CHAPTER 4

The role of poly (ADP-ribose) polymerase during Cd^{2+} induced cell death

Introduction

PARP-1, is a nuclear enzyme that catalyzes the formation of poly (ADP-ribose) on its target proteins using NAD^+ as a substrate (D' Amours *et al.*, 1999). Binding of damaged DNA to PARP-1 results in poly (ADP-ribosylation) of many nuclear proteins including PARP-1 itself, histones, and transcription factors (D' Amours *et al.*, 1999). In addition to DNA repair, PARP1 is also implicated in cell death. Over activation of PARP has been emphasized as one of the pathogenic mechanisms of induced Parkinson's disease (Mandir *et al.*, 1999), amyotrophic lateral sclerosis (Kim *et al.*, 2003, 2004) and ischaemic stroke (Eliasson *et al.*, 1997; Endres *et al.*, 1997, 1998a,b; Love *et al.*, 1998, 1999b; Strosznajder *et al.*, 2003), traumatic brain injury (Wallis *et al.*, 1996; Whalen *et al.*, 1999; LaPlaca *et al.*, 2001; Besson *et al.*, 2003) and inflammatory diseases (Virag & Szabo, 2002). PARP expression was increased in autopsied ischaemic human brain and PARP knockout mice showed increased resistance to ischaemic brain damage. These studies provide good examples to show the important role of PARP in cell death induced by ischaemic stroke and other stress signals (Eliasson et al., 1997; Love et al., 1998, 1999).

PARP-1 mediated cell death pathway may be caspase-independent and morphologically distinct from classical apoptosis (Hong *et al.*, 2004). Key events in this cell-death pathway include mitochondrial depolarization, mitochondrial permeability transition and mitochondrial release of apoptosis-inducing factor (AIF). AIF, upon release from the mitochondria, translocates into the nucleus, where it triggers nuclear DNA fragmentation. The PARP activation- induced translocation of AIF plays a critical role in MEF (Mouse embryonic fibroblast) and neuronal cell death upon exposure to MNNG (Virag *et al.*, 1998). However, signal/s which is/are involved during nuclear-mitochondria cross talk is yet to be addressed.

It is hypothesised that overactivation of the enzyme may stampede the cells into cellular suicide by depleting cellular NAD^+ and ATP stores (Virag and Szabo, 2002). As the ADP-ribose donor for PARP-1-catalyzed PARylation reactions, NAD^+ plays a central role in determining the function and activity of PARP-1. The synthesis of

NAD^+ occurs in multiple cellular compartments, including the nucleus, which may be the most relevant source of NAD^+ for PARP-1 (Berger *et al.*, 2004; Rongvaux *et al.*, 2003). This has led to the 'suicide hypothesis', in which rapid catabolism of NAD^+ by PARP-1 activation affect cellular energy metabolism and, ultimately, lead to cell death (Berger *et al.*, 1986; Berger 1983). Contrary to this general concept it was reported that no significant changes in NAD^+ levels were observed despite of PARP activation (Paschen et al., 2000; Goto et al., 2002).

Earlier lab studies described events during oxidative stress induced PARP mediated cell death in *D. discoideum* (Rajawat, 2010). The role of PARP was intercepted by benzamide or by PARP antisense. It was demonstrated that oxidative stress induced cell death was also partially rescued by PARG inhibition. We put forth the idea that *D. discoideum* exhibits paraptosis which is mediated by PARP. PARP and AIF are the major players governing *D. discoideum* cell death kinetics during paraptosis and necrosis induced by oxidative stress (Rajawat, 2010). Similarly, role of PARP during cell death induced by different stresses such as UV-C, staurosporine (STS), starvation was also studied. UV-C and starvation mimic the oxidative stress induced cell death. However, STS induced paraptosis that is characterized by AIF mediated DNA fragmentation was not affected with PARP inhibition. Thus PARP is a dispensable player of paraptosis (Mir, 2011).

Our present study addresses the role of PARP during Cd^{2+} induced cell death in cellular slime mold *D. discoideum* with an aim to find the link for nuclear mitochondrial cross talk during this type of cell death. Pharmacological agents like benzamide a potent inhibitor of Poly(ADP-ribose) polymerase (PARP), gallotannin a inhibitor of Poly(ADP-ribose) glycohydrolase (PARG), exogenous NAD^+ supplementation, and ALLN (Ac-LLnL-CHO, MG-101, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal, N-Acetyl-Leu-Leu-Norleu-al) a calpain inhibitor to explore the protective effect of these agents against oxidative stress induced cell death.

Results

4.1 Standardization of NAD^+ dose for supplementation studies during Cd^{2+} induced oxidative stress in *D. discoideum* cells

Doses of benzamide (1 mM), gallotannin (50 μM) and ALLN (50 μM) for cell death studies were already standardized in the lab (Rajawat, 2010). To standardize the NAD^+ concentration for replenishment studies, *D. discoideum* cells were treated with different concentrations of NAD^+ (1, 10 and 20 mM) and trypan blue exclusion assay was done to assess cell death. 1 to 10 mM NAD^+ doses did not induce cell death while 20 mM NAD^+ led to ~40% cell death (Fig. 4.1). Based on these results 10mM NAD^+ was used for further studies.

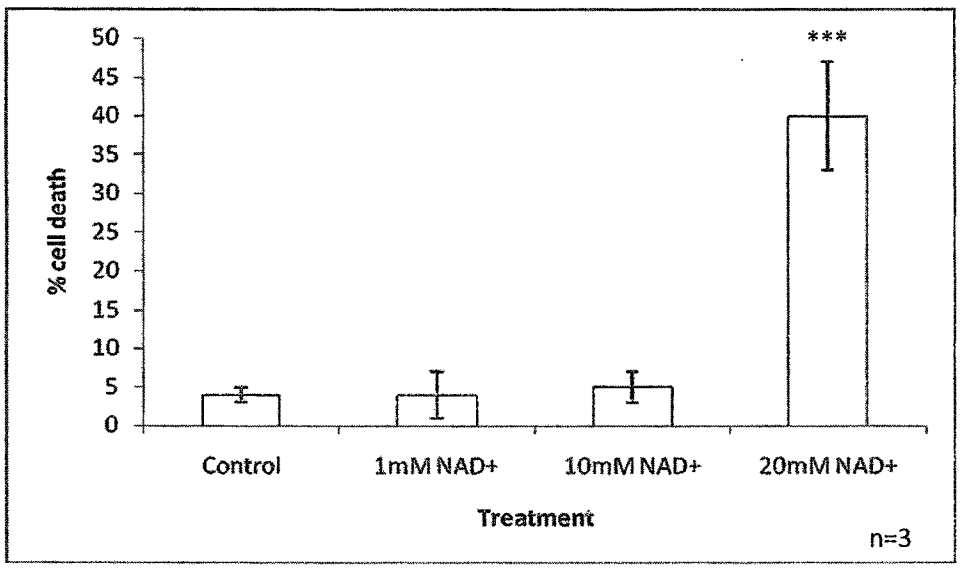

Figure 4.1. Dose dependent effect of NAD^+ supplementation on *D. discoideum* cell death. Results are the mean ± SE of three independent experiments. *** p value <0.001 compared to control.

4.2 Effect of NAD^+ supplementation or PARP inhibition (benzamide) or PARG inhibition (gallotannin) on Cd^{2+} induced cell death

D. discoideum cells were supplemented with 1 and 10 mM NAD^+ for 2 hrs prior to oxidative stress (H_2O_2 or Cd^{2+}) and cell death was monitored by trypan blue assay.

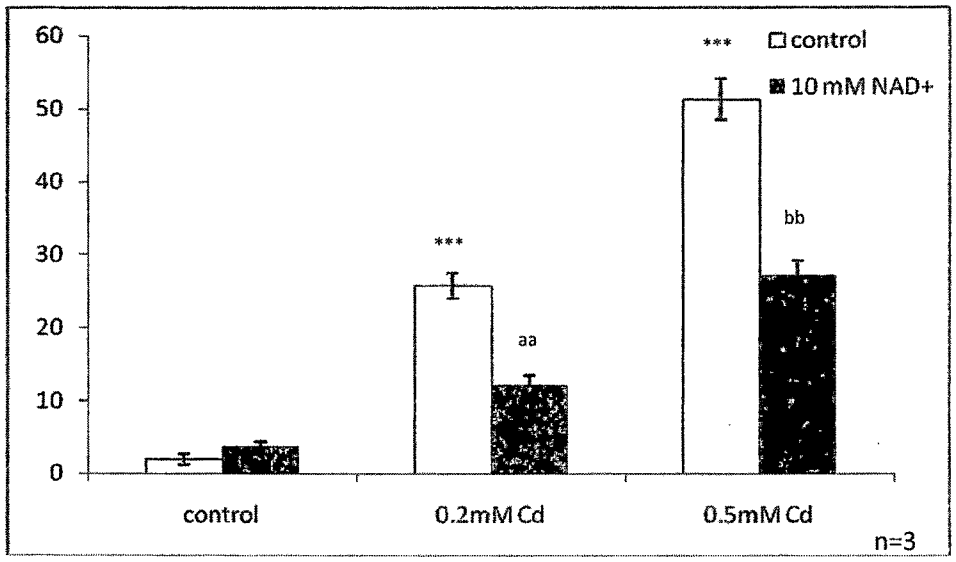

Figure 4.2.1 Effect of NAD^+ supplementation on Cd^{2+} induced cell death. *** p value <0.001compared to control; aa & bb p value <0.01compared to 0.2mM and 0.5mM Cd^{2+} respectively.

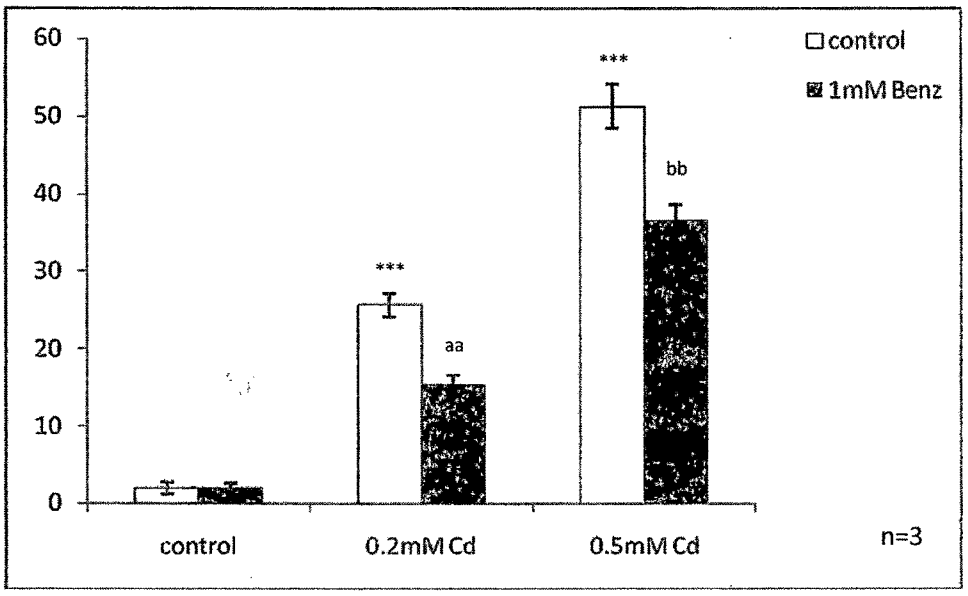

Figure 4.2.2 Effect of PARP inhibition on Cd^{2+} induced cell death. *** p value <0.001compared to control; aa & bb p value <0.01compared to 0.2mM and 0.5mM Cd^{2+} respectively.

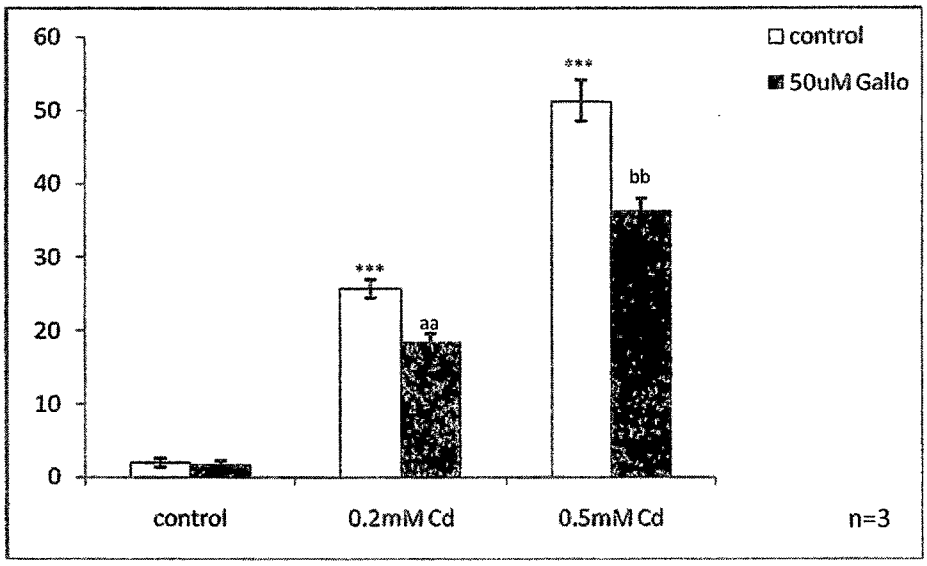

Figure 4.2.3 Effect of gallotannin on Cd^{2+} induced cell death. *** p value <0.001compared to control; aa & bb p value <0.01compared to 0.2mM and 0.5mM Cd^{2+} respectively.

As can be seen in figs. 4.2.1-4.2.3, NAD^+ supplementation or PARP inhibition (benzamide), or PARG inhibition (gallotannin) pre treatment partially intercepted Cd^{2+} induced cell death in *D. discoideum* cells. Cell death with Cd^{2+} was found to be reduced from 25% (paraptotic dose) to 12%, 15%, and 18% in cells pretreated with 10mM NAD^+, 1 mM benzamide and 50 μM gallotannin respectively and cell death after necrotic dose treatment was rescued from 50% to 30%, 35%, and 48% in 10mM NAD^+, 1mM benzamide and 50μM gallotannin respectively.

Similarly *D. discoideum* cells were supplemented with 1 and 10 mM NAD^+ for 2 hrs prior to H_2O_2 treatment and cell death was monitored by trypan blue assay. As can be seen in fig. 4.2.4 cell death was found to be reduced from 25% to 15% with 1 mM NAD^+ and 25% to 8% with 10 mM NAD^+ while in case of necrotic dose of H_2O_2 cell death was reduced from 50% to 40% with 1 mM NAD^+ and 50% to 30% with 10 mM NAD^+ pre treatment. ~~Thus NAD^+ supplementation rescued cell death in a dose dependent manner~~. Thus NAD^+ supplementation rescued cell death in a dose dependent manner.

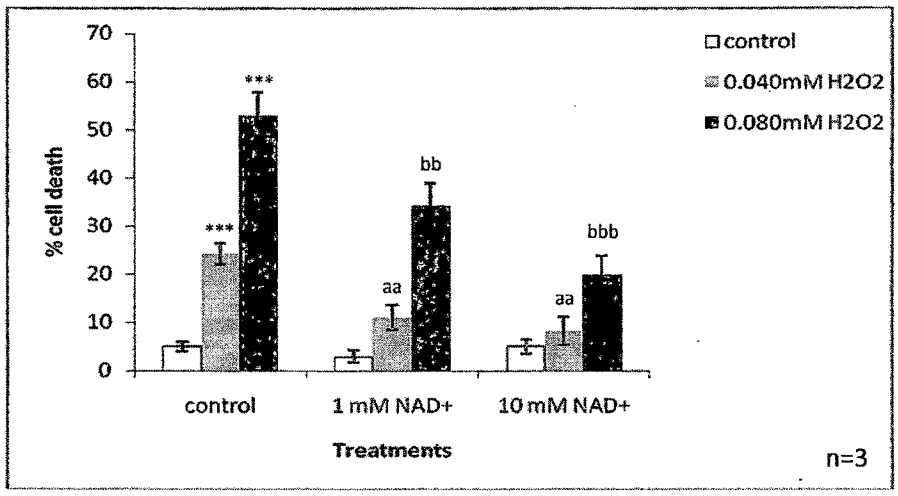

Figure 4.2.4 Effect of NAD^+ supplementation on H_2O_2 induced cell death. *** p value <0.001compared to control; aa & bb p value <0.01compared to 0.04 and 0.08mM H_2O_2 respectively; bbb p value <0.001compared to 0.08mM H_2O_2.

Similarly PARP and PARG inhibition reduced the percent of paraptotic cell death from 25 to14 and 16% while the percent of necrotic cell death was reduced from 50 to 30 and 34% respectively (Figs. 4.2.5 and 4.2.6).

