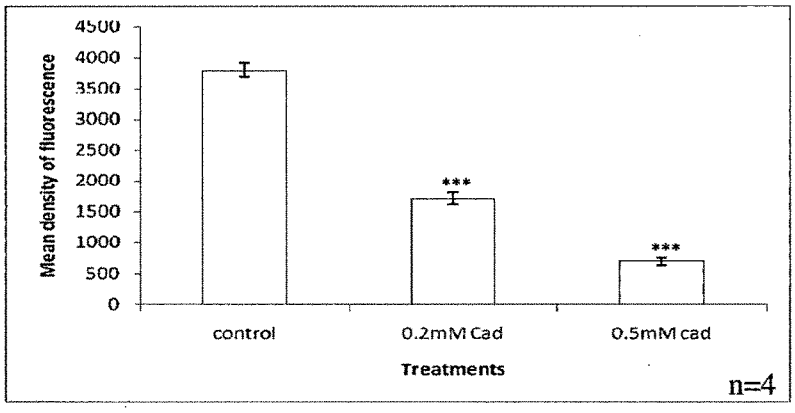


Figure 3.1.8a Effect of Cd^{2+} induced changes in MMP monitored by $DiOC_6$ using fluorometry. Cd^{2+} led to decrease in Mitochondrial membrane potential after 3 hours. Data (mean ± S.E.) are from four independent experiments. *** p value <0.001 as compared to control.

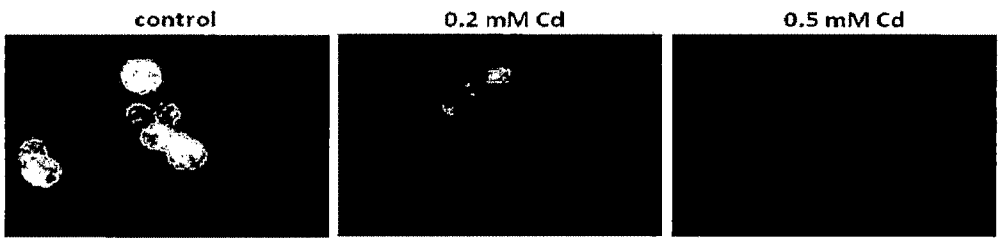


Figure 3.1.8b Effect of Cd^{2+} on MMP changes as monitored by $DiOC_6$ using fluorescence microscopy. Live cells accumulate the dye in mitochondria hence fluorescence intensity was found to be high in control and decreased with Cd^{2+} stress. Photographs were taken with 60X objective.

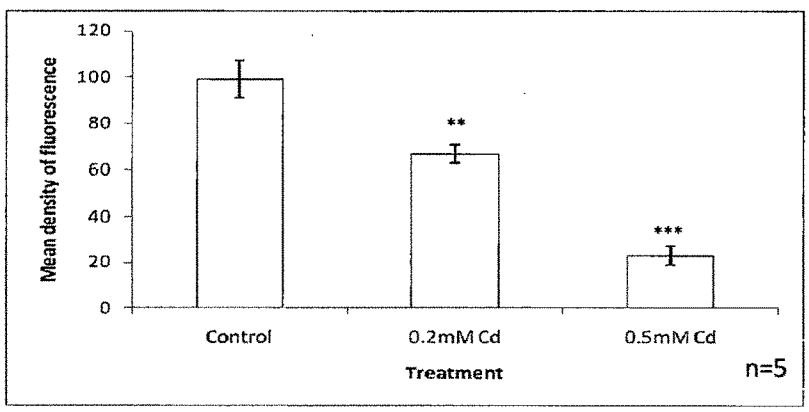


Figure 3.1.8c Densitometric analysis: Cd^{2+} induced changes in MMP. Cd^{2+} led to decrease in mitochondrial membrane potential. Data (mean ± S.E.) are from five independent experiments. ** p value <0.01; *** p value <0.001 as compared to control.

3.1.9 Effect of GSH on Cd^{2+} induced changes in MMP

We have also studied the effect of GSH on Cd^{2+} induced changes in MMP. As can be seen in Fig. 3.1.9a, paraptotic and necrotic doses of Cd^{2+} induced reduction of MMP could be prevented significantly in GSH pre treated *D. discoideum* cells.

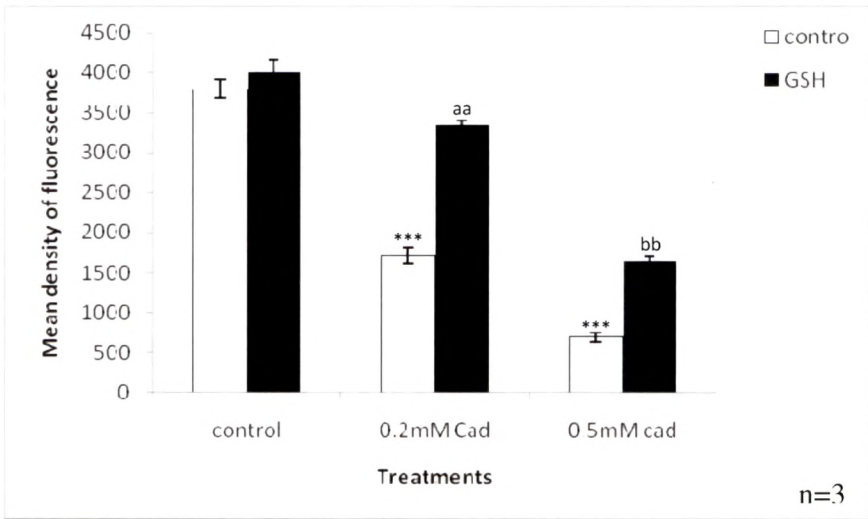


Figure 3.1.9a Effect of GSH on Cd^{2+} induced changes in MMP by fluorometry. 1mM GSH restored the mitochondrial membrane potential partially at both paraptotic and necrotic doses. *** p value <0.001 as compared to control; aa & bb p value b<0.01 compared to 0.2 and 0.5 mM Cd^{2+} respectively.

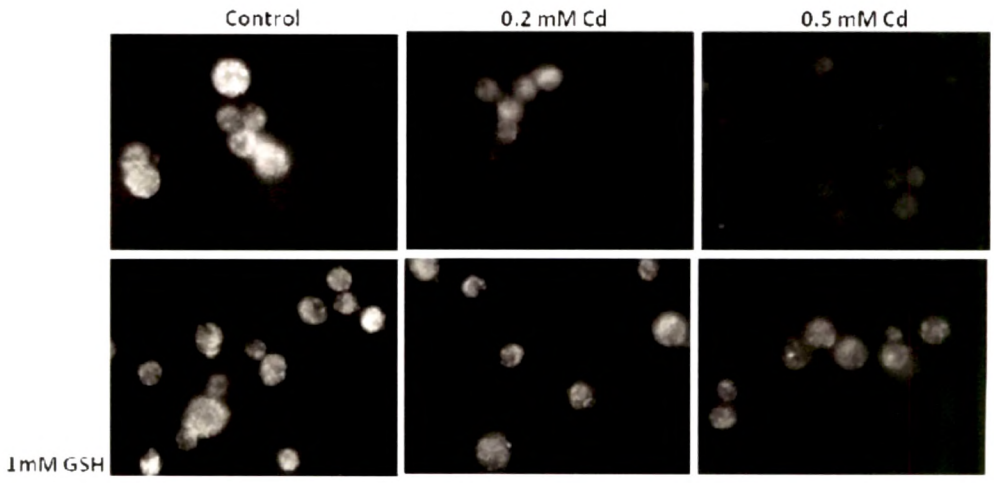


Figure 3.1.9b Effect of GSH on Cd^{2+} induced changes in MMP by fluorescence microscopy. 1mM GSH partially restored the changes in MMP. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

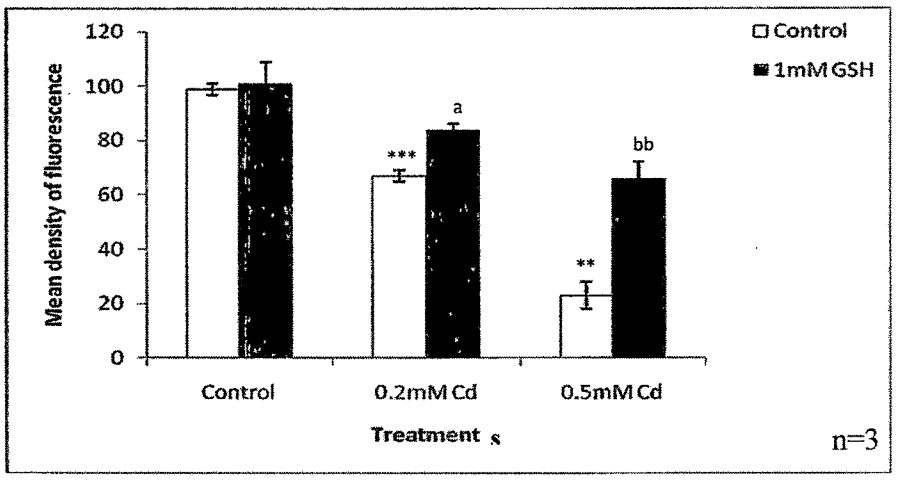


Figure 3.1.9c Densitometric analysis: Effect of GSH pre treatment on Cd^{2+} induced changes in MMP. ** p value <0.01; *** p value <0.001 as compared to control; a p value<0.05 compared to 0.2 mM Cd^{2+}; bb p value <0.01 compared to 0.5 mM Cd^{2+} respectively.

3.1.10 Cd^{2+} induced changes in development

We have also studied the effect of Cd^{2+} on development in *D. discoideum*. Cd^{2+} showed dose dependent toxic effect on *D. discoideum* development. 0.2 mM Cd^{2+} treated cells displayed developmental block at loose aggregate stage while with 0.5 and 1 mM dose complete developmental block was seen till one week. Interestingly, 1 mM GSH showed strong protection against Cd^{2+} induced developmental arrest. As shown in the Table 3.1.10 and Fig. 3.1.10, GSH pretreatment showed complete restoration in 0.2 and 0.5 mM Cd^{2+} induced developmental block. However, it could not completely restore the developmental block induced by 1 mM Cd^{2+}.

Cd^{2+} (mM)	LA (h)	TA (h)	SF (h)	FB (h)
control	8	12	18	24
0.2 mM	24	-	-	-
0.5 mM	36	-	-	-
1 mM	-	-	-	-

Table 3.1.10a: Effect of Cd^{2+} on *D. discoideum* development LA= loose aggregate, TA=Tight aggregate, SF=Slug formation, FBF= Fruiting body formation, - = No development till one week

GSH (1mM) $^{+}Cd^{2+}$ (mM)	LA	TA	SF	FB
Control	8	12	18	24
0.2 mM	8	12	24	36
0.5 mM	8	12	24	36
1 mM	12	-	-	-

Table 3.1.10b Effect of GSH on Cd^{2+} induced changes on *D. discoideum* development. LA= loose aggregate, TA=Tight aggregate, SF=Slug formation, FBF= Fruiting body formation, - = No development till one week

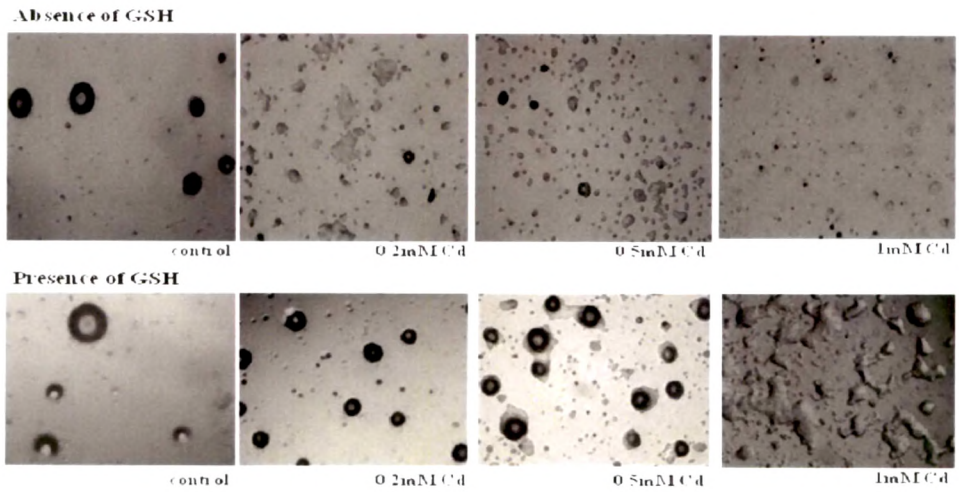


Figure 3.1.10a, Effect of GSH on Cd^{2+} induced developmental changes. Photographs at 12 hr, taken with 4X objective.

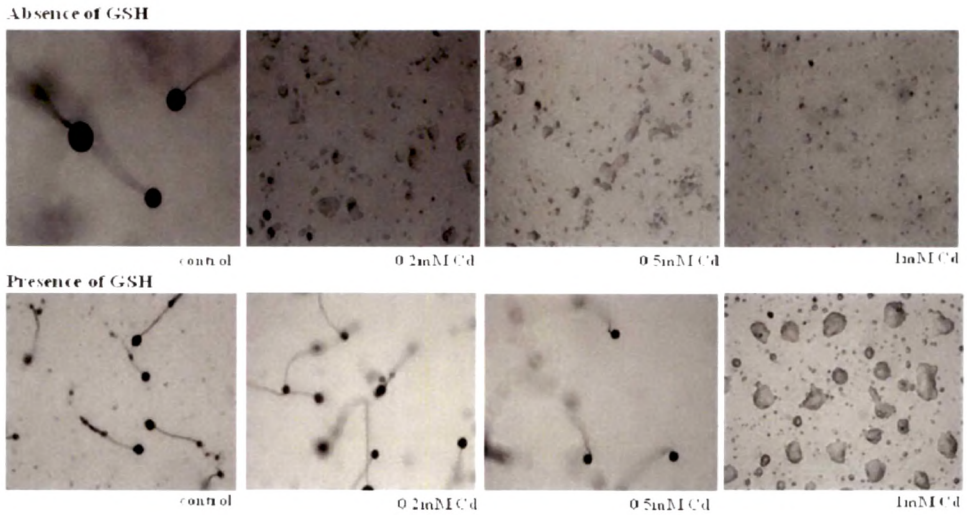


Figure 3.1.10b, Effect of GSH on Cd^{2+} induced developmental changes. Photographs at 24 hrs, taken with 4X objective.