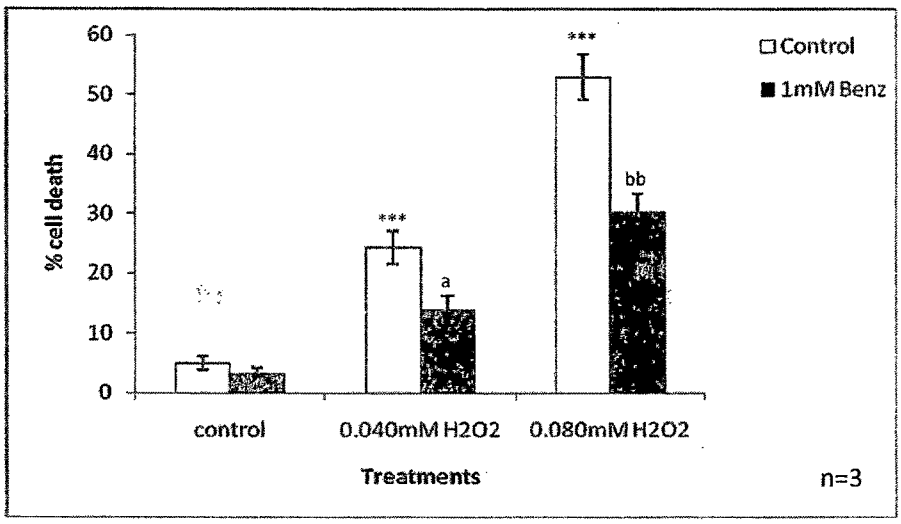

Figure 4.2.5 Effect of PARP inhibition on H_2O_2 induced cell death. *** p value <0.001compared to control; bb p value <0.01compared to 0.08mM H_2O_2; a p value <0.05compared to 0.04mM H_2O_2.

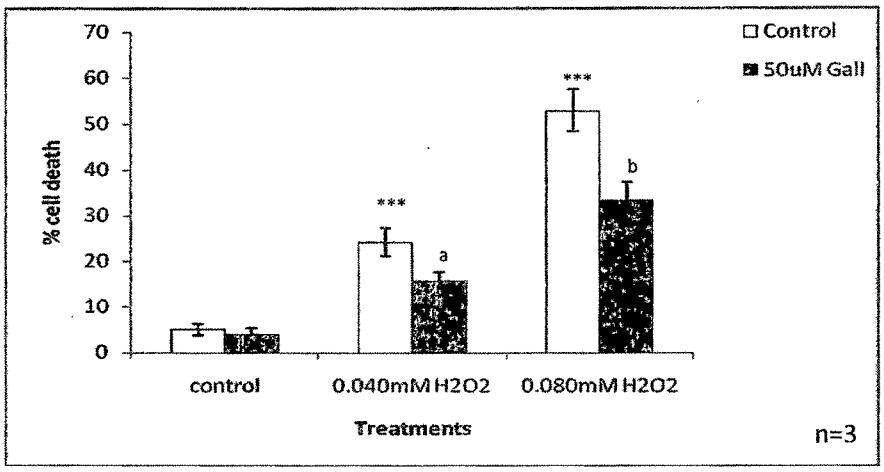

Figure 4.2.6 Effect of PARG inhibition on H_2O_2 induced cell death. *** p value <0.001 compared to control; a and b p value <0.05 compared to 0.04 and 0.08mM H_2O_2 respectively.

Calpains, a family of Ca^{2+}-dependent cysteine proteases reside in the cytosol in inactive form. In response to increased levels of cytosolic Ca^{2+}, calpains translocate to the intracellular membranes and are activated by autocatalytic hydrolysis. To analyse the role of calpain in this PARP mediated cell death of *D. discoideum,* studies were done using its peptide inhibitor, ALLN. Calpain inhibition reduced the percent of paraptotic cell death from 25 to 19% while the percent of necrotic cell death was reduced from 50 to 40% (Fig. 4.2.7).

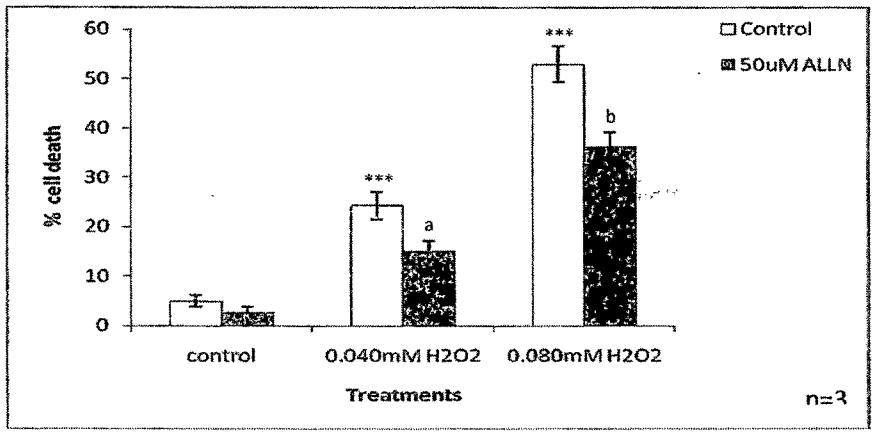

Figure 4.2.7 Effect of Calpain inhibition on H_2O_2 induced cell death. *** p value <0.001compared to control; a and b p value <0.05 compared to 0.04 and 0.08mM H_2O_2 respectively.

4.3 Effect of NAD^+, benzamide, gallotannin or ALLN pretreatment on Cd^{2+} induced ROS production

NAD^+ supplementation, PARP inhibition, PARG inhibition and calpain inhibition significantly rescued paraptotic and necrotic cell death induced by Cd^{2+} and H_2O_2 in *D. discoideum*. To find out whether these agents act by reducing the ROS that was produced under oxidative stress.

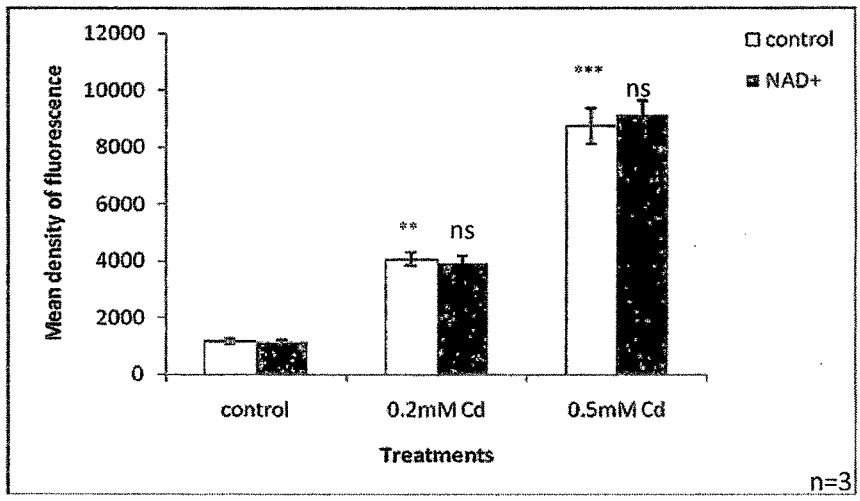

Figure 4.3.1: Effect of NAD^+ on Cd^{2+} induced ROS production. *** p value <0.001; ** p value <0.01 compared to control; ns=non significant as compared to respective Cd^{2+} dose.

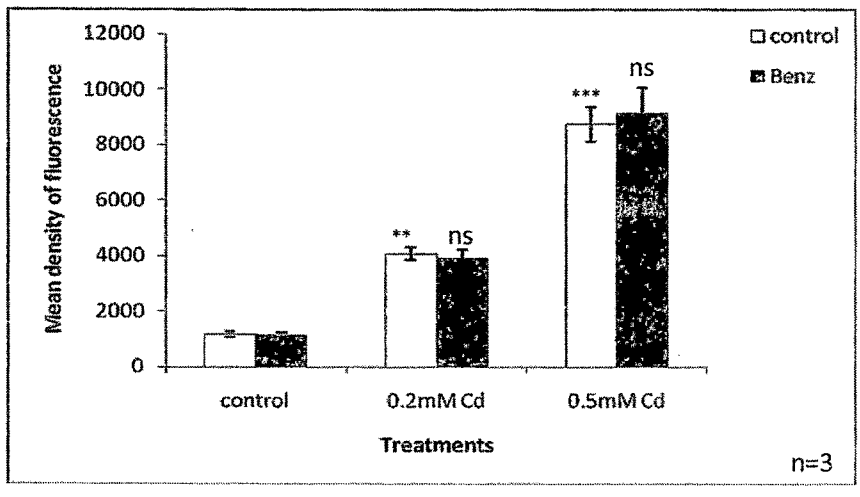

Figure 4.3.2 Effect of PARP inhibition on Cd^{2+} induced ROS production. *** p value <0.001; ** p value <0.01 compared to control; ns=non significant as compared to respective Cd^{2+} dose.

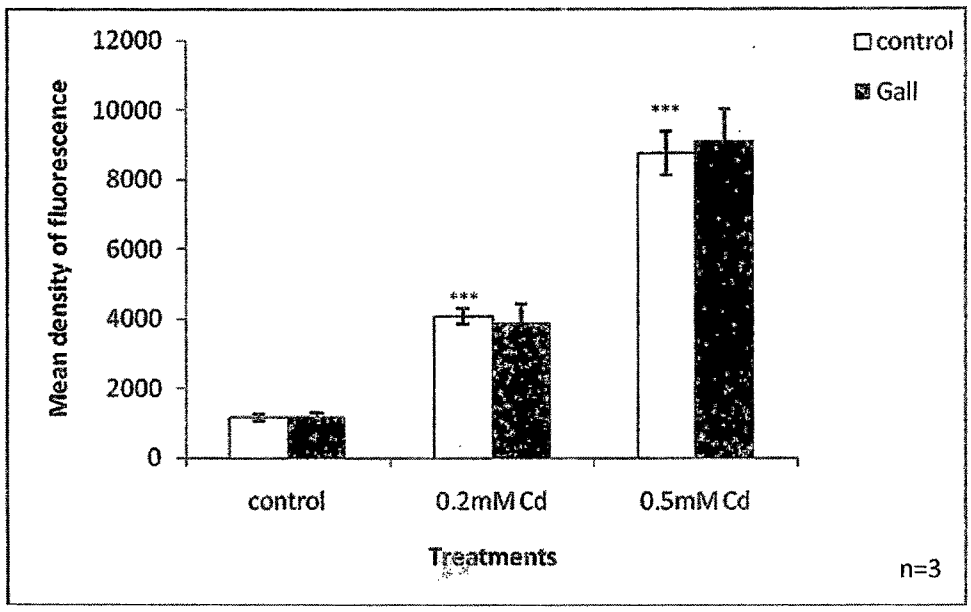

Figure 4.3.3: Effect of gallotannin on Cd^{2+} induced ROS production. *** p value <0.001 compared to control; ns=non significant as compared to respective Cd^{2+} dose.

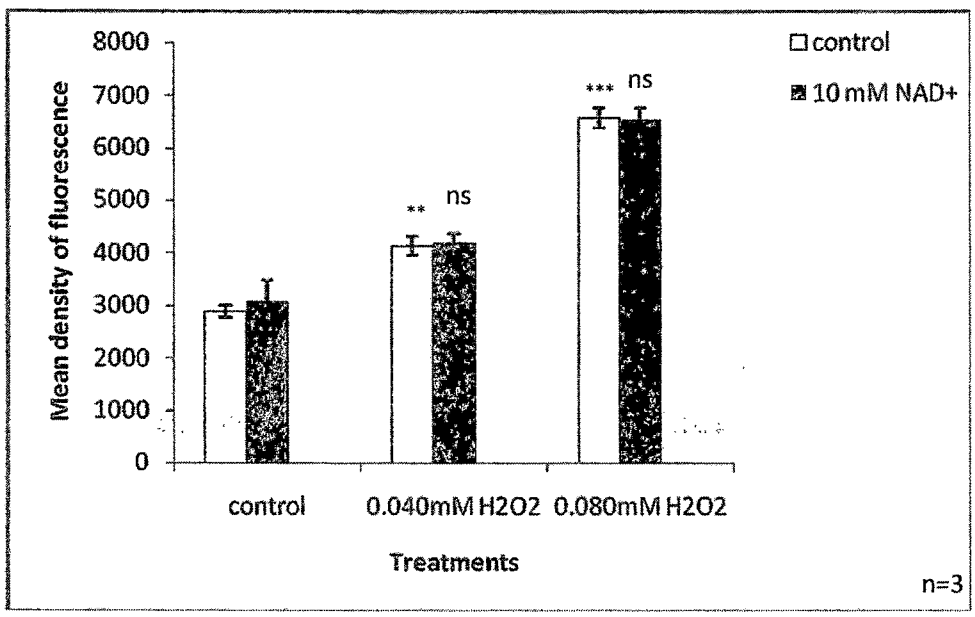

Figure 4.3.4: Effect of NAD^+ supplementation on H_2O_2 induced ROS production. *** p value <0.001; ** p value <0.01 compared to control; ns= non-significant as compared to respective control.

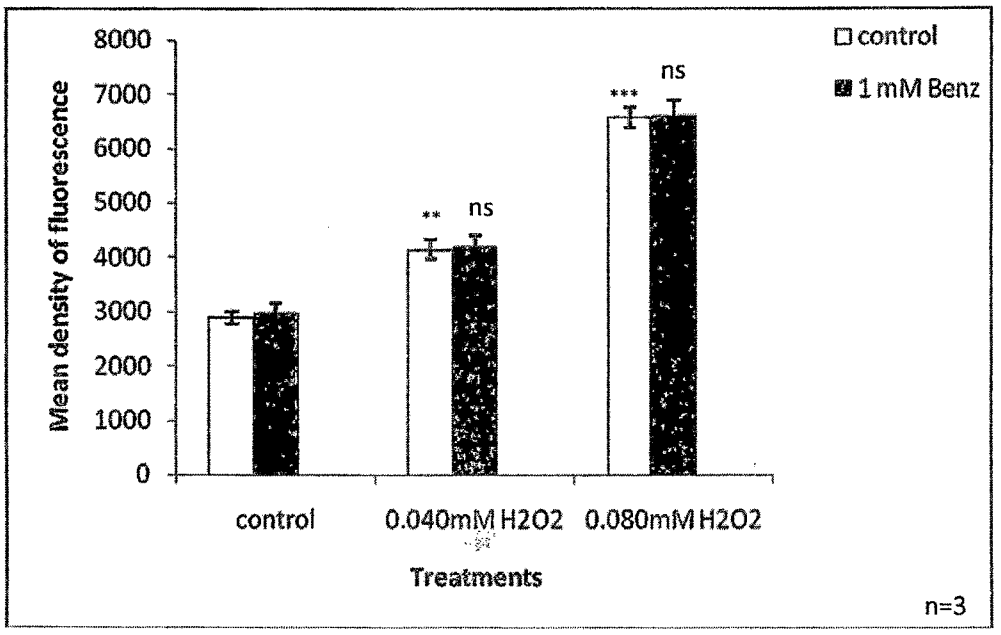

Figure 4.3.5: Effect of benzamide on H_2O_2 induced ROS production. *** p value <0.001; ** p value <0.01; ns= non-significant as compared to respective control.

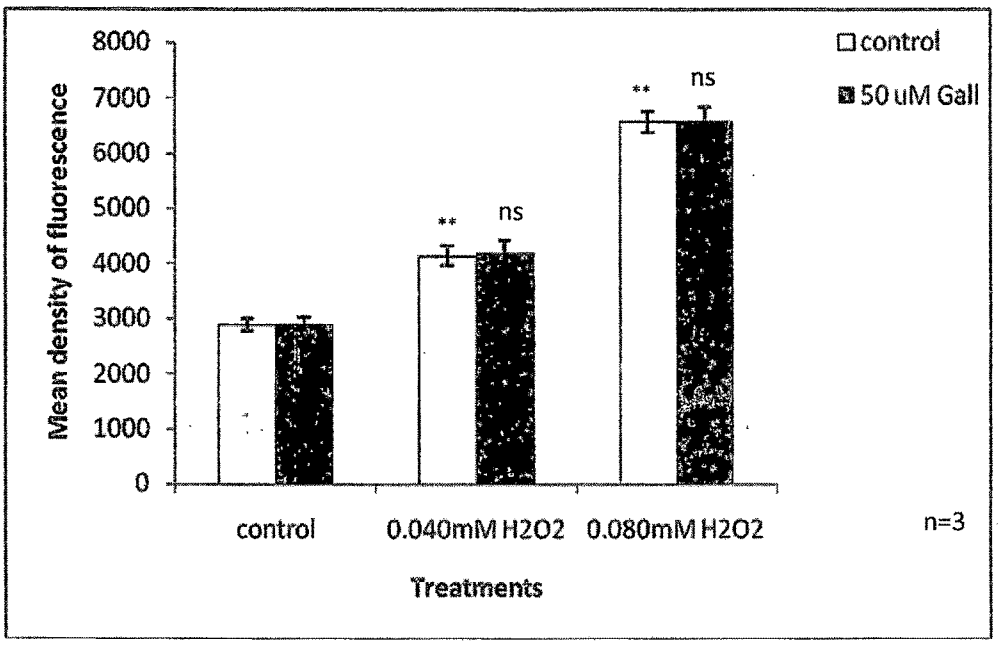

Figure 4.3.6: Effect of gallotannin on H_2O_2 induced ROS production. ** p value <0.01; ns= non-significant as compared to respective control.