3.2 Oxidative stress induction by exogenous addition of H_2O_2

3.2.1 Effect H_2O_2 on *Dictyostelium discoideum* cell death

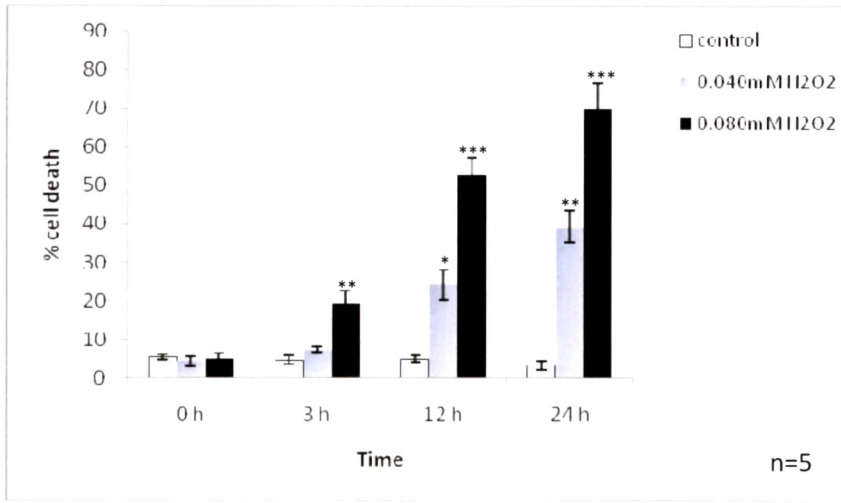


Figure 3.2.1a Dose and time dependent effect of cumene H_2O_2 on cell death. Data (mean ± S.E.) are from five independent experiments. * p value <0.05; ** p value <0.01; *** p value <0.001 as compared to control of respective time point.

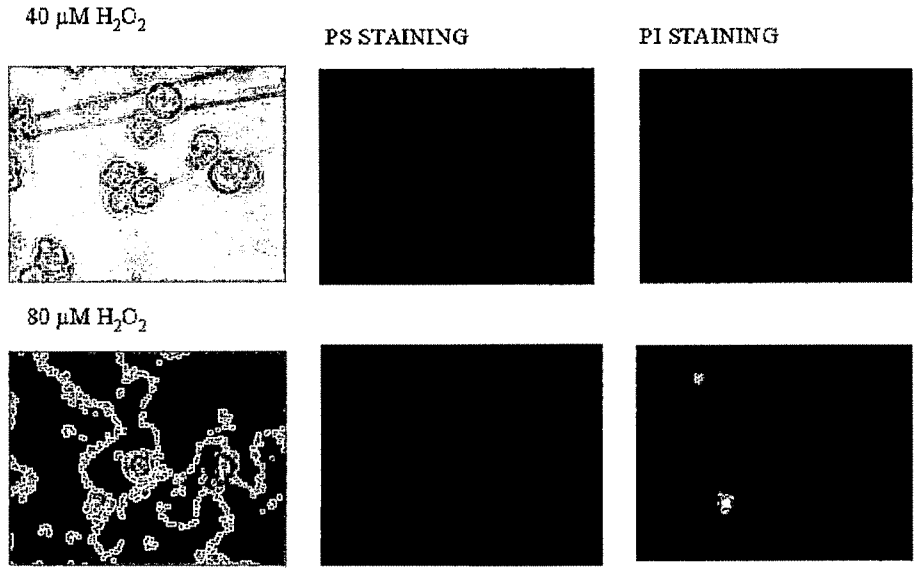


Figure 3.2.1b H_2O_2 induced cell death by PS-PI dual staining. PS exposure is seen at 5 hours while PI staining at 12 hours with 0.04 mM H_2O_2 (paraptotic dose) while 0.08 mM H_2O_2 was found to be necrotic as both AnnexinV-FITC and PI staining were observed at 3 hours. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

As per the above results Cd^{2+} induced cell death could be via oxidative stress induction. Henceforth we used cumene H_2O_2 as a positive control in our studies. Peroxides are often used as models to induce oxidative damage in cells *in vitro*. Cumene H_2O_2 penetrates inside the cells easily and causes damage of DNA and ultimately cell death. To find out the paraptotic and necrotic doses of H_2O_2, *D. discoideum* cells were treated with different doses of cumene H_2O_2 in HL5 medium and % cell death was measured by trypan blue exclusion assay. Cell death was found to be 25% and 50% at 12 hours after treatment with 0.04mM and 0.08mM H_2O_2 respectively (Fig. 3.2.1a). As can be seen in Fig 3.2.1b. 0.08mM cumene H_2O_2 treated cells exhibited both PS exposure and PI staining at 3 hours, in contrast 0.04mM cumene H_2O_2 was found to be paraptotic as PS exposure was exhibited by 50% cells after 5 hours while PI was not observed till 12 hours of oxidant treatment. Thus, based on Annexin V-PI dual staining results, 0.04mM and 0.08mM cumene H_2O_2 doses were found to be paraptotic and necrotic respectively.

3.2.2 Effect H_2O_2 on intracellular Reactive Oxygen Species (ROS) production

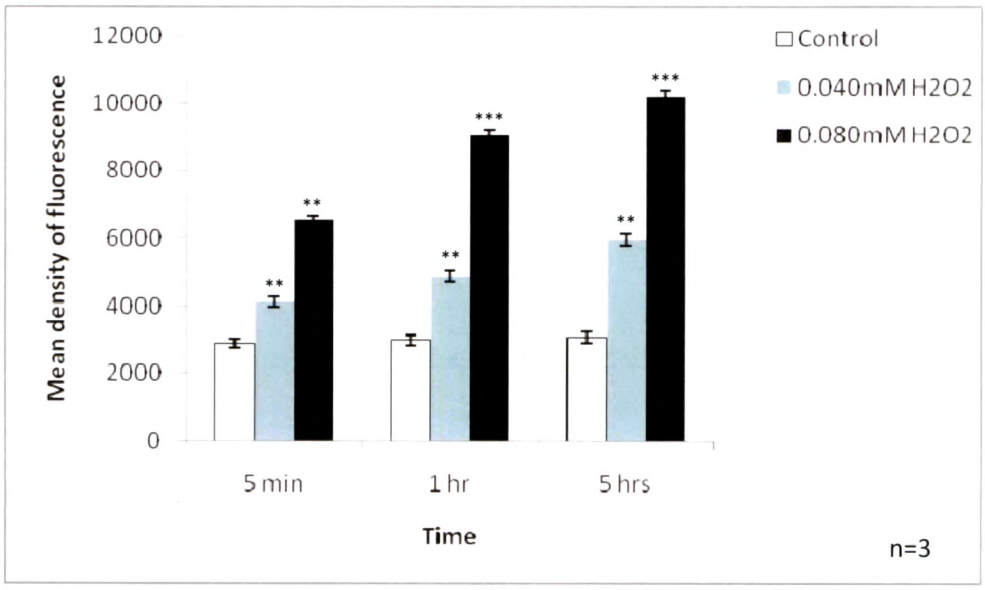


Figure 3.2.2a Effect of H_2O_2 on ROS production detected by fluorometry. H_2O_2 led to dose dependent increase in ROS production. Data (mean ± S.E.) are from three independent experiments. ** p value <0.01;*** p value <0.001 as compared to control.

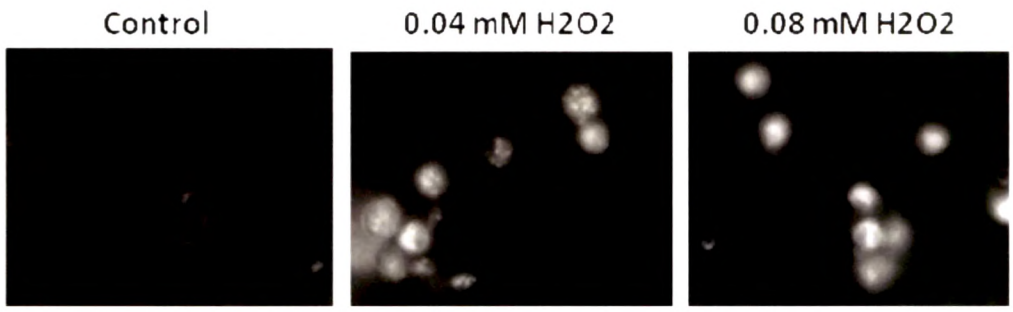


Figure 3.2.2b Effect of H_2O_2 on ROS production detected by fluorescence microscopy. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

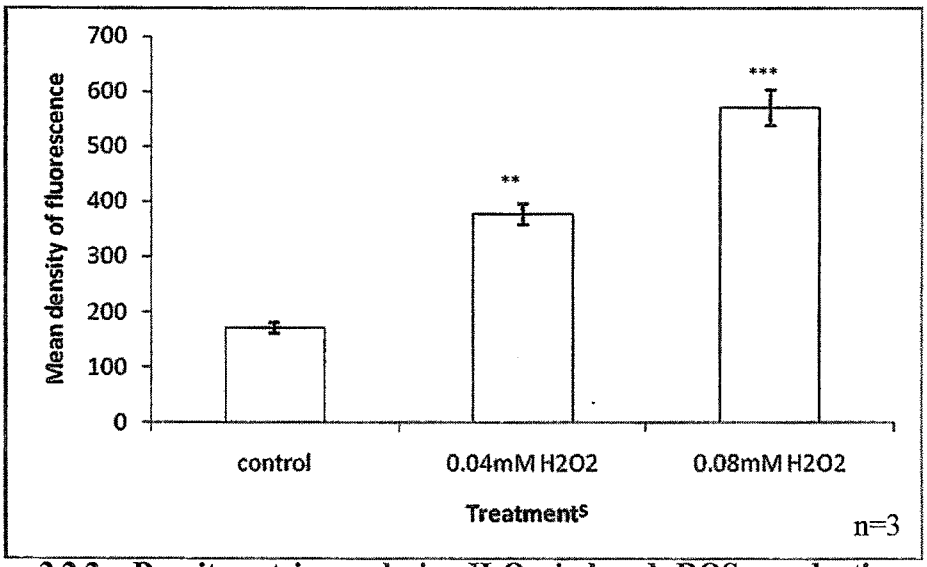


Figure 3.2.2c Densitometric analysis: H_2O_2 induced ROS production using DCFDA dye. H_2O_2 led to dose dependent increase in ROS production within 5 minutes. Data (mean ± S.E.) are from three independent experiments. *** p value <0.001 as compared to control.

Intracellular ROS generation was determined for paraptotic and necrotic doses of cumene H_2O_2. Figures 3.2.2a,b,c show increase in ROS generation within *D. discoideum* cells with increase in dose of H_2O_2.

3.2.3 PARP activation induced by H_2O_2

PARP being one of the immediate DNA damage sensors, DNA damage is necessary and sufficient to induce activation of PARP. PARP activity increased initially and peaked at 10 minutes post exposure to paraptotic and necrotic doses of cumene H_2O_2 (Figs. 3.2.3a, b). Earlier lab reports also showed no significant difference in PARP activity at 15 minutes time point after oxidant treatments. At necrotic doses, peak PARP activity was seen at 5 minutes post oxidant treatments. High poly ADP-ribose levels were maintained for a few minutes and basal level was regained after 10 min of hydroxylamine and H_2O_2 exposure during paraptosis (Rajawat, 2010).

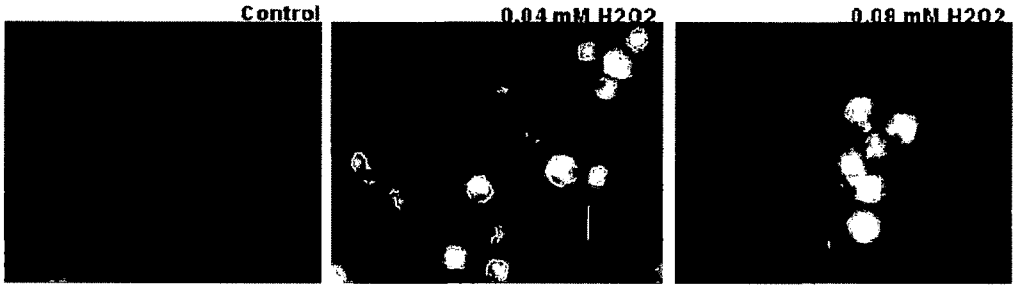


Figure 3.2.3a Effect of H_2O_2 induced PARP activation in *D. discoideum*. Data are representative of at least five independent experiments. Photographs were taken with 60X objective.

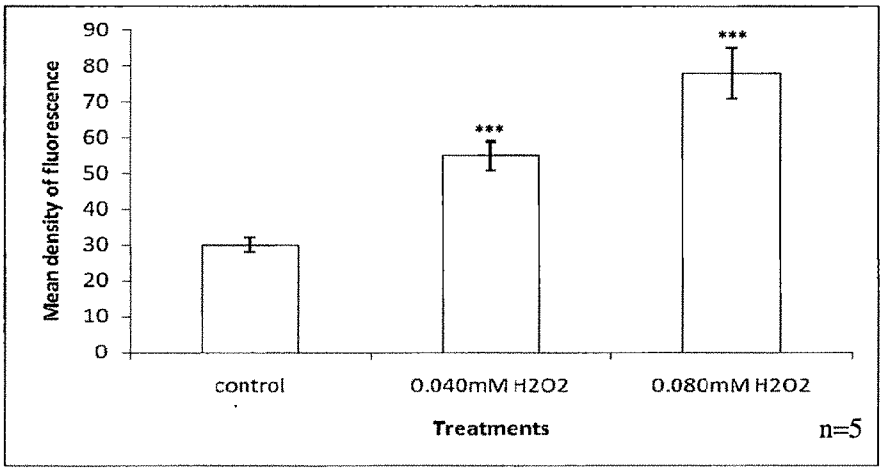


Figure 3.2.3b Densitometric analysis: H_2O_2 induced PARP activation. Cd^{2+} led to increase in PARP activation within 10 minutes of stress. Data (mean ± S.E.) are from five independent experiments. *** p value <0.001 as compared to control.

3.2.4 Effect of H_2O_2 on NAD^+ content

Depleted NAD^+ is one of the probable candidate signals responsible for the downstream events in the paraptotic pathway. Thus PARP activation was also confirmed by monitoring cellular NAD^+ levels in control and H_2O_2 treated cells and the results are shown in Fig. 3.2.4. Our results showed 0.04mM (paraptotic dose) and 0.08mM (necrotic dose) cumene H_2O_2 treated *D. discoideum* cells also exhibited a sharp decline (62% and 80% reduction respectively) in NAD^+ levels within 1 hour of H_2O_2 treatment (Figure 3.2.4).