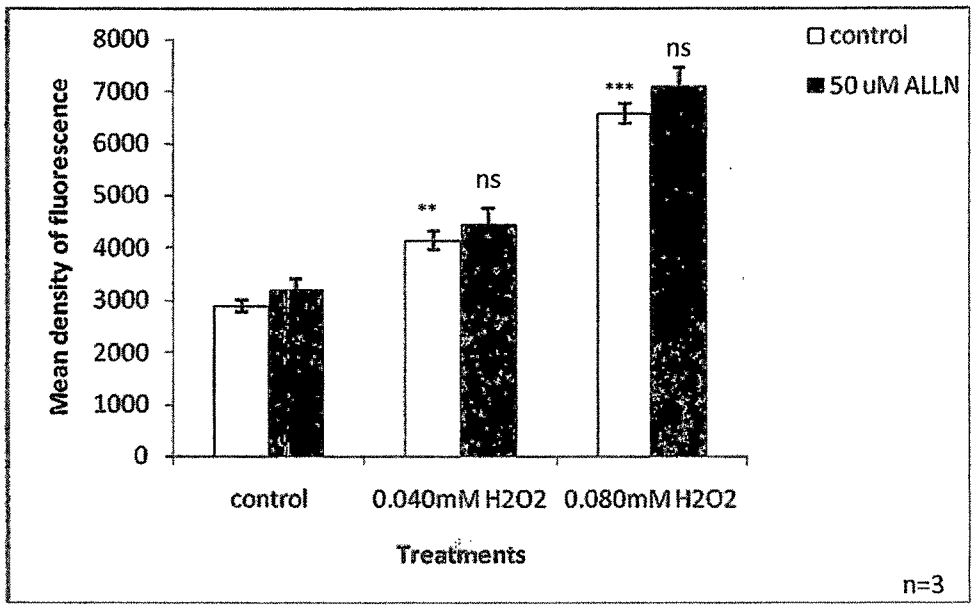

Figure 4.3.7: Effect of ALLN on H_2O_2 induced ROS production. *** p value <0.001; ** p value <0.01; ns= non-significant as compared to respective control.

Effect of various pretreatments on ROS production was monitored. As can be seen from the Figs. 4.3.1-4.3.7, NAD^+ (1 & 10mM) supplementation, benzamide (1mM), gallotannin (50μM) and ALLN (50μM) treatments had no effect on the ROS production during Cd^{2+} or cumene H_2O_2 induced cell death. Thus all these agents show the rescuing effect by acting at a downstream step of Cd^{2+} or cumene H_2O_2 induced cell death.

4.4 PARP activation induced by oxidative stress

Activation of PARP is seen in response to DNA damage. PARP activity in *D. discoideum* cells was assayed at 5 min after oxidant treatment. PARP activity increased initially and peak activity was seen at 10 minutes post exposure to paraptotic and necrotic doses of cumene H_2O_2 (Figs. 4.4.1-4.4.7). It was observed that NAD^+ supplementation increased the PARP activity significantly.

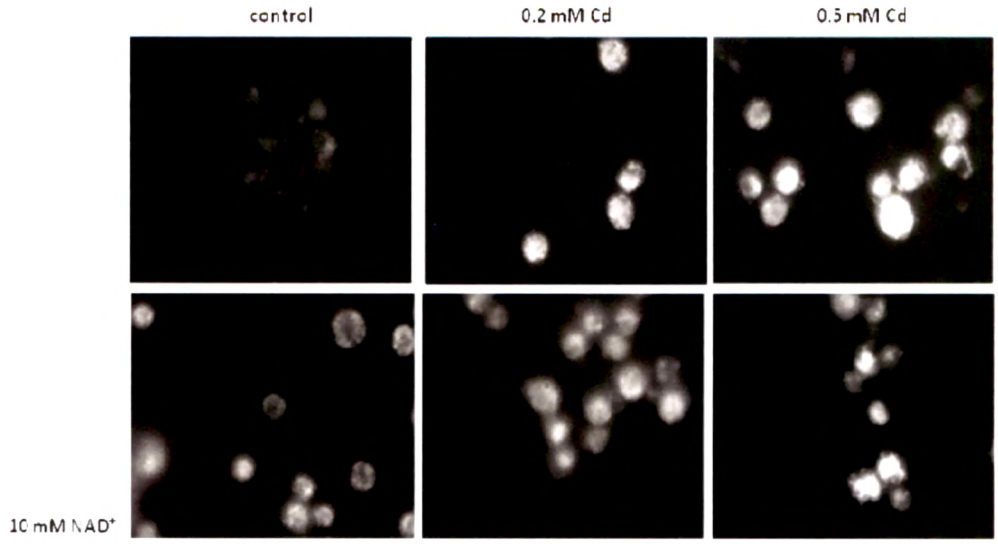

Figure 4.4.1a: Effect of NAD^+ supplementation on Cd^{2+} induced PARP activation

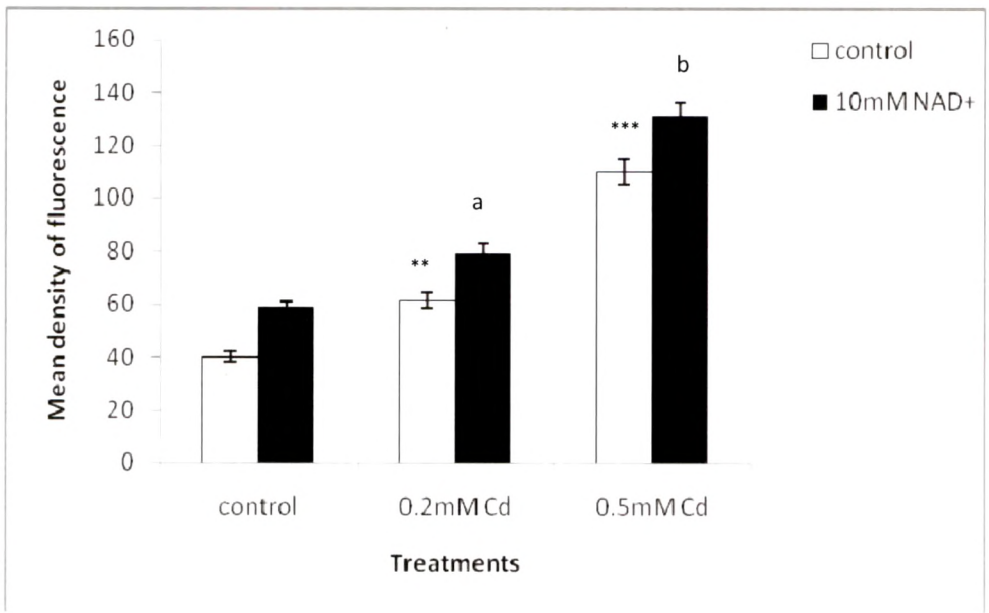

Figure 4.4.1b Densitometric analysis: Cd^{2+} induced PARP activation with NAD^+ supplementation. *** p value <0.001; ** p value <0.01 as compared to control; a p value<0.05 as compared to 0.2 mM Cd^{2+}; b p value<0.05 as compared to 0.5 mM Cd^{2+}.

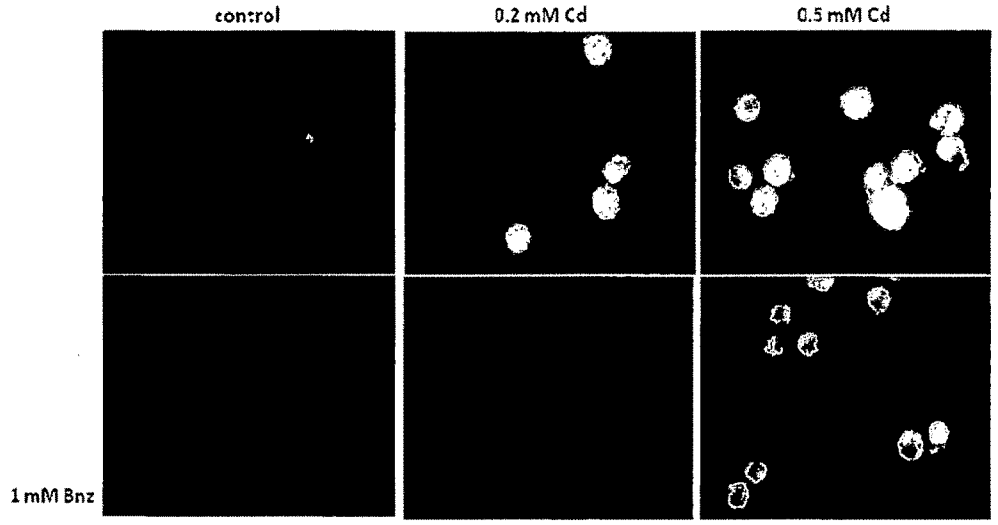

Figure 4.4.2a: Effect of benzamide on Cd^{2+} induced PARP activation

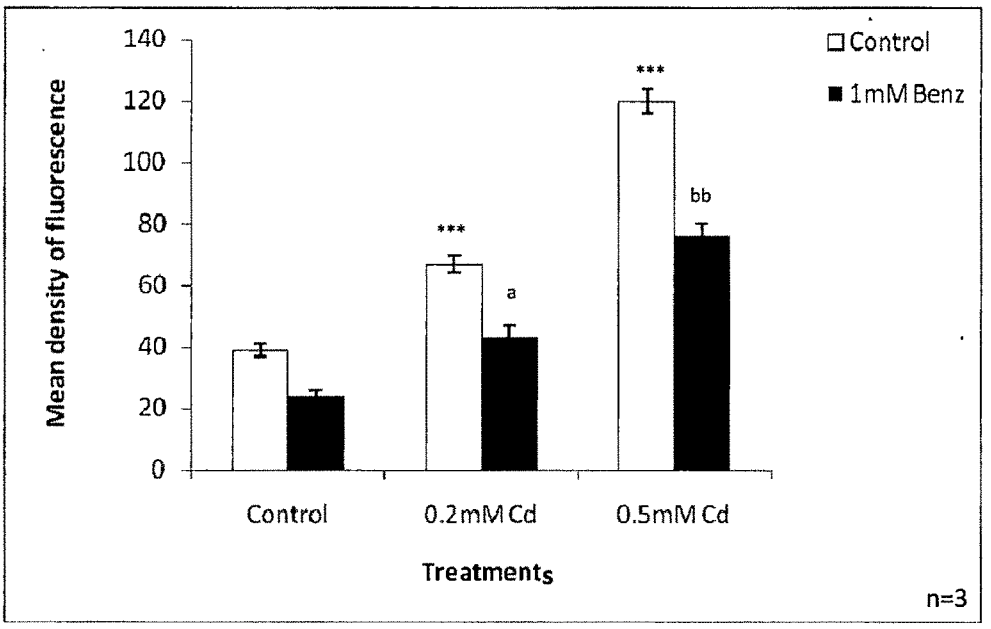

Figure 4.4.2b: Densitometric analysis: Cd^{2+} induced PARP activation and benzamide pre treatment. *** p value <0.001 compared to control; a p value <0.05 compared to 0.2mM Cd^{2+}; bb p value <0.01 compared to 0.5mM Cd^{2+}.

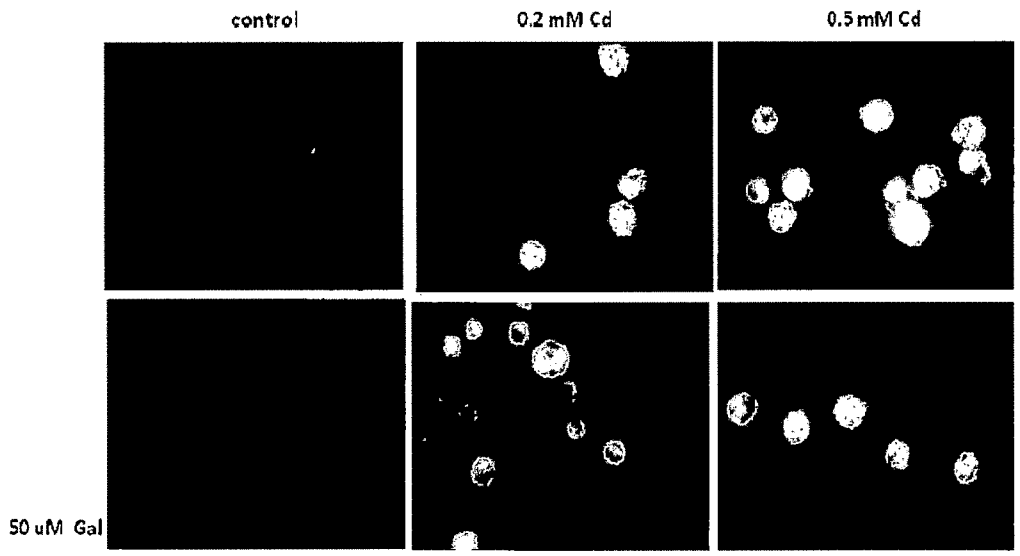

Figure 4.4.3a: Effect of gallotannin on Cd^{2+} induced PARP activation

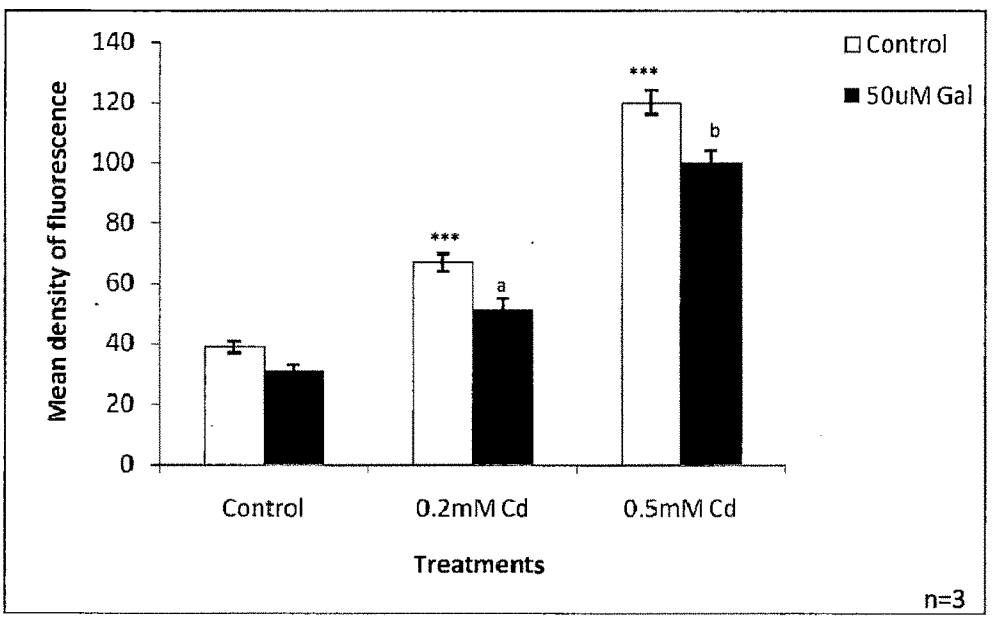

Figure 4.4.3b: Densitometric analysis: Effect of gallotannin on Cd^{2+} induced PARP activation. *** p value <0.001 compared to control; a & b p value <0.05 compared to 0.2mM and 0.5mM Cd^{2+} respectively.