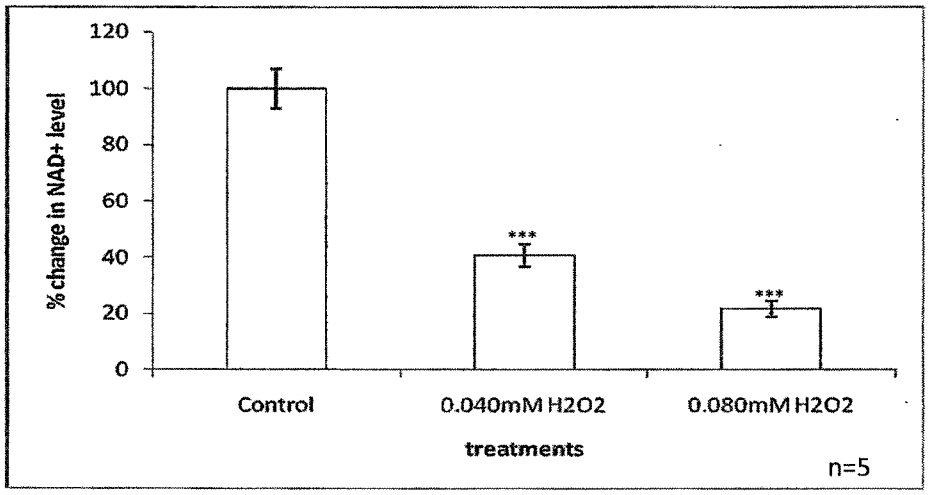


Figure 3.2.4 Effect of H_2O_2 on cellular NAD^+ levels. H_2O_2 led to dose dependent decrease in NAD^+ levels. Data (mean ± S.E.) are from five independent experiments. *** p value <0.001 as compared to control.

3.2.5 Effect of H_2O_2 on mitochondrial membrane potential (MMP)

As mention above, H_2O_2 treated *D. discoideum* cells were also monitored for MMP changes by using $DiOC_6$ dye. As can be seen in Fig. 3.2.5 reduction in MMP in H_2O_2 treated *D. discoideum* cells was monitored fluorimetrically and by fluorescence microscopy. H_2O_2 treatment also led to changes in MMP.

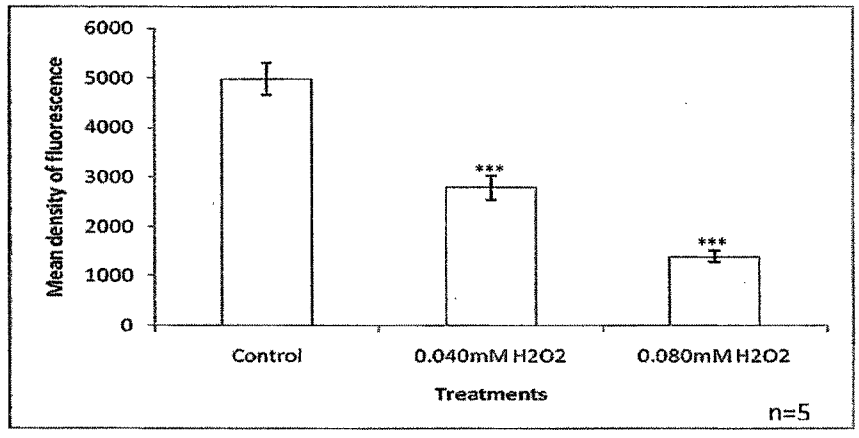


Figure 3.2.5a Effect of H_2O_2 induced changes in MMP by fluorometry. H_2O_2 led to decrease in Mitochondrial membrane potential. Data (mean ± S.E.) are from five independent experiments. *** p value <0.001 as compared to control.

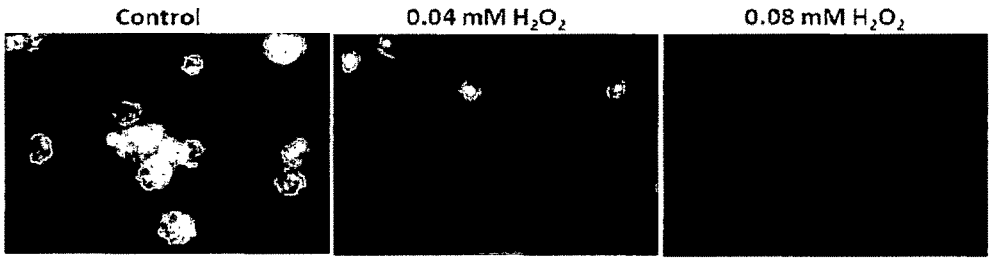


Figure 3.2.5b Effect of H_2O_2 induced changes in MMP by fluorescence microscopy. Data are representative of at least five independent experiments. Photographs were taken with 60X objective.

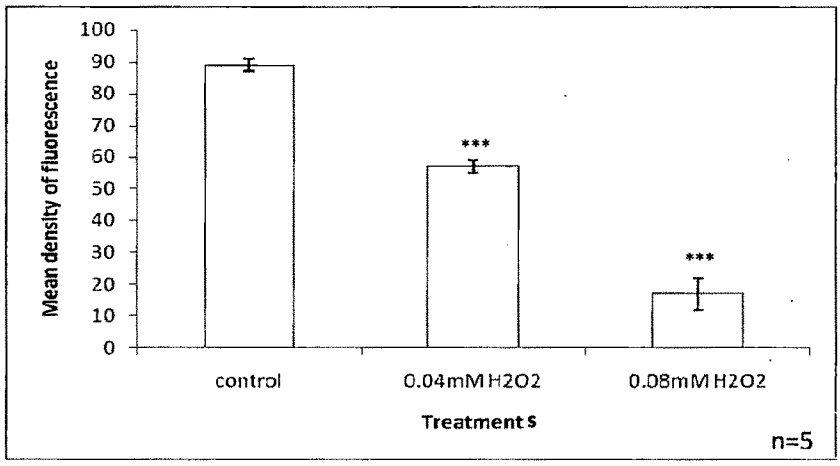


Figure 3.2.5c Densitometric analysis: H_2O_2 induced changes in MMP. H_2O_2 led to decrease in Mitochondrial membrane potential at 3hrs. Data (mean ± S.E.) are from five independent experiments. *** p value <0.001 as compared to control.

Discussion

Cadmium as a cell death inducer and cell death characterization

Both oxidative stress and inhibition of repair of oxidative DNA damage undoubtedly play a role in Cd^{2+} carcinogenesis (Waisberg *et al.*, 2003; Liu *et al.*, 2009). Although Cd^{2+} does not participate in redox cycle and therefore does not directly promote Fenton reaction, it nevertheless increases cellular levels of reactive oxygen species (ROS) (Lieberthal *et al.*, 1998; Oh and Lim, 2006). Depletion of antioxidant defense might seem a plausible mechanism, but Cd^{2+} also facilitates adaptive increase in levels of glutathione, the Cd^{2+}-binding protein metallothionein, and catalase that are

protective against peroxidative damage (Liu *et al.*, 2009; Lieberthal *et al.*, 1998). Displacement of Fenton-active metals from other sites and inhibition of mitochondrial electron transport are also other plausible mechanisms for Cd^{2+} toxicity (Templeton and Cherian, 1991).

Kinetic studies of cell death parameters were done using Cd^{2+} as cell death inducer. As monitored by trypan blue exclusion method for Cd^{2+} LD_{50} was found to be 0.5mM and our PS-PI dual staining results suggest that, 0.2mM and 0.5mM Cd^{2+} yielded paraptotic and necrotic cell death, as *D. discoideum* does not show apoptotic cell death due to the absence of caspases (Olie *et al.*, 1998). We have further confirmed that Cd^{2+} induces paraptotic or necrotic cell death via ROS generation (Figure 3.12a,b,c; 3.13 and 3.14). This study demonstrates several important findings that help in understanding the sequence of events happening during Cd^{2+} induced PARP mediated cell death in *D. discoideum*. ROS could be beneficial or exhibit pathophysiological role as cell death inducer and severity of the stress determines whether cells undergo necrosis or apoptosis (Nosseri *et al.*, 1994; Palomba *et al.*, 1996). Dose dependent effect of Cd^{2+} was carried out to select paraptotic and necrotic doses. Cd^{2+} and H_2O_2 at different concentrations can induce different types of cell death in *D. discoideum*. These results are parallel with a murine macrophage like tumor cell line which exhibits apoptosis and necrosis when exposed to lower and higher doses of H_2O_2 respectively (Schraufstatter *et al.*, 1986).

It has been reported that 0.01 mM Cd^{2+} treatment showed caspase independent cell death while 0.05 mM Cd^{2+} induced necrotic cell death in mesangial cells (Douglas *et al.*, 2010). During our studies Cd^{2+} yielded caspase independent (paraptotic) cell death by 0.2 mM Cd^{2+} (exposure for 1 hour) while 0.5 mM Cd^{2+} (exposure for 1 hour) showed necrosis in *D. discoideum* (Fig. 3.1.1). This finding suggests that *D. discoideum* cells are atleast 10-50 fold more resistant to Cd^{2+} than mesanglial cells.

Reduced glutathione and other thiol containing proteins play a key role in cellular protection against Cd^{2+} toxicity. Elevation of GSH levels has been demonstrated to be

protective against Cd^{2+}-induced lethality in rats (Singhal *et al.*, 1987). The ionic species, Cd^{2+}, which is regarded as being responsible for Cd^{2+}-induced toxicity, can be scavenged by glutathione to prevent its interaction with critical cellular targets. In addition, the GSH redox cycle, which includes GSH, glutathione peroxidase and glutathione reductase, plays important role in the detoxification of ROS that are generated by Cd^{2+} so as to protect cells from the potential toxicity (Meister and Anderson, 1983). It has been demonstrated that exposure to Cd^{2+} results in induction of the genes for γ-glutamylcysteine synthetase (γ-GCS), glutathione-S-transferases (GST-α and GSTπ) and the elevated synthesis of GSH so as to result in rapid and efficient detoxification of Cd^{2+} as well as the ROS generated from this metal (Chin and Templeton, 1993; Eneman *et al.*, 2000). However, either continuous exposure or exposure to toxic doses of Cd^{2+} may overwhelm the cellular supply of GSH and the related defense system so as to result in toxicity, including carcinogenesis (Liu *et al.*, 1990). In general, the expression of antioxidant genes such as those coding for the synthesis of superoxide dismutase and catalase is repressed by Cd^{2+} (Casalino *et al.*, 1997; Hussain *et al.*, 1987; Liu *et al.*, 2002; Ognjanovic *et al.*, 1995; Shukla *et al.*, 1989) and thus offers the underlying reason for Cd^{2+}-induced lipid peroxidation, oxidative stress, and the associated toxicity. In contrast, there are some reports suggesting an increase in activity of SOD and catalase in tissues adapted to Cd^{2+} by prolonged exposure (Kostic *et al.*, 1993; Sarkar, 1995; Zikic *et al.*, 1998). These findings reinforce the conclusions from the data on Cd^{2+}-induced ROS generation.

Our results suggest that Cd^{2+} induced cell death could be via ROS generation in *D. discoideum* (Fig. 3.2.1) hence we have studied cumene H_2O_2 induced cell death. Interestingly, GSH confers strong protection against Cd^{2+} induced ROS production (Fig. 3.1.4), MMP changes (Fig. 3.1.9), cell death (Fig. 3.1.2) and development (and 3.1.10). *D. discoideum* showed significant induction of either *cat*A mRNA or CatA enzyme activity under oxidative stress, however, cat A mutant was found to be 160 fold more sensitive to H_2O_2 (Garcia *et al.*, 2003). 2-5 fold more catalase activity was found in *D. discoideum* compared to other organisms and this could also confer resistance to oxidative stress to this organism (Madigan and Katz, 1989). Furthermore, we have reported an increase in glutathione peroxidase (GPx) activity

under oxidative stress (Katoch and Begum, 2003). Thus presence of high level of catalase throughout its life cycle and significant induction of GPx activity could confer *D. discoideum* cells higher resistance to oxidative stress. Furthermore, *D. discoideum* has a high content of unsaturated fatty acids making up 75-90% of the fatty acids of the organism and of the membrane (Weeks and Herring, 1980). However, the fatty acids are monounsaturated or with two double bonds and hence are less susceptible to lipid peroxidation compared to polyunsaturated fatty acids. Thus non-significant changes in lipid peroxidation (LPO) levels (Katoch and Begum, 2003) under oxidative stress could also offer an explanation for the high resistance of *D. discoideum* to oxidative stress.

3.1.5 Induction of DNA damage by Cd^{2+} exposure leading to PARP activation

ROS generated by Cd^{2+} treatment are known to cause DNA lesions; the most abundant being base modification and phosphorylation of gamma H2AX protein (Minami *et al.*, 2005). Also there is evidence that Cd^{2+} interferes with several DNA repair mechanisms including the repair of oxidative DNA damage. Phosphorylation of H_2AX (γH2AX) at Ser139 is known to play a very early and important role in the cellular response to DNA double strand breaks and is mediated by ataxia telangiectasia mutated kinase (ATM) (Burma *et al.*, 2001; Sedelnikova *et al.*, 2003). To explore the effect of Cd^{2+} on *D. discoideum* DNA cells were treated with Cd^{2+} and DNA damage was assessed using anti-H2AX antibody. Immunofluorescence results revealed that treatment with increasing doses of Cd^{2+} resulted in increased H2AX phosphorylation within 5 minutes (Figure 3.1.5), suggesting an early DNA damage induction post Cd^{2+} stress induction.

At low concentrations of Cd^{2+}, which do not generate oxidative base modifications as such, have been demonstrated to inhibit the repair of oxidative damage in mammalian cells (Dally and Hartwig, 1997). Regarding nucleotide-excision repair, Cd^{2+} inhibited the removal of thymine dimers generated by UV-irradiation by interfering with the first step of this repair pathway, i.e. the incision at the DNA lesion. Cd^{2+} at low concentrations inhibited the specific binding of repair proteins to damaged DNA, and this effect was restored by zinc. Furthermore, Cd^{2+} inhibited the bacterial repair

enzyme formamido–pyrimidine–glycosylase and the specific DNA binding of the mammalian protein XPA that is essential for DNA damage recognition during nucleotide-excision repair (Asmuss *et al.*, 2000). Both proteins are members of the family of zinc-finger proteins, and the inhibitory effect of Cd^{2+} was assigned to a substitution of zinc by Cd^{2+} (Hartwig, 2001). Buchko *et al.* (2000) used spectroscopic methods to study the effect of Cd^{2+} on the structure of the XPA protein. In spite of the observed complex formation of Cd^{2+} with the four cysteine sulphur atoms of the DNA binding zinc finger region, there were no structural differences between the zinc and the Cd^{2+} forms of XPA. Nevertheless, Cd^{2+} still could interfere with the DNA binding of XPA by binding to surface residues of this protein.

Cd^{2+} was effective as an inhibitor of the expression of 8-oxoguanine DNA glycosylase both *in vivo* in rats exposed to aerosols of Cd^{2+} acetate and *in vitro* in a rat lung epithelial cell line (Potts *et al.*, 2003). This down-regulation was observed both at the mRNA and the protein levels, and it was observed by Cd^{2+} concentrations that caused no loss in viability in the cell culture (10uM).