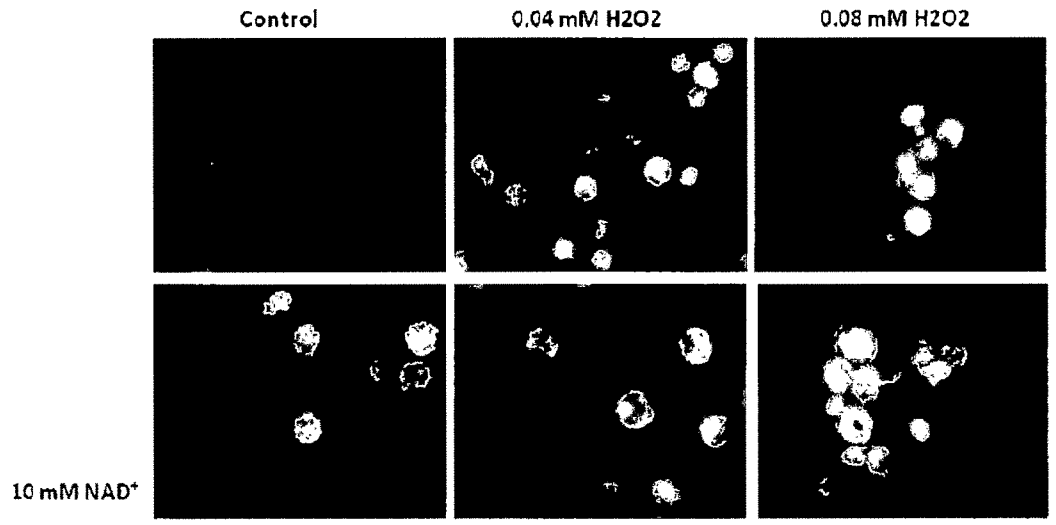

Figure 4.4.4a Effect of NAD^+ supplementation on H_2O_2 induced PARP activation

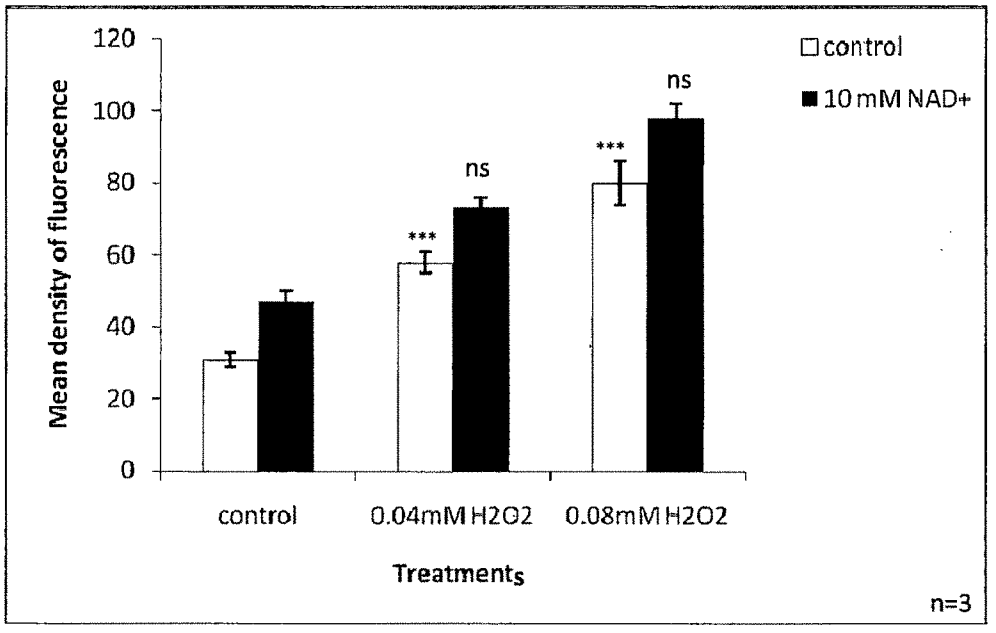

Figure 4.4.4b: Densitometric analysis: H_2O_2 induced PARP activation with NAD^+ supplementation. *** p value <0.001; ns= non-significant as compared to respective control.

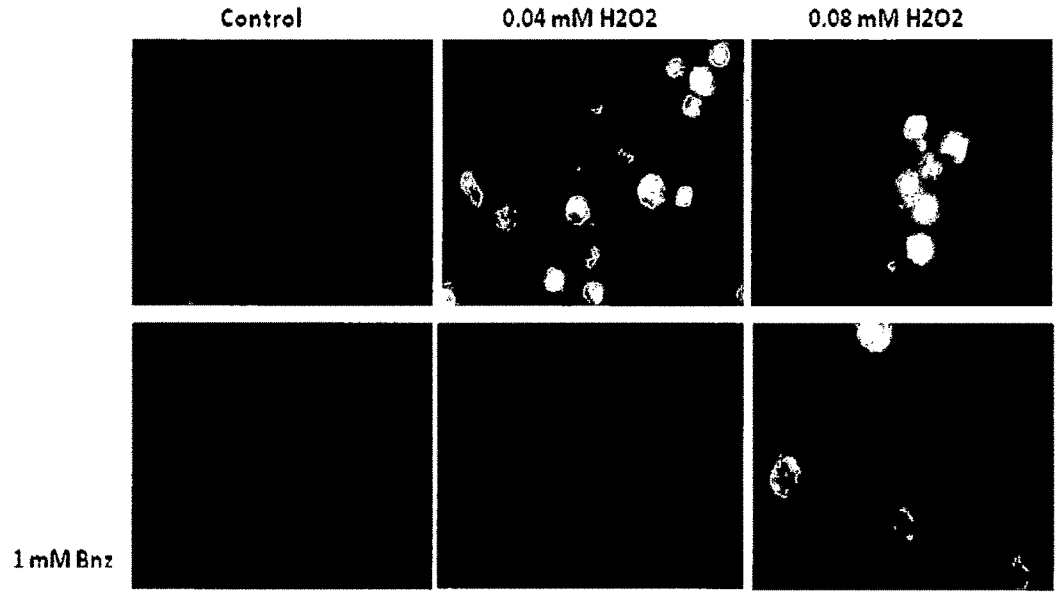

Figure 4.4.5a: Effect of benzamide on H_2O_2 induced PARP activation

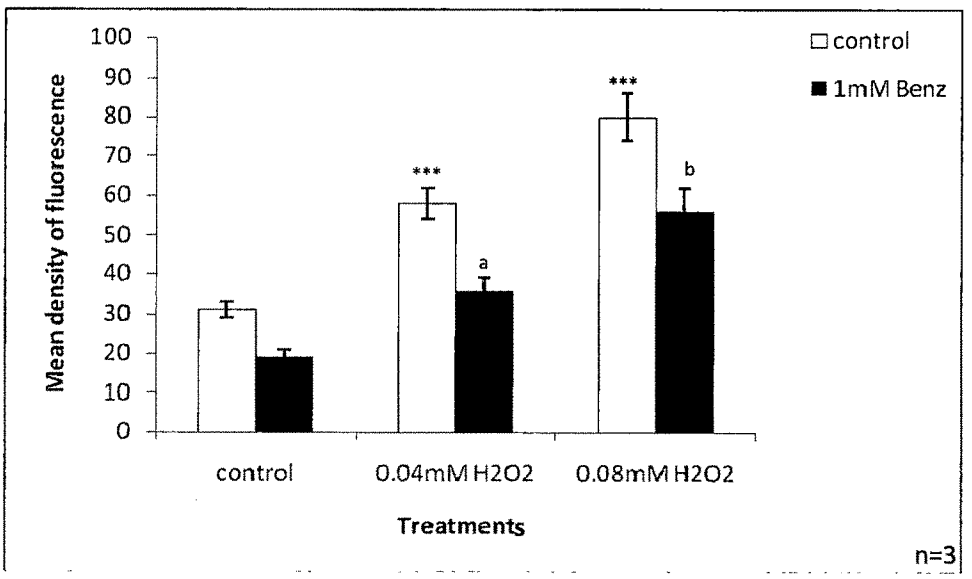

Figure 4.4.5b: Densitometric analysis: H_2O_2 induced PARP activation in the presence of benzamide. *** p value <0.001 compared to control; a & b p value <0.05 compared to 0.04mM and 0.08mM H_2O_2 respectively.

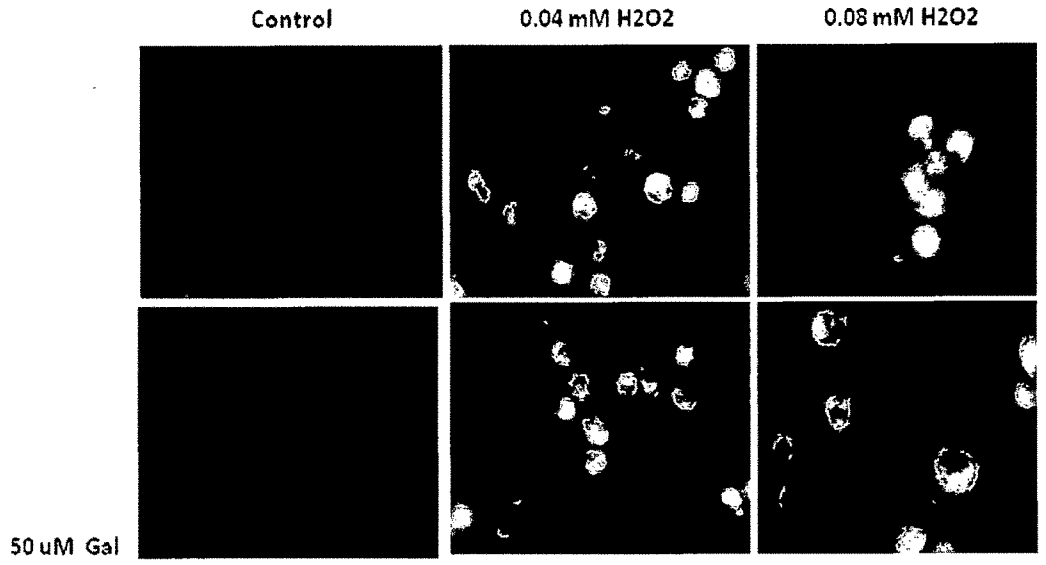

Figure 4.4.6a: Effect of gallotannin on H_2O_2 induced PARP activation

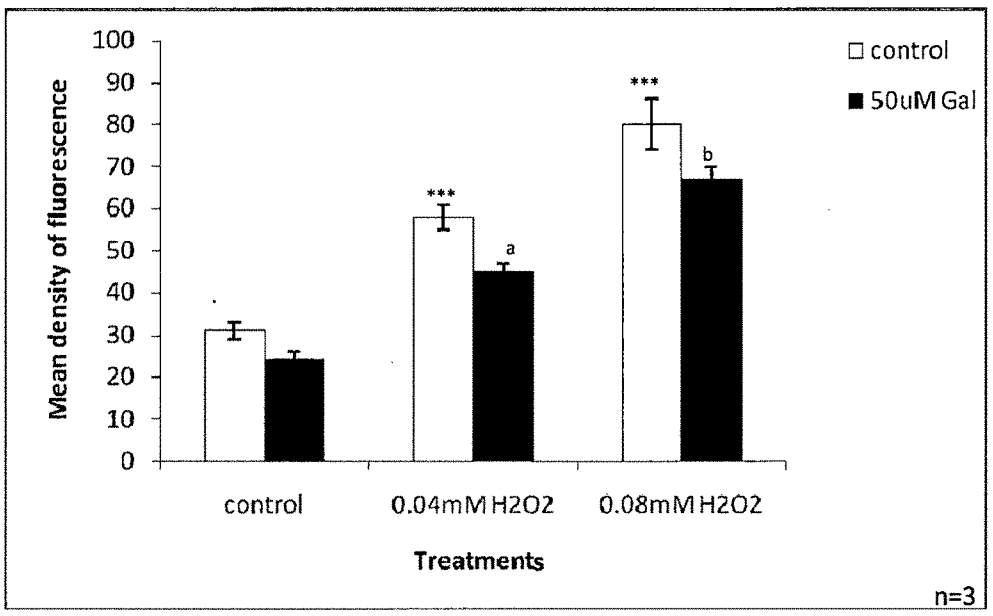

Figure 4.4.6b: Densitometric analysis: Effect of gallotannin on H_2O_2 induced PARP activation. *** p value <0.001 compared to control; a & b p value <0.05 compared to 0.04mM and 0.08mM H_2O_2 respectively.

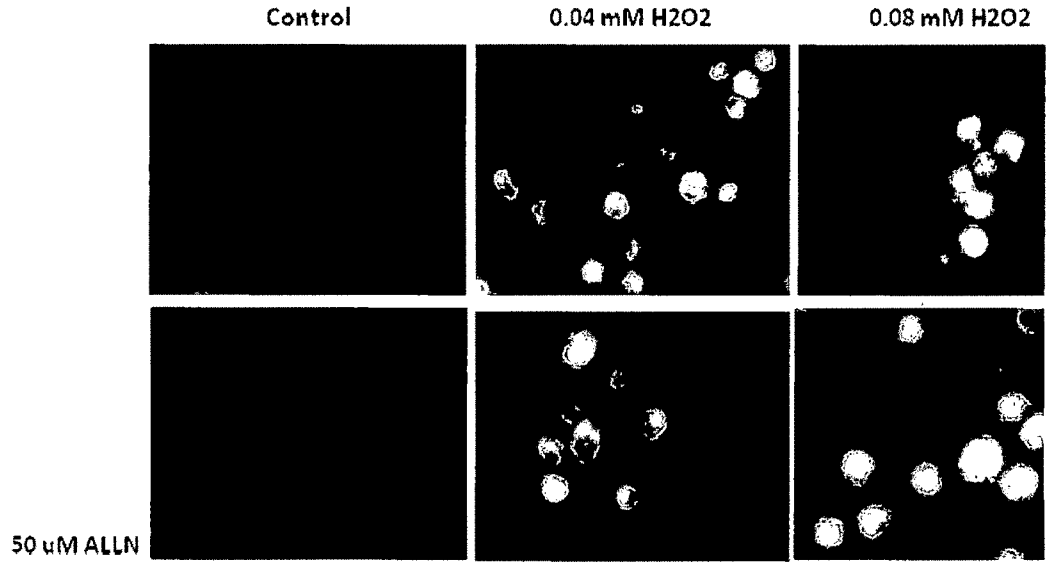

Figure 4.4.7a: Effect of ALLN on H_2O_2 induced PARP activation

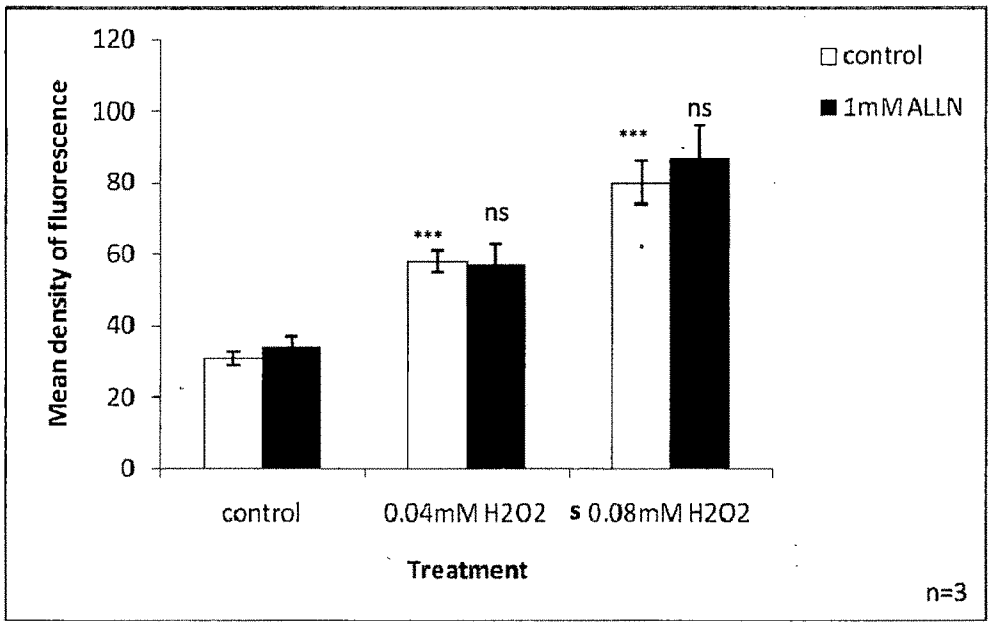

Figure 4.4.7b: Densitometric analysis: Effect of ALLN on H_2O_2 induced PARP activation. *** p value <0.001; ns= non-significant as compared to respective control.

However inhibition of calpain, did not affect the oxidative stress induced PARP activation, suggesting calpain activation is a downstream event during cell death (Fig. 4.4.7).

4.5 Effect of NAD^+, benzamide, gallotannin or ALLN pre treatment on oxidative stress induced 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 signalling molecule during oxidative stress induced PARP mediated cell death. As can be seen in Figs. 4.5.1-4.5.7 Cd^{2+} and H_2O_2 induced depletion of NAD^+ could be rescued significantly by all pharmacological agents except ALLN.

62% NAD^+ depletion could be seen in paraptotic (LD_{25}) dose of H_2O_2. *D. discoideum* cells were supplemented with 1 and 10 mM NAD^+ for 2 hrs prior to oxidative stress (H_2O_2) and NAD^+ levels were checked. 41 and 68% restoration could be seen in 1mM and 10 mM NAD^+ pre treatment respectively. However 78% NAD^+ depletion could be seen in necrotic (LD_{50}) dose of H_2O_2 which can be restored to 38 and 50% in 1mM and 10mM NAD^+ pre treated cells respectively. Similarly 10mM NAD^+ also restored NAD^+ levels when Cd^{2+} was used as the oxidative stress inducer.

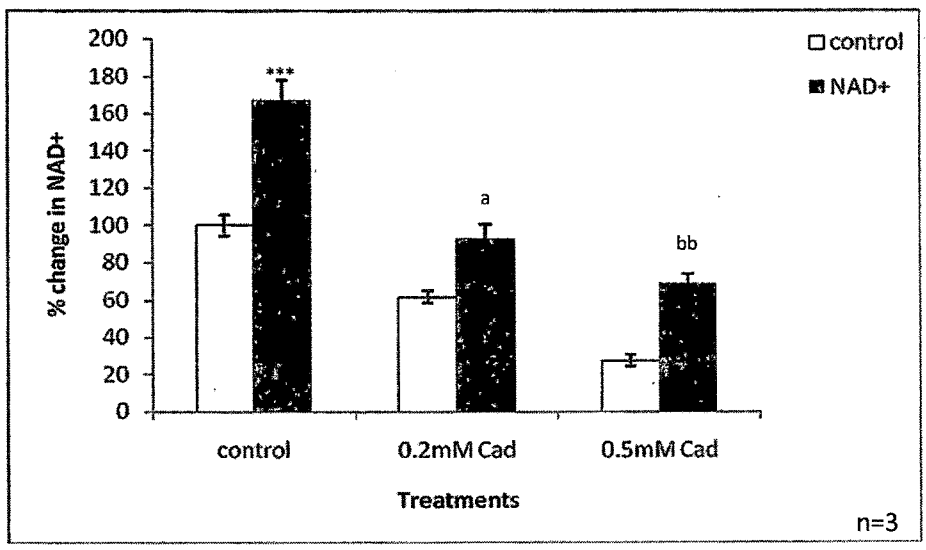

Figure 4.5.1: Effect of exogenous addition of 10 mM NAD^+ on Cd^{2+} induced NAD^+ depletion. *** p value <0.001 compared to control; a p value <0.05 compared to 0.2mM Cd^{2+}; bb p value <0.01 compared to 0.5mM Cd^{2+}.

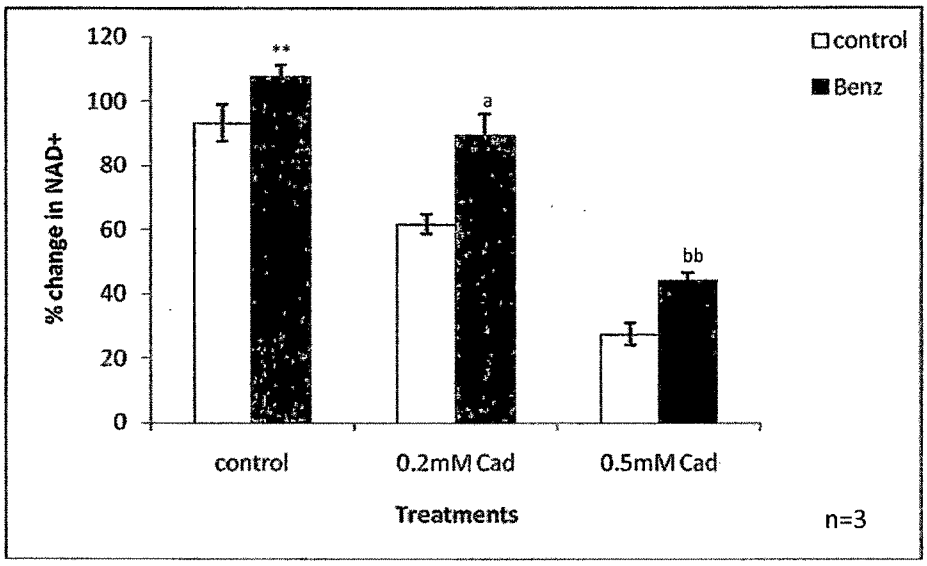

Figure 4.5.2: Effect of PARP inhibition on Cd^{2+} induced NAD^+ depletion. *** p value <0.001; ** p value <0.01 compared to control; a p value <0.05 compared to 0.2mM Cd^{2+}; bb p value <0.01 compared to 0.5mM Cd^{2+}.

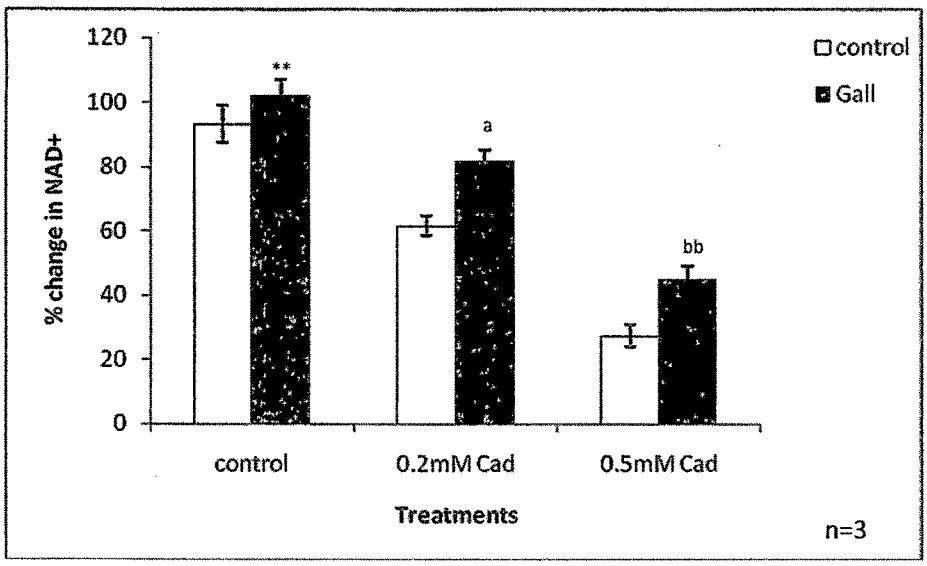

Figure 4.5.3: Effect of PARG inhibition on Cd^{2+} induced NAD^+ depletion. *** p value <0.001; ** p value <0.01 compared to control; a p value <0.05 compared to 0.2mM Cd^{2+}; bb p value <0.01 compared to 0.5mM Cd^{2+}.

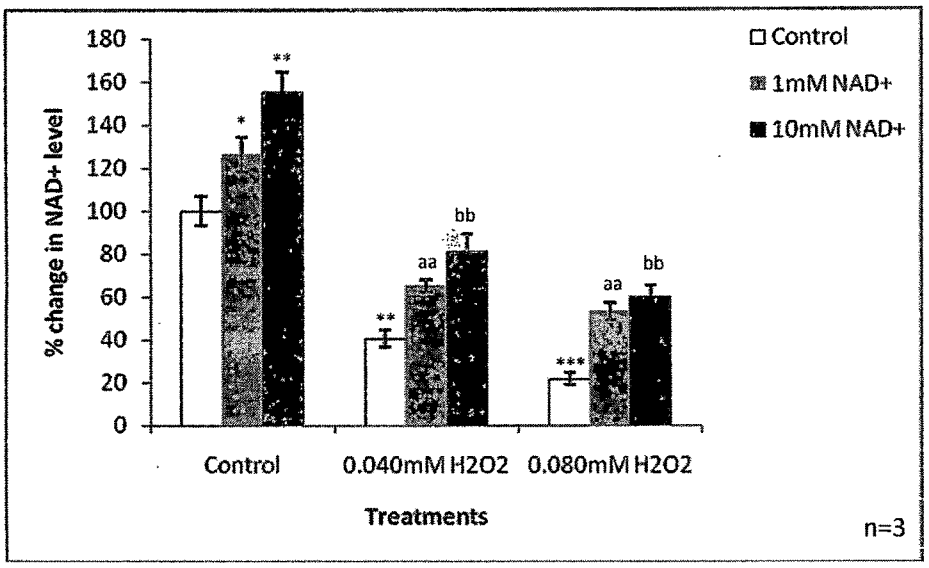

Figure 4.5.4: Effect of exogenous addition of NAD^+ on H_2O_2 induced NAD^+ depletion. *** p value <0.001; ** p value <0.01; * p value <0.05 compared to control; aa p value <0.01 compared to 0.04mM H_2O_2; bb p value <0.01 compared to 0.08mM H_2O_2.

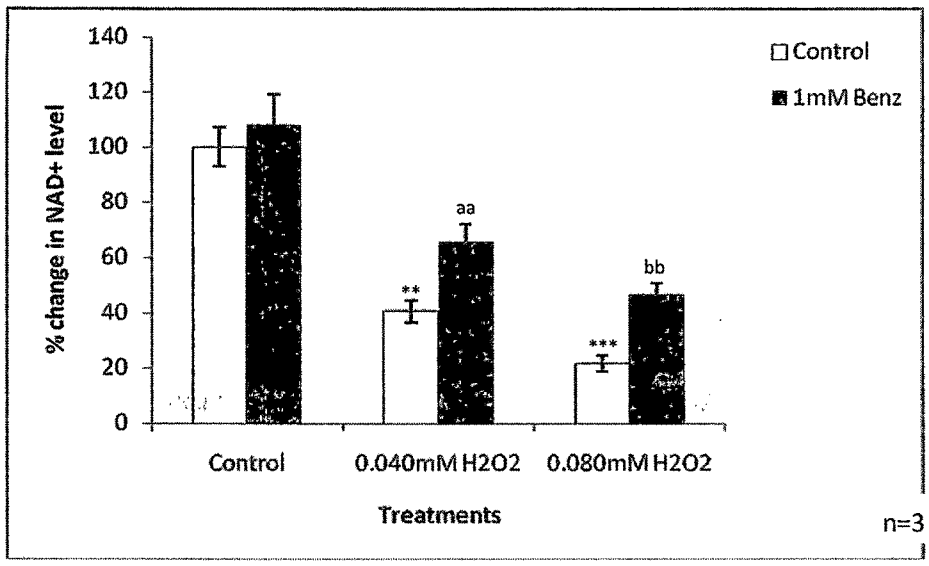

Figure 4.5.5: Effect of PARP inhibition on H_2O_2 induced NAD^+ depletion. *** p value <0.001; ** p value <0.01compared to control; aa p value <0.01 compared to 0.04mM H_2O_2; bb p value <0.01 compared to 0.08mM H_2O_2.

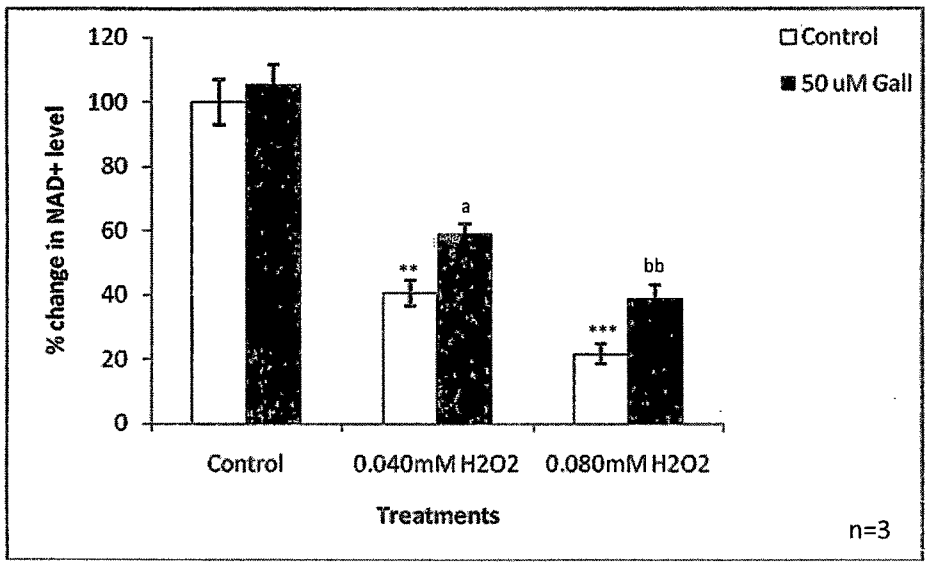

Figure 4.5.6: Effect of PARG inhibition on H_2O_2 induced NAD^+ depletion. *** p value <0.001; ** p value <0.01compared to control; a p value <0.05 compared to 0.04mM H_2O_2; bb p value <0.01 compared to 0.08mM H_2O_2.

Interestingly 62% NAD^+ depletion in paraptotic dose of H_2O_2 could be restored to 31 and 28% in Benzamide (Fig.4.5.5), and Gallotannin (Fig.4.5.6) pre treatment respectively while 78% NAD^+ depletion in necrotic dose of H_2O_2 which could be restored to 32 and 25% in Benzamide (Fig.4.5.5), and Gallotannin (Fig.4.5.6) pre treatment respectively. Similarly NAD^+ depletion was also prevented when Cd^{2+} was used as cell death inducer (Figs. 4.5.1-4.5.3).

4.6 Effect of NAD^+, benzamide, gallotannin or ALLN pre treatment on oxidative stress induced MMP changes

Using the mitochondrial membrane potential ($\Delta\psi_m$)-sensitive fluorescent dye $DiOC_6$, we have observed changes in the MMP ($\Delta\psi_m$) of cultured *D. discoideum* cells under oxidative stress condition. These fluctuations in $\Delta\psi_m$ appear to represent partial, transient depolarizations of mitochondria. However mitochondria being decision maker of life and death, mitochondrial membrane potential is the first event to be sensing NAD^+ depletion. Thus if we control NAD^+ depletion, mitochondria may function normally.

As can be seen in Figs. 4.6.1-4.6.7 paraptotic and necrotic doses of Cd^{2+} and H_2O_2 induced reduction of MMP could be prevented significantly by treatment with all pharmacological agents. However NAD^+ supplementation showed maximal rescue in MMP changes as compared to any other pharmacological agents.

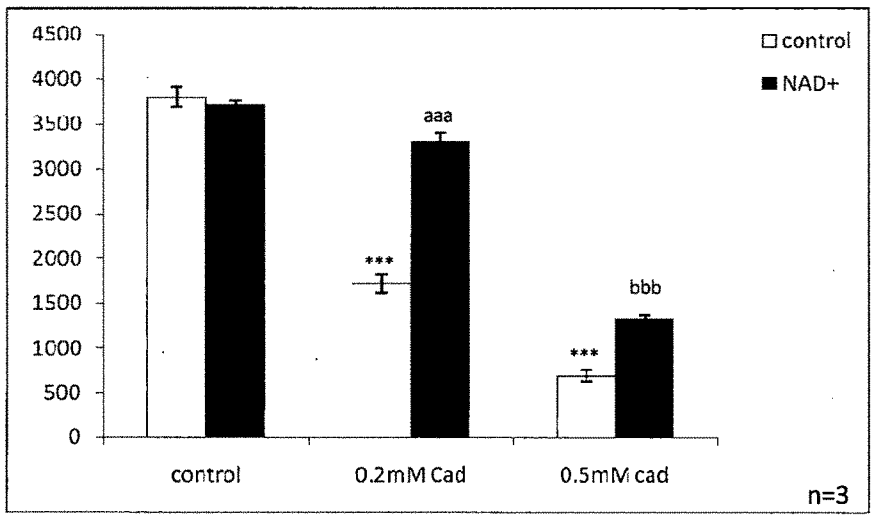

Figure 4.6.1a: Effect of exogenous addition of NAD^+ on Cd^{2+} induced MMP changes by fluorometry. *** p value <0.001 compared to control; aaa & bbb p value <0.001 compared to 0.2mM Cd^{2+} and 0.5mM Cd^{2+} respectively.

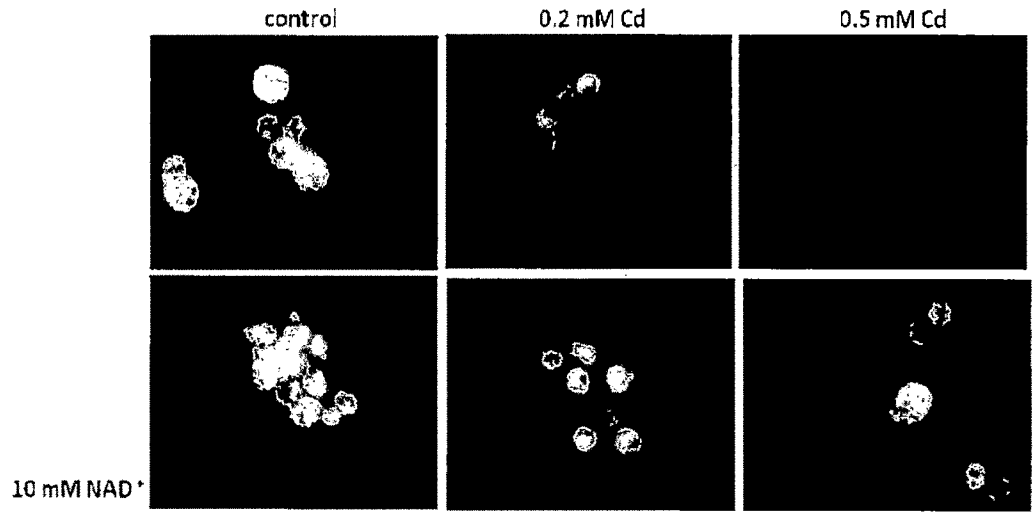

Figure 4.6.1b: Effect of exogenous addition of NAD^+ on Cd^{2+} induced MMP changes by fluorescence microscopy

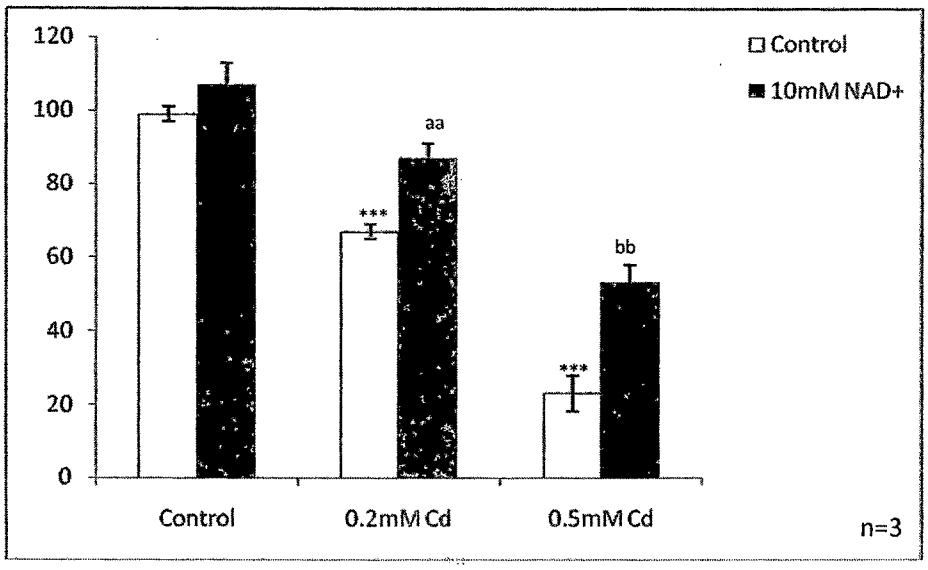

Figure 4.6.1c: Densitometric analysis: Effect of NAD^+ supplementation on Cd^{2+} induced changes in MMP. *** p value <0.001 compared to control; aa p value <0.01 compared to 0.2mM Cd^{2+}; bb p value <0.01 compared to 0.5mM Cd^{2+}.