Jin *et al.* (2003) found that chronic exposure of yeast to environmentally relevant Cd^{2+} resulted in extreme hypermutability because Cd^{2+} reduced the capacity for mismatch repair, and in extracts of human cells Cd^{2+} inhibited at least one step leading to mismatch removal. $Cd^{2+}Cl2$ concentrations (10–1000 nm) on DNA integrity in Hepatocyte (HepG2) cells have been reported, where Lopez-Ortal *et al.* (1999), showed DNA single-strand breaks formation in a human fetal hepatic cell line WRL-68, even below 10 nm $Cd^{2+}Cl2$ concentration. Based on these reports we propose that Cd^{2+} at 0.2mM-0.5mM concentration showed DNA damage in *D. discoideum* (Fig. 3.1.5a,b). Earlier lab reports also showed basal PARP activity at 15 minutes time point after oxidant/UV-C treatment. At necrotic doses, peak PARP activity was seen at 5 minutes post oxidant/UV-C treatment. High poly ADP-ribose levels were maintained for a few min and basal level was regained after 10 min of hydroxylamine and cumene H_2O_2 exposure during paraptosis (Rajawat, 2010 and Mir, 2011).

3.1.6 PARP activation leads to mitochondrial changes via NAD^+ depletion

It has been reported that nuclear NAD^+ pool can transport in cytosol or *vice versa* thus NAD^+ depletion is one of the best parameters to be checked for nuclear-mitochondrial cross talk. As per our hypothesis NAD^+ may act as currency coin or depletion of NAD^+ may serve as an important signal during oxidative stress induced PARP mediated cell death. Consequent to PARP activation cellular NAD^+ as well as ATP pools deplete, later as a result of cellular attempt to replenish the reduced NAD^+ levels of the cells. Depleted NAD^+ is one of the probable candidate signals responsible for the downstream events in the paraptotic/necrotic pathway.

Depletion of NAD^+, an essential cofactor for cellular energy production that participates in vital signalling pathways, is a major cause of cell death caused by genotoxic stress (Yang *et al.*, 2007). Hence effect of NAD^+ supplementation on oxidative stress induced cell death was also studied.

Mitochondria and the nucleus play critical roles in cell death. The signalling between these two organelles is dynamic. Understanding the signalling process between the nucleus and the mitochondria in cell death programs could lead to more options in the development of new therapeutic targets. The molecular mechanisms accounting for PARP-dependent cell death are still being investigated but changes in mitochondrial membrane potential leading to the release of AIF and its translocation to the nucleus appears to be essential in this death process. PARP-mediated depletion of NAD^+ and ATP or the signals due the PARP over activation that trigger AIF translocation, however, factors and the mechanism of mitochondrial AIF release remain to be explored.

Our present work suggests that the activation of PARP (within 10 min) and reduction in mitochondrial membrane potential (within 3 hrs) could occur at post exposure of oxidative stress. Interestingly NAD^+ depletion could occur in 1 hr post exposure of Cd^{2+} and H_2O_2 in *D. discoideum* (Figs. 3.1.7, 3.1.8 and 3.1.9). Based on these results it is possible that NAD^+ might act as signalling molecule or as messenger in nuclear-

mitochondria cross talk during oxidative stress induced PARP mediated cell death in *D. discoideum* cells.

Intracellular NAD^+ is segregated into cytosolic and mitochondrial pools that do not readily interchange (Lisa *et al.*, 2001), and previous reports suggest that PARP-1 activation leads to an initial, preferential depletion of the cytosolic NAD^+ pool. Cytosolic NAD^+ is an essential co-factor at the lactate dehydrogenase step and at the glyceraldehyde-3-phosphate dehydrogenase step of glycolysis. Glycolysis is almost completely blocked in astrocytes after PARP-1 activation, and this blockade is reversed by NAD^+ repletion. It has also been shown that astrocytes (and neurons) can be rescued from PARP-1-induced cell death by metabolic substrates such as pyruvate that bypass glycolysis (Ying *et al.*, 2003). These substrates are oxidized in the mitochondrial tricarboxylic acid cycle, suggesting that the mitochondrial NAD^+ pool is preserved for a period of time after PARP-1 activation (Alano *et al.*, 2004). These observations are consistent with the fact that mitochondrial NAD^+ is not normally accessible to the cytosol and therefore cannot normally serve as substrate for PARP-1. It has been proposed, however, that MPT permits the egress of mitochondrial NAD^+ (Lisa *et al.*, 2001) and this may lead to potentially irreversible loss of NAD^+ from the mitochondrial compartment.

Mitochondria under physiological conditions also play active roles in the maintenance of normal cellular functioning. A key feature of mitochondria that allows them to participate in cell survival is proton pumping across the impermeable inner membrane. This generates an electrochemical gradient, composed of $\Delta\psi_m$ (mitochondrial membrane potential) and ΔpH (pH gradient), which is used for ATP synthesis, ADP–ATP exchange, uptake of respiratory substrates and inorganic phosphate, transport of K^+, Na^+, and anions to regulate volume, and regulation of protons to control heat production (Bernardi, 1999). Mitochondria also play protective roles by buffering cells against high concentrations of calcium (Budd and Nicholls, 1996; White and Reynolds, 1997; Stout *et al.*, 1998) and sequestering proapoptotic proteins, such as AIF and cyt c (Green and Reed, 1998; Desagher and Martinou, 2000).

Importantly, MNNG-treated astrocytes in which NAD^+ was maintained near normal levels did not exhibit MPT or cell death, suggesting that NAD^+ is essential in the link between PARP-1 activation and MPT. NAD^+ does not directly influence MPT, but NAD^+ depletion may promote MPT by several indirect mechanisms. ATP and ADP are important endogenous inhibitors of MPT, (Zoratti *et al.*, 2001), and these purines are consumed during NAD^+ synthesis (Zang *et al.*, 1994; Berger 1985; Ha *et al.*, 1999). Another factor promoting MPT may be impaired substrate delivery to mitochondria, because NAD^+ is an essential for flux though the glycolytic pathway to the mitochondria (Ying *et al.*, 2002). Impaired substrate delivery may also contribute to $\Delta\psi_m$ depolarization, which, in turn, promotes MPT (Bernardi *et al.*, 1996).

Inhibition of MPT with either cyclosporin A or Bcl-2 overexpression has been previously shown to block AIF release in a cell-free mitochondria system and in tumor cell lines (Métrailler-Ruchonnet *et al.*, 2007). Because AIF is normally localized within the mitochondrial intermembrane space (Green, 1998), this suggests the possibility that MPT may provide a route for AIF release (Porter *et al.*, 2006). It is also possible; however, that MPT or the associated collapse of mitochondrial membrane potential leads to AIF release by more indirect routes. Although many studies suggest that PARP-1 is localised exclusively to the nucleus, one report suggests that PARP-1 may also be localized to mitochondria (Rossi *et al.*, 2009). PARP-1 localization to mitochondria could permit direct interactions between PARP-1 and mitochondrial components involved in MPT.

Based on these reports and our present data, we suggest that, both NAD^+ depletion and MPT are necessary downstream events in the sequence of events leading from PARP-1 activation to cell death. The findings support a complex interaction between the nuclear and mitochondrial cell death programs during PARP-1 activation, whereby nuclear DNA damage triggers PARP-1 activation and NAD^+ depletion, NAD^+ depletion leads to reduction of mitochondrial membrane potential, in turn leads to nuclear AIF translocation and cell death in *D. discoideum* under oxidative stress induction by Cd^{2+} or H_2O_2.