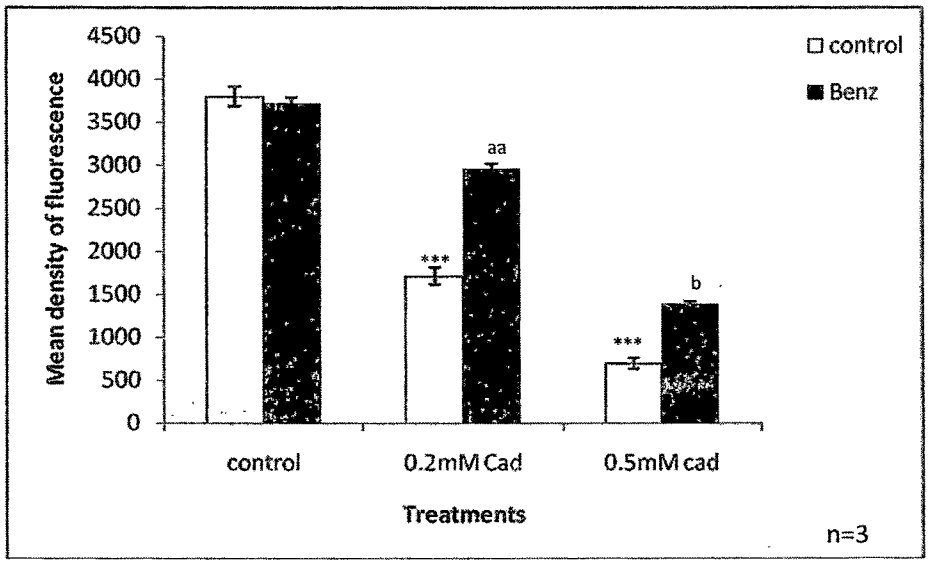

Figure 4.6.2a: Effect of benzamide on Cd^{2+} induced changes in MMP by fluorometry. *** p value <0.001 compared to control; aa p value <0.01 compared to 0.2mM Cd^{2+}; b p value <0.05 compared to 0.5mM Cd^{2+}.

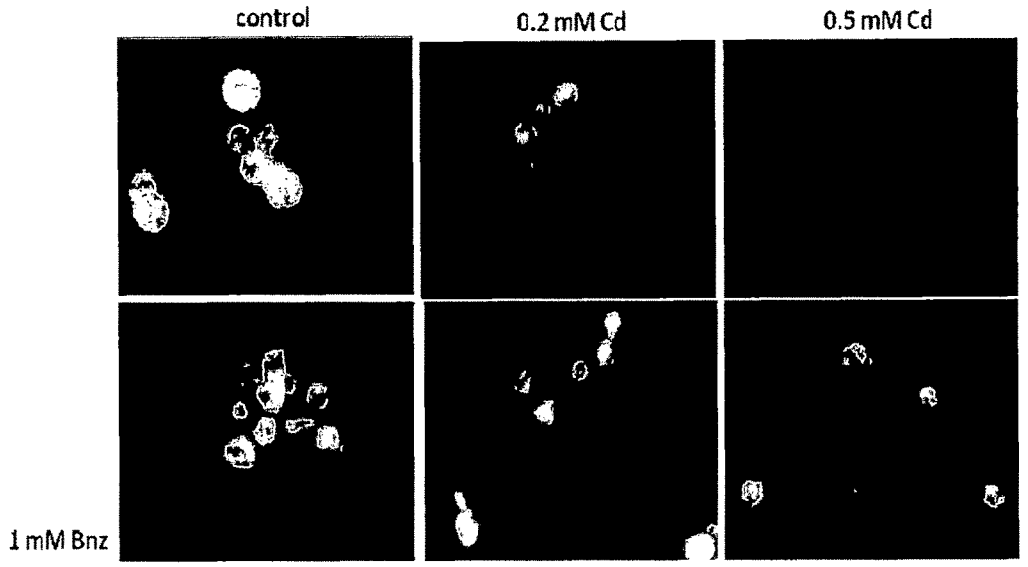

Figure 4.6.2b: Effect of benzamide on Cd^{2+} induced changes in MMP by fluorescence microscopy.

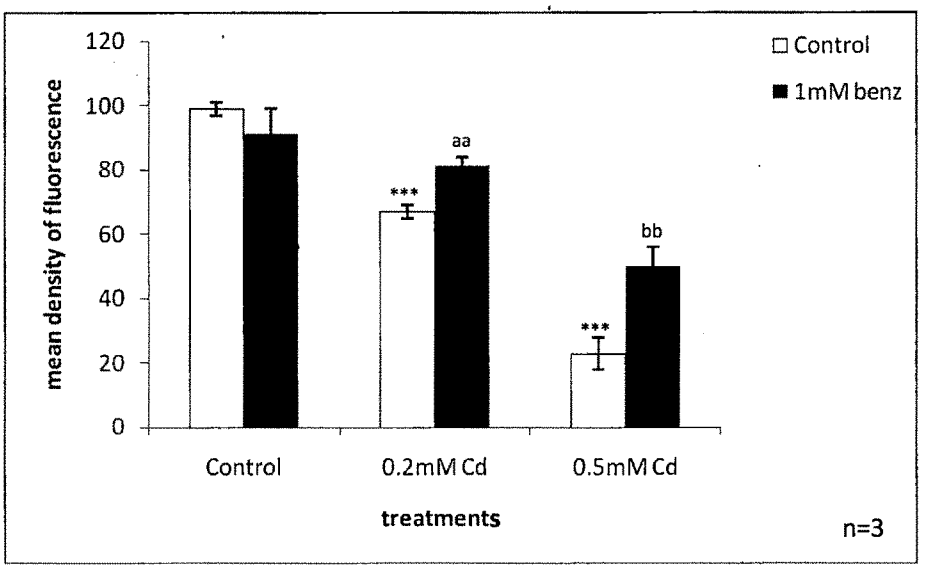

Figure 4.6.2c: Densitometric analysis: Effect of benzamide pre treatment on Cd^{2+} induced changes in MMP. *** p value <0.001 compared to control; aa & bb p value <0.01 compared to 0.2mM Cd^{2+} and 0.5mM Cd^{2+} respectively.

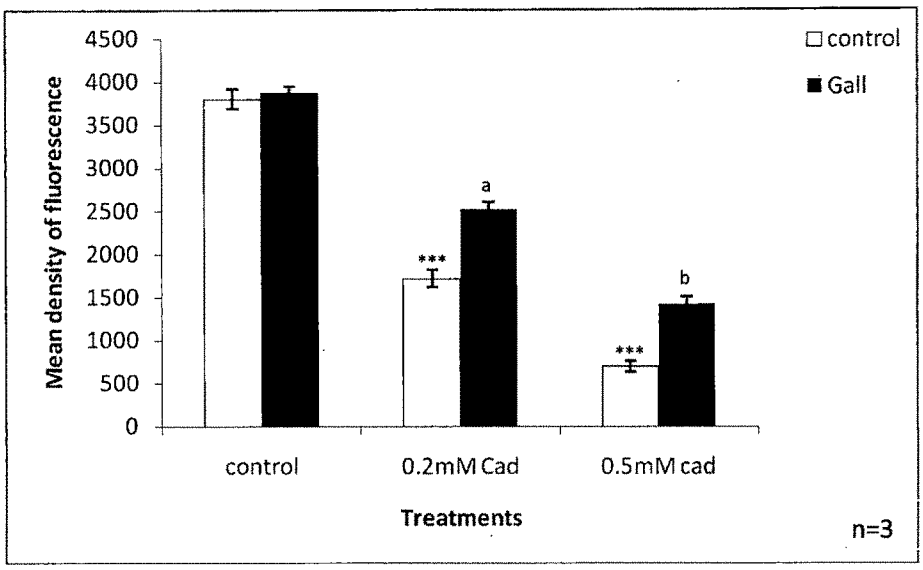

Figure 4.6.3a: Effect of gallotannin on Cd^{2+} induced changes in MMP by fluorometry. *** p value <0.001 compared to control; a p value <0.05 compared to 0.2mM Cd^{2+}; b p value <0.05 compared to 0.5mM Cd^{2+}.

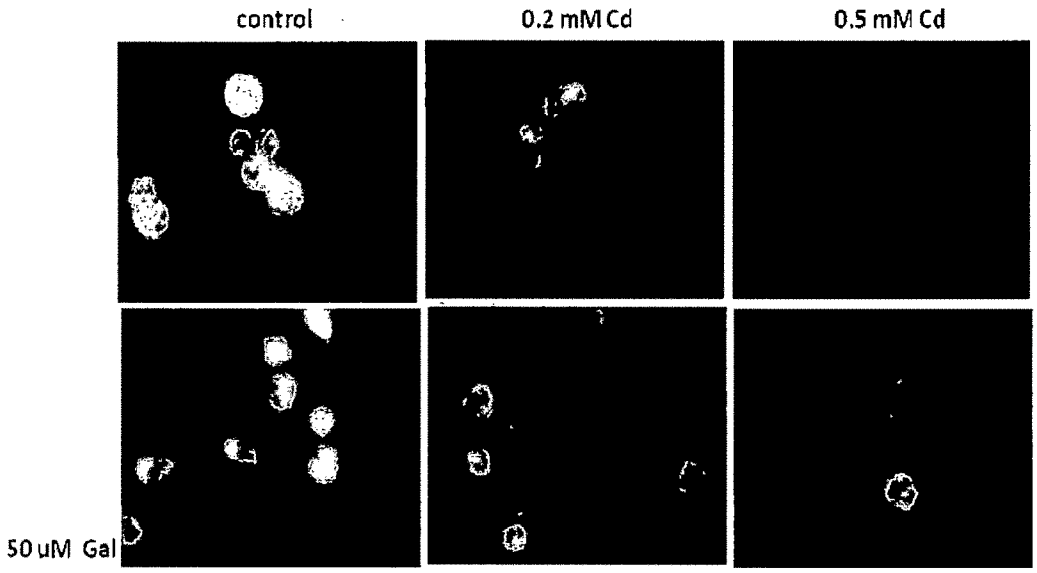

Figure 4.6.3b Effect of gallotannin pre treatment on Cd^{2+} induced changes in MMP by fluorescence microscopy

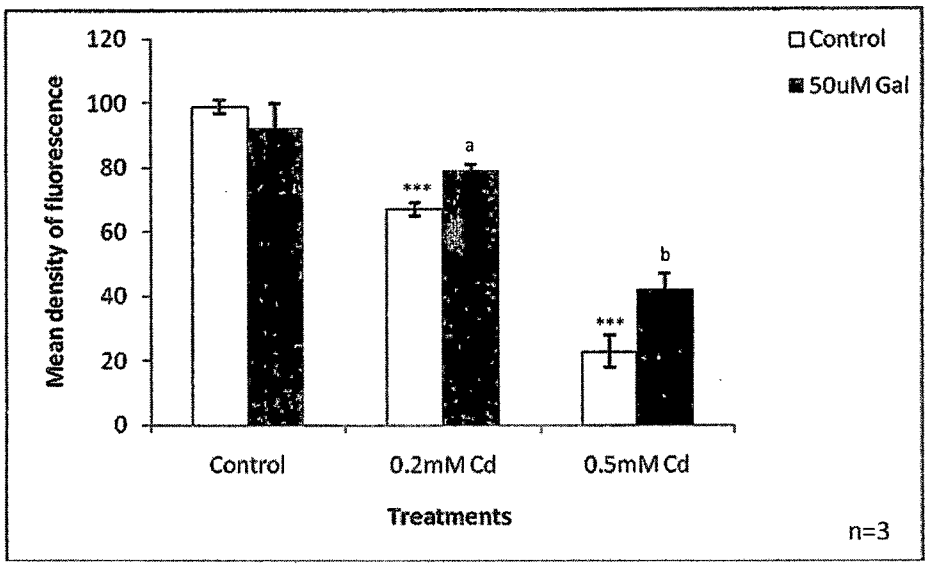

Figure 4.6.3c: Densitometric analysis: Effect of gallotannin pre treatment on Cd^{2+} induced changes in MMP. *** p value <0.001 compared to control; a p value <0.05 compared to 0.2mM Cd^{2+}; b p value <0.05 compared to 0.5mM Cd^{2+}.

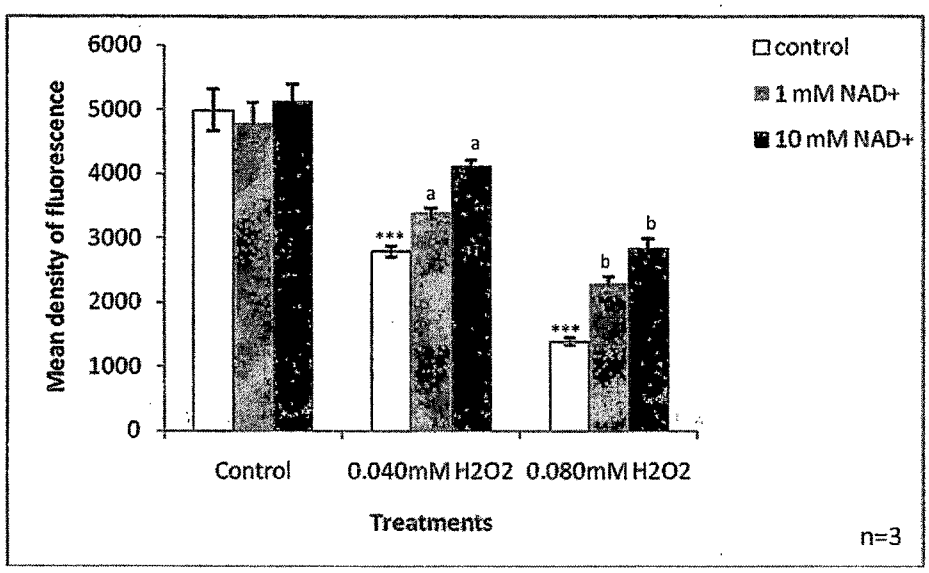

Figure 4.6.4a Effect of exogenous addition of NAD^+ on H_2O_2 induced MMP changes by fluorometry. *** p value <0.001 compared to control; a p value <0.05 compared to 0.04mM H_2O_2; b p value <0.05 compared to 0.08mM H_2O_2.