Cd^{2+} does not catalyze Fenton-type reactions (as like H_2O_2) because it does not accept or donate electrons under physiological conditions, and it is only weakly genotoxic. However inhibition of DNA repair by Cd^{2+} is likely to play an important role in carcinogenesis (Waalkes *et al.*, 2003). At noncytotoxic concentrations, Cd^{2+} inhibited base excision repair of DNA damaged by light in HeLa cells (Hartwig *et al* 2002; Dally *et al.*, 1997). In nucleotide excision repair, Cd^{2+} inhibited the first step, which is recognition of DNA damage (Hartmann *et al.*, 1998). It has been shown that Cd^{2+} inhibits the binding to DNA of the xeroderma pigmentosum group A protein, XPA, a protein necessary for the recognition of DNA damage (Asmuss *et al.*, 2000). Thus, there is ample evidence that Cd^{2+} can inhibit DNA repair at multiple levels, leading to genome instability. Interestingly, *D. discoideum* exhibit unusual DNA repair and antioxidant system, explaining the use of higher dose of Cd^{2+} (0.2mM and 0.5mM) during our studies. However our data suggest that Cd^{2+} induced cellular toxicity via oxidative stress generation in *D. discoideum.* Cd^{2+} activates the calcium channel Cch1/Mid1, which also contributes to Cd^{2+} entry into the cell. While cumene H_2O_2 penetrates more easily in cell membrane as well as organelles membranes. Furthermore cumene H_2O_2 and its derivatives peroxynitrite or hydroxyl radicals are known to be direct genotoxic has been well documented, which explaining cumene H_2O_2 requires in micro molar concentration to induce similar level of toxicity in *D. discoideum.*

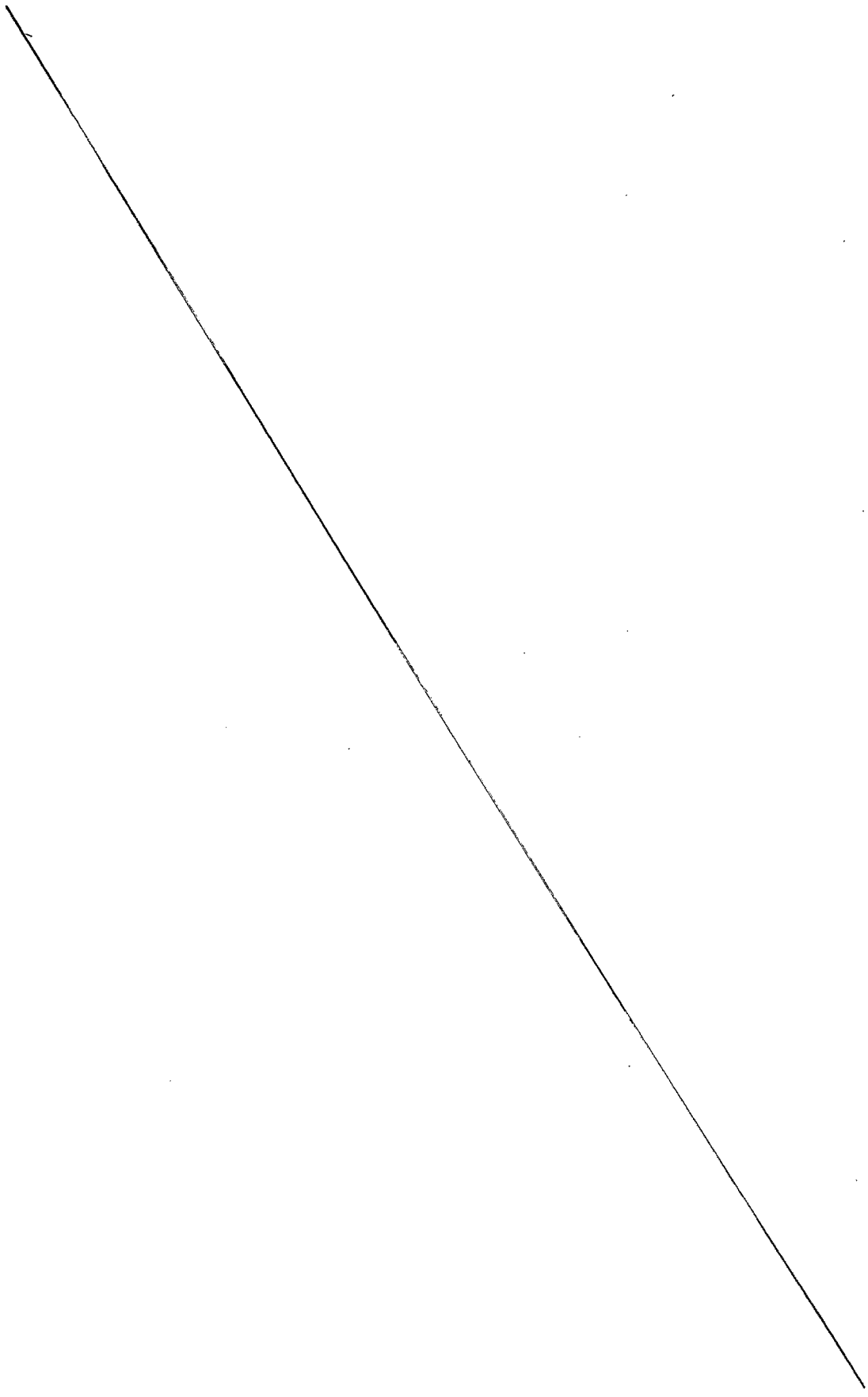
From our results it is clear that H_2O_2 treatment could generate significant ROS i.e. one fold and two fold induction could be seen within a 5 min in paraptotic and necrotic dose of H_2O_2 respectively, that are measurable with DCFDA dye. Similarly Cd^{2+} treatment also yielded DCFDA measurable ROS after 5 min treatment. Therefore 0.04mM H_2O_2 and 0.2mM Cd^{2+} exposed *D. discoideum* cells yielded ROS within 5 min of treatment that are sufficient to take the cells in paraptotic mode of cell death. Similarly 0.08 mM H_2O_2 or 0.5mM Cd^{2+} treatment resulted in high levels in ROS, thus making *D. discoideum* cells to exhibit necrotic cell death. It is interesting to note that Cd^{2+} treatment, as well as H_2O_2 exposed *D. discoideum* cells within 10 min could generate significant ROS formation leading to DNA damage that further results in

PARP activation followed by MMP changes and cell death of paraptotic or necrotic types.

Cd^{2+} induced changes in *D. discoideum* development

Multicellular organisms have evolved sophisticated systems by which cells regulate their respective functions co-ordinately. *Dictyostelium*, as a social amoeba, represents an unusual mode of multicellularity by which previously independent cells group together to form a multicellular organism, but requires a similar degree of communication between different cells as do multicellular plants and animals. *Dictyostelium* utilizes superoxide as a signalling intermediate at the onset of its development from unicellular to multicellular existence (Bloomfield *et al.*, 2003). However ROS are required only in moderate levels for pre aggregation phase of developmental.

Cadmium induces sub cellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves (Romero *et al.*, 2004). These ROS cause DNA damage and thereby mediate cytotoxic effects. Similar effects were also observed in our experiments where exposure of *D. discoideum* to cadmium resulted in significant DNA damage. Cadmium showed a dose dependent toxic effect on the development. Glutathione is the first line of defense against cadmium toxicity (Anderson *et al.*, 1987). It forms complex with several heavy metals and thus might function to protect cells against metal toxicity. Such a role has been ascribed to the cysteine-rich protein metallothionein, which binds cadmium and certain other heavy metal ions avidly (Vallee *et al.*, 2003). Glutathione is required for growth and prespore cell differentiation in *D. discoideum* (Kim *et al.*, 2005) and also glutathione is important to scavenge ROS in the cells. This is also supported by our study in which cadmium treatment is showing developmental block or delay in a dose dependent manner, while GSH pretreated cells showed restoration in Cd^{2+} induced delay in development (Figures 3.1.10a, b). The effect of catB mutant of *D. discoideum* resembles the effects observed after cadmium treatment, as in both cases delayed or block development at progression of aggregation to slug formation stage was observed. These results suggest cadmium might be affecting CatB activity/expression during development (Garcia et al., 2003).



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