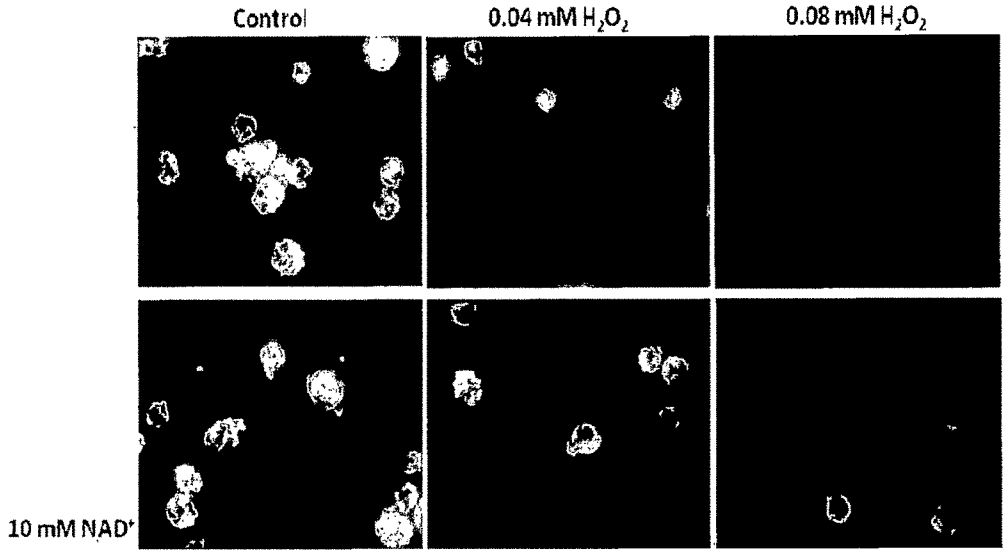

Figure 4.6.4b Effect of exogenous addition of NAD^+ on H_2O_2 induced MMP changes by fluorescence microscopy

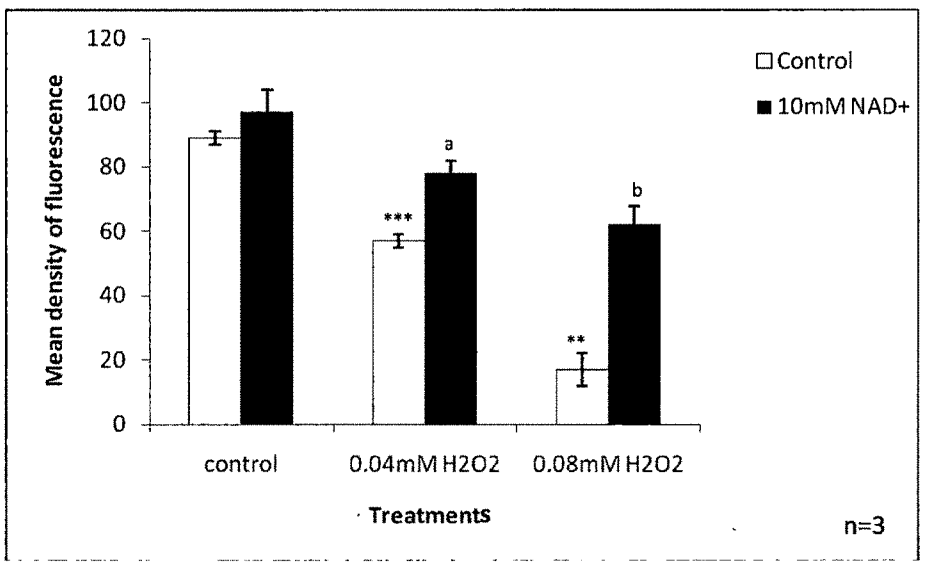

Figure 4.6.4c Densitometric analysis: Effect of NAD^+ supplementation on H_2O_2 induced changes in MMP. *** p value <0.001; ** p value <0.01 compared to control; a p value <0.05 compared to 0.04mM H_2O_2; b p value <0.05 compared to 0.08mM H_2O_2.

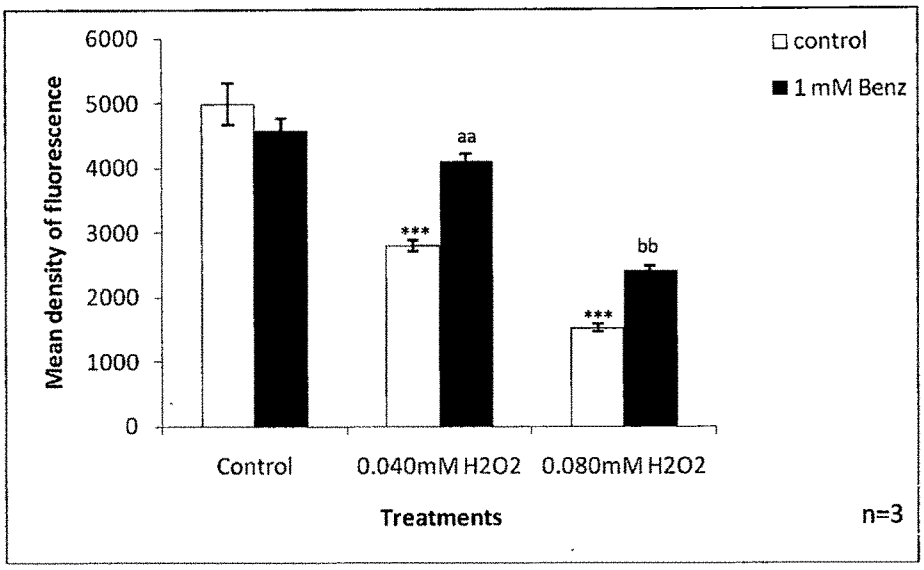

Figure 4.6.5a: Effect of PARP inhibition on H_2O_2 induced MMP changes by fluorometry. *** p value <0.001 compared to control; aa p value <0.01 compared to 0.04mM H_2O_2; bb p value <0.01 compared to 0.08mM H_2O_2.

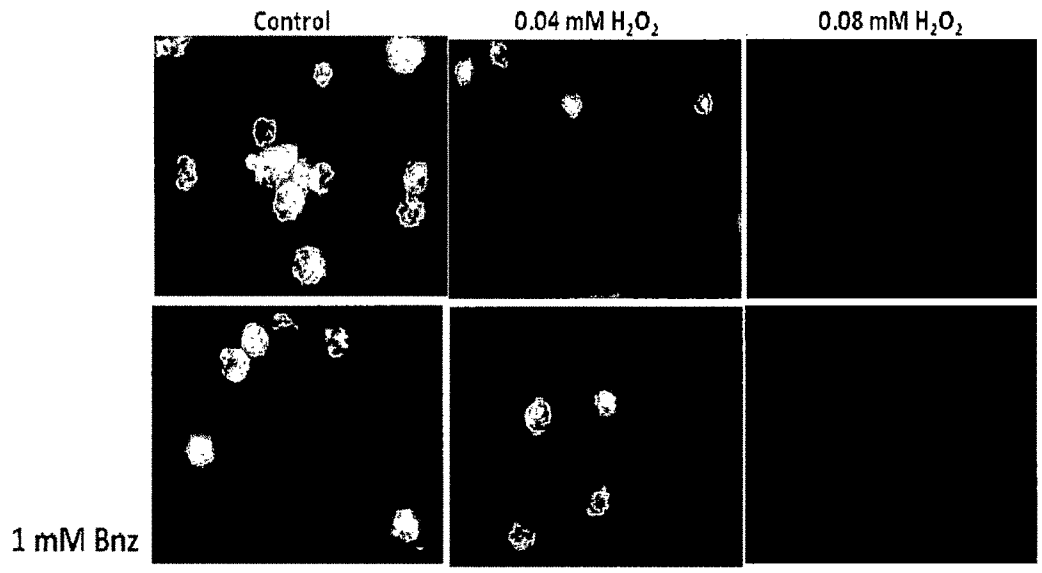

Figure 4.6.5b: Effect of PARP inhibition on H_2O_2 induced MMP changes by fluorescence microscopy

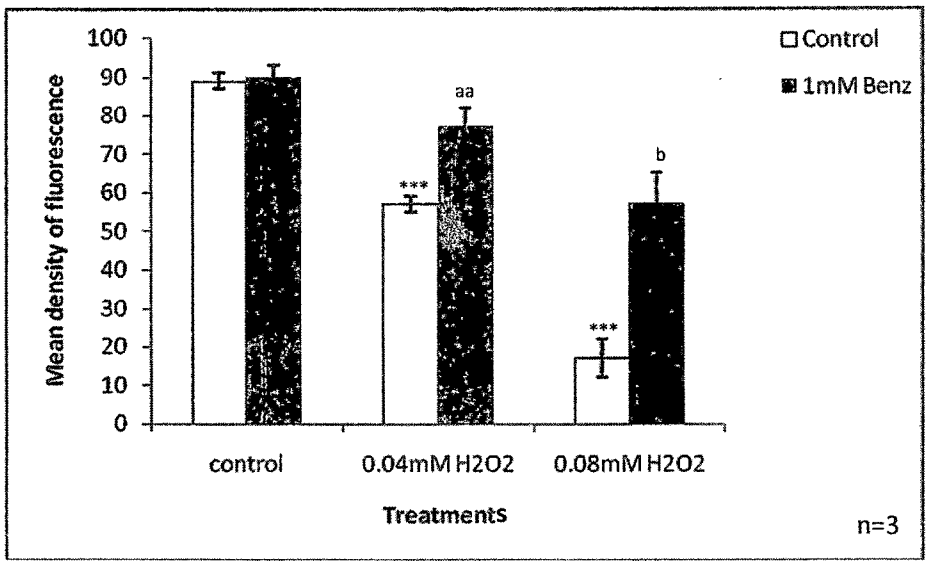

Figure 4.6.5c: Densitometric analysis: Effect benzamide pre treatment on H_2O_2 induced changes in MMP. *** p value <0.001 compared to control; aa p value <0.01 compared to 0.04mM H_2O_2; b p value <0.05 compared to 0.08mM H_2O_2.

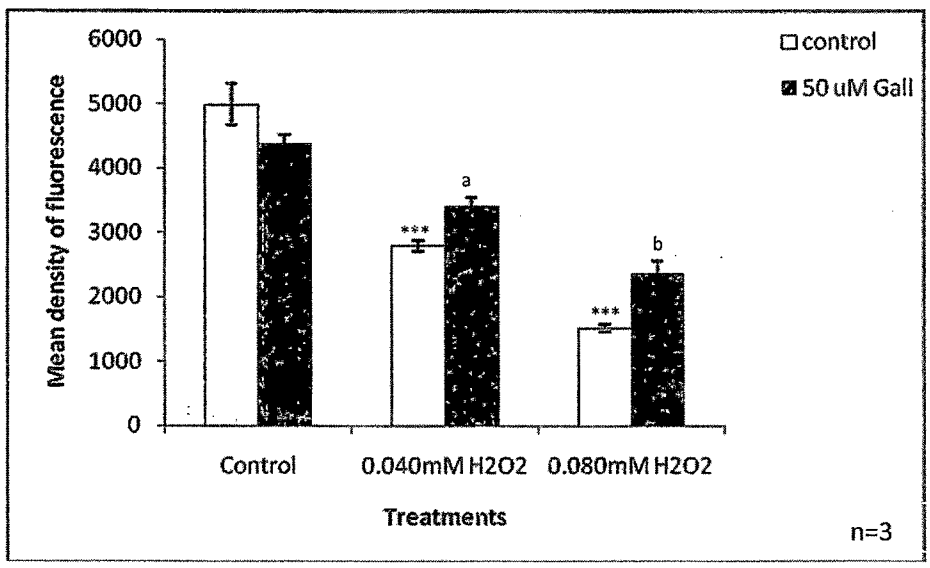

Figure 4.6.6a: Effect of PARG inhibition on H_2O_2 induced MMP changes by fluorometry. *** p value <0.001 compared to control; a p value <0.05 compared to 0.04mM H_2O_2; b p value <0.05 compared to 0.08mM H_2O_2.

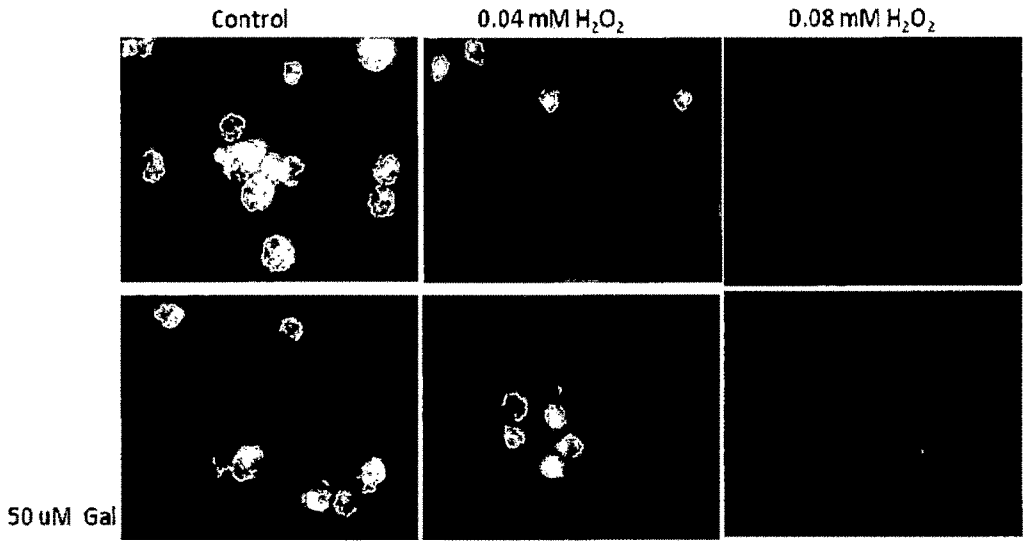

Figure 4.6.6b: Effect of PARG inhibition on H_2O_2 induced MMP changes by fluorescence microscopy

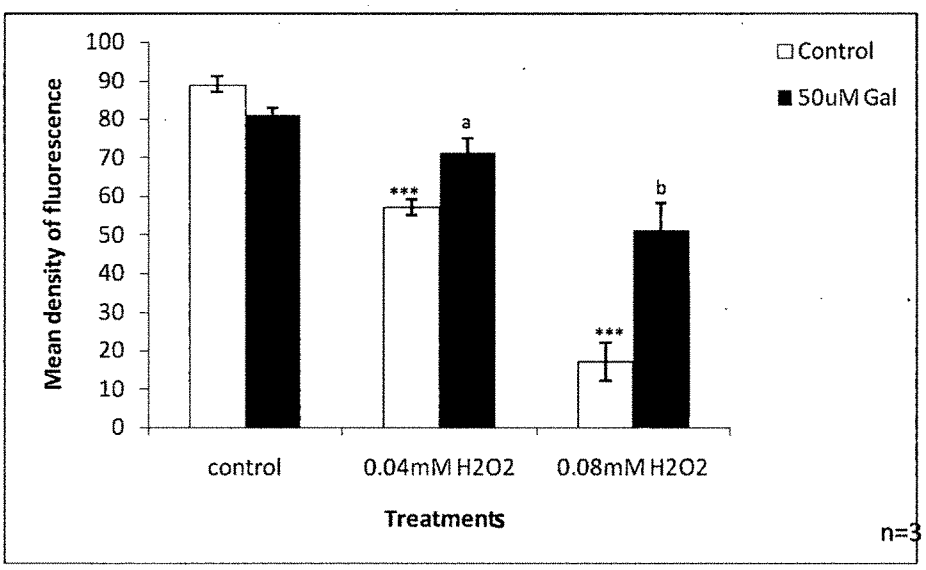

Figure 4.6.6c: Densitometric analysis: Effect of gallotannin pre treatment on H_2O_2 induced changes in MMP. *** p value <0.001compared to control; a p value <0.05 compared to 0.04mM H_2O_2; b p value <0.05 compared to 0.08mM H_2O_2.

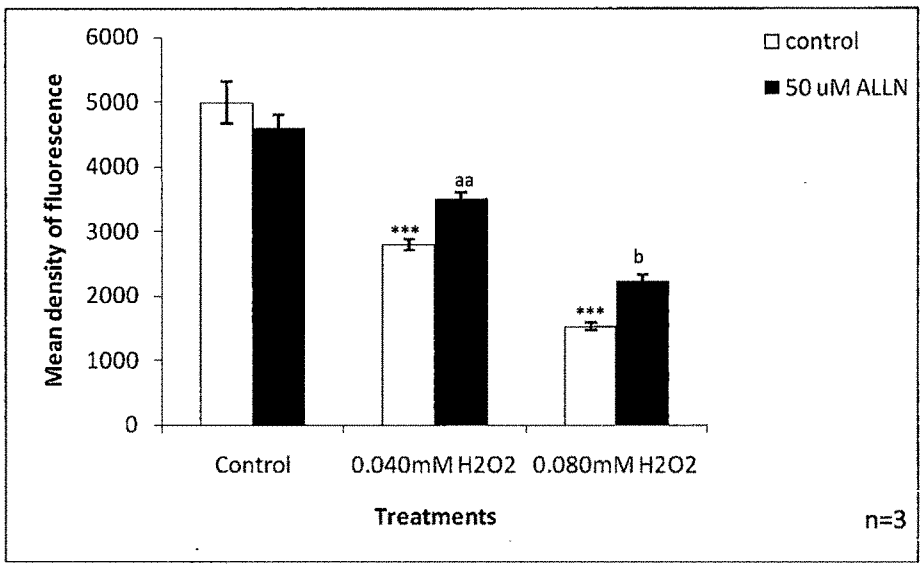

Figure 4.6.7a: Effect of ALLN pre treatment on H_2O_2 induced MMP changes by fluorometry. *** p value <0.001 compared to control; aa p value <0.01 compared to 0.04mM H_2O_2; b p value <0.05 compared to 0.08mM H_2O_2.

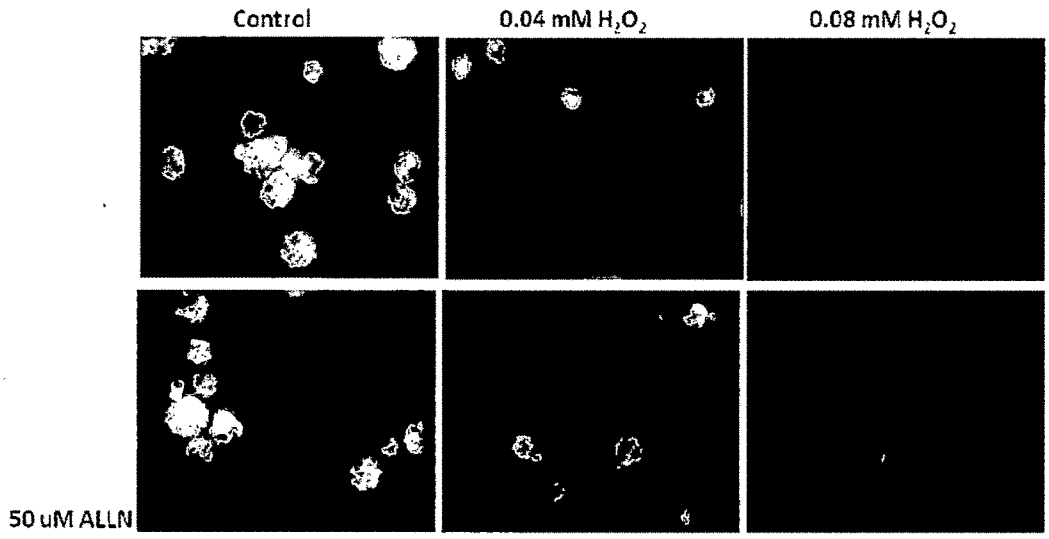

Figure 4.6.7b: Effect of ALLN on H_2O_2 induced MMP changes by fluorescence microscopy

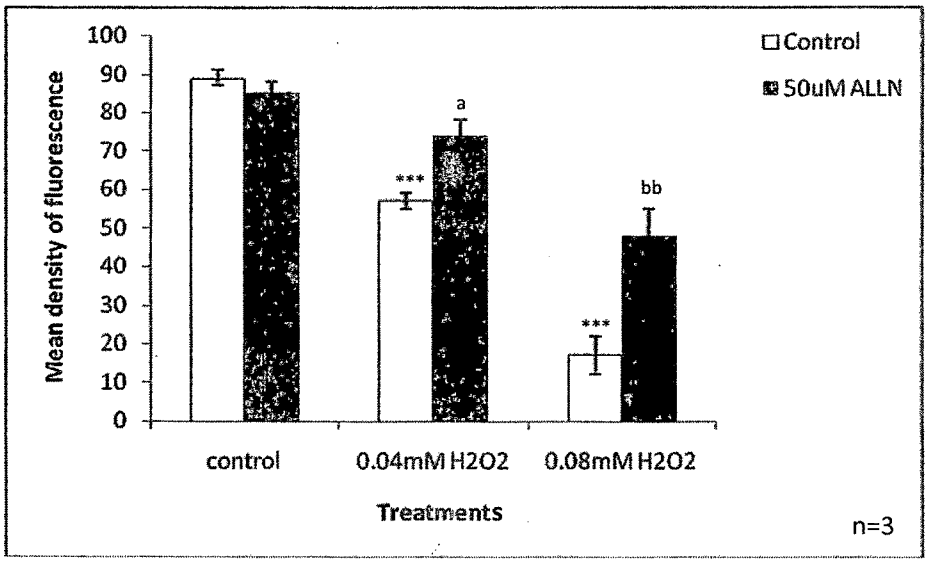

Figure 4.6.7c: Densitometric analysis: Effect of ALLN pre treatment on H_2O_2 induced changes in MMP. *** p value <0.001 compared to control; a p value <0.05 compared to 0.04mM H_2O_2; bb p value <0.01 compared to 0.08mM H_2O_2.

4.7 Heterologous expression of *Sf*-1 caspase using pA15-cas (constitutive promoter) in *D. discoideum*

As caspases are absent in *D. discoideum*, we wanted to study its effects if expressed in this organism. The pA15-cas clone which was available in the lab (contains *Spodoptera frugiperda (Sf-1)* caspase under constitutive promoter-pAct) was confirmed by *ClaI* and *XhoI* digestion.

After confirmation of the clone it was tranfected into *D. discoideum* cells. The transfectants could not survive however, the positive control of transfection pA15-GFP grew properly. Further, these positive control cells when subjected to development showed fruiting bodies with green fluorescence suggesting successful transfection.

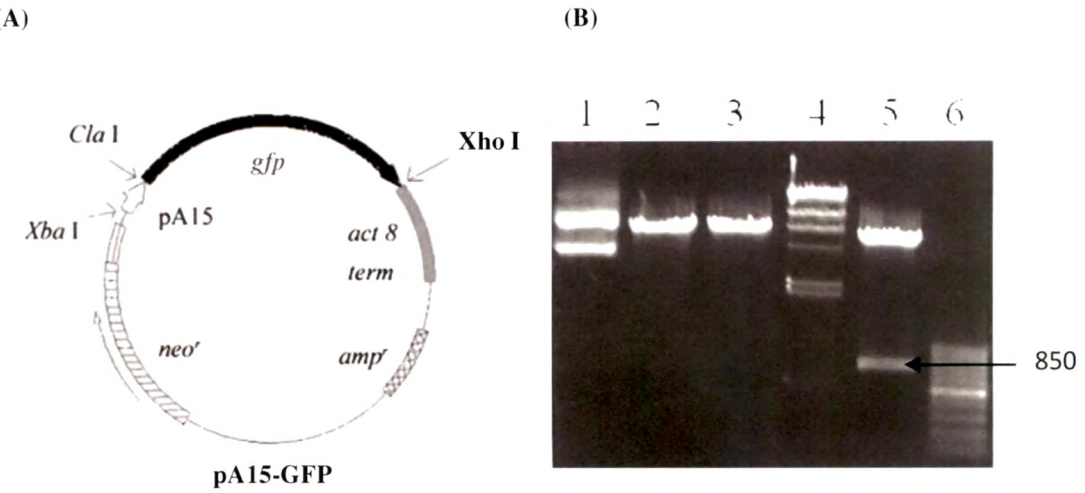

Figure 4.7.1: Vector and clones used for heterologous expression of caspase in *D. discoideum*.

(A) Map of the *D. discoideum* expression vector used for heterologous expression of caspase.

(B) Confirmation of construct pA15-*cas* by restriction digestion pattern: 1) Undigested clone pA15-cas; 2) *Cla*I digested pA15-cas; 3) *Xho*I digested pA15-cas; 4) Marker λ *Hind*III; 5) 850bp release by *Cla*I and *Xho*I double digest of pA15-cas; 6) Marker 100bp ladder.

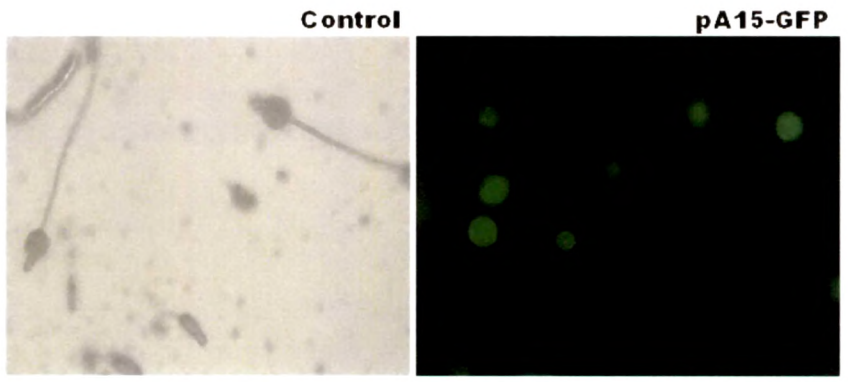

Figure 4.7.2: Development of *D. discoideum* cells transfected with pA15-GFP. Developmental photographs were taken at 24 hrs of post starvation.

Discussion

Cd^{2+} induced cell death in *D. discoideum*

Programmed cell death may take the form of apoptotic or nonapoptotic types. While caspases mediate apoptosis, the mediators of nonapoptotic cell death programs are less well characterized. Here, we report that paraptosis, an alternative, nonapoptotic cell death program that can be induced by Cd^{2+}, is mediated by PARP. Although pharmacological inhibition of PARP during conditions of oxidative stress induced by different agents is found to be beneficial, its inhibition could lead to adverse effects due to its involvement in various physiological processes. However, PARG inhibition with gallotannin could serve as a better option in curing diseases involving PARP overactivation.

The role of PARP in apoptotic death in several eukaryotic systems is well characterized, but the role of PARP and PARG in paraptotic cell death is yet to be addressed. Families of cysteine proteases called caspases (Cysteinyl–ASPartyl ProteASES) carry out apoptosis in higher eukaryotes. Functional caspases are not present in *D. discoideum* (Uren *et al.*, 2000). Although ~~paracapase~~ paracaspase gene is reported in *D. discoideum*, knock out of paracaspase did not affect developmental cell death (Bouffay *et al.*, 2004). Thus *D. discoideum* is one an excellent model to study the effect of heterologous expression of caspase on cell death of this organism during its unicellular and multicellular (developmental) phases. PARP over activation leading to mitochondrial changes is well reported, however the nuclear-mitochondrial cross talk is yet to be known. Present study addresses this aspect of paraptosis where *D. discoideum* cells were pretreated independently with NAD^+ supplementation, benzamide (PARP inhibitor), gallotannin (PARG inhibitor) or ALLN (Calpain inhibitor) each prior to oxidant (Cd^{2+} or cumene H_2O_2).

10 mM NAD^+ supplementation showed reduction in Cd^{2+} induced cell death from 25% to 12% (paraptotic dose) and 50% to 30% respectively (necrotic dose) (Fig. 4.2.1). Cell death with 1mM NAD^+ supplementation was found to be reduced from

25% to 15% and 50% to 40% in paraptotic and necrotic doses of cumene H2O2 induced cell death respectively (Fig. 4.2.4). These results suggest NAD^+ could rescue oxidative stress induced cell death in a dose dependent manner. It has been reported that extracellular application of NAD^+ was able to restore intracellular NAD^+ levels and counteract cell death induced by FK866, suggesting NAD^+ addition may affect physiological NAD^+ homeostasis (Billington *et al.*, 2008). It has also been shown that NAD^+ when exogenously applied to the culture medium of neurons, it can be transported across the axonal membrane (Araki *et al.*, 2004). This is also supported by our results where addition of 20 mM NAD^+ led to significant cell death in control cells (Fig. 4.1). This implies that exogenous NAD^+ enters D. discoideum cells; however the underlying mechanism of cell death by excess NAD^+ is yet to be understood.

To study the involvement of PARP during Cd^{2+} induced cell death, *D. discoideum* cells were pretreated with 1 mM benzamide and cell death was monitored. PARP inhibition showed reduction in cell death from 25% to 15% and 50% to 35% at paraptotic and necrotic dose of Cd^{2+} respectively (Fig. 4.2.2). Similar trend was also observed with H_2O_2 induced cell death also (Fig. 4.2.5). These results suggest that PARP plays very important role in *D. discoideum* cell death induced by oxidative stress. We recently published PARP's involvement in developmental cell death also (Rajawat *et al.*, 2007; 2011). Interestingly PARG inhibition by gallotannin also showed ~40 % and ~32 % rescue in Cd^{2+} induced paraptotic and necrotic cell death respectively (Fig. 4.2.3). These data indicate that PARG also plays important role in *D. discoideum* cell death.

Altogether these results imply that PARP, PARG and NAD^+ depletion contribute significantly and may be synergistically to *D. discoideum* cell death induced by oxidative stress (induced by Cd^{2+} or H_2O_2). Ours is the first report on the role of PARP in *D. discoideum* cell death, where we have shown the involvement of PARP during oxidative stress mediated delayed development and also during normal aggregation (Rajawat *et al.*, 2007; 2011).

Evidence suggests that MNNG-induced DNA damage leads to calpain activation through PARP-1. Calpain in turn activates Bax resulting in its translocation from cytosol to mitochondria, where it facilitates the release of AIF from the mitochondrial intermembrane space to the cytosol. Activated calpain also regulates AIF release by cleaving the membrane-anchored AIF to the soluble and cell death-active form, tAIF (Moubarak *et al.,* 2007). Calpain inhibition also rescues cell death induced by H_2O_2 (Fig. 4.2.7). On the other hand, although AIF mediated cell death pathway is present predominantly role of calpain during developmental cell death in *D. discoideum* is yet to be addressed.

ROS production and cell death in *D. discoideum*

In aerobic organisms the energy needed to fuel biological functions is produced in the mitochondria via the electron transport chain. In addition to energy, reactive oxygen species (ROS) that have the potential to cause cellular damage are produced by this chain. Mitochondria are a major source of ROS that may lead to DNA-damage response, including activation of p53 and PARP. While activation of p53 may cause apoptosis and cell cycle arrest, on the other hand hyperactivation of PARP leads to cell death.

The amount of ROS produced in oxidatively stressed *D. discoideum* cells was measured fluorometrically as well as with fluorescence microscope using DCFDA dye. Both the doses of Cd^{2+} produced ROS in a dose and time dependent manner. ROS estimations were done with various pre-treatments to find out if NAD^+ supplementation, benzamide and gallatonin rescued H_2O_2 induced paraptotic and necrotic cell death in *D. discoideum* by scavenging the ROS. Benzamide and gallotannin have been reported as ROS scavengers too (Virag and Szabo; 2002); however, in this study none of these agents (including NAD^+ and ALLN) showed reduction in ROS levels induced by Cd^{2+} or H_2O_2(Figs. 4.3.1-4.3.7); suggesting unique and indirect mechanism of action of these agents. Also these results suggest ROS production to be an event upstream to PARP activation. Similar results were

obtained when same experiments were done using H_2O_2 as stress inducer. However GSH pre treatment during Cd^{2+} stress prevented ROS production significantly (Fig. 3.7). These results suggest that GSH could block cell death at an upstream step.

PARP activation is the upstream event during paraptotic and necrotic cell death

Effect of NAD^+ supplementation, benzamide, gallotannin and ALLN pretreatments on PARP activity was measured by indirect immunofluroscence method immediately after 10 min of oxidative stress, as peak PARP activity is found at this time point (Rajawat, 2010) after oxidative stress induction. There was a dose dependent effect of oxidative stress on PARP activation at 10 min. Not surprisingly, NAD^+ being a substrate for PARP, its supplementation showed increased PARP activation in a dose dependent manner (Figs. 4.4.1 and 4.4.4), whereas pretreatment with benzamide and gallotannin showed decreased PARP activity as benzamide and gallotannin are inhibitors of PARP and PARG respectively (Figs. 4.4.2, 4.4.3, 4.4.5 and 4.4.6). Although all these agents i.e. benzamide, gallaotannin and NAD^+ had opposing effect on PARylation. This implies that PARP activation may not be the actual culprit leading to cell death. Significant rescue in cell death with exogenous NAD^+ despite of increasing PARP activity further project PARP as an **"Innocent killer protein"** (as its actual role is to recruit DNA repair machinery upon DNA damage). Interestingly inhibition of calpain, did not affect the oxidative stress induced PARP activation, suggesting calpain activation is a downstream event in cell death cascade.

NAD^+ depletion leads to MMP changes in *D. discoideum*

Irrespective of the morphological features of end-stage cell death (that may be apoptotic, necrotic, autophagic, or mitotic), mitochondrial outer membrane permeabilization (MOMP) is frequently the decisive event that delimits the frontier between survival and death. Thus mitochondrial membranes constitute the battleground on which opposing signals combat to seal the cell's fate. Local players that determine the propensity to MOMP include the pro- and antiapoptotic members of the Bcl-2 family, proteins from the mitochondrial permeability transition pore complex, as well as a plethora of interacting partners including mitochondrial lipids.

Intermediate metabolites, redox processes, sphingolipids, ion gradients, transcription factors, as well as kinases and phosphatases link lethal and vital signals emanating from distinct subcellular compartments to mitochondria. Thus mitochondria integrate a variety of proapoptotic signals. Once MOMP has been induced, it causes the release of catabolic hydrolases and activators of such enzymes (including those of caspases) from mitochondria. These catabolic enzymes as well as the cessation of the bioenergetic and redox functions of mitochondria finally lead to cell death, meaning that mitochondria coordinate the late stage of cellular demise. Pathological cell death induced by ischemia/reperfusion, intoxication with xenobiotics, neurodegenerative diseases, or viral infection also relies on MOMP as a critical event. The inhibition of MOMP constitutes an important strategy for the pharmacological prevention of unwarranted cell death. Conversely, induction of MOMP in tumor cells constitutes the goal of anticancer chemotherapy.

Several studies suggest that mitochondria play a central role in the execution of programmed cell death (Chandra *et al.*, 2002). Oxidative stress has been reported to cause mitochondrial dysfunction directly by promoting rapid loss of MMP (mitochondrial membrane potential) (Cipriani *et al.*, 2005). When cells take up paraptosis, mitochondria undergo an initial priming phase associated with hyperpolarization which further leads to an effector phase, during which mitochondria swell and release proapoptotic proteins (Rego *et al.*, 2001). Our lab results have shown that subsequent to PARP activation mitochondrial membrane potential change was observed which increased (non significantly) initially at 1 hour, but reduced at 3 hours and was almost lost by 5 hours post 1 mM hydroxylamine stress. Conversely, in necrosis the MMP increased in 1 hour and then decreased at 2 hours post oxidative stress, much earlier compared to paraptotic dose (Rajawat, 2010).

Paraptotic cells exhibit a change in mitochondrial membrane potential compared to control cells. This change in MMP was measured fluorimetrically and by fluorescence microscopy using $DiOC_6$ dye (Figs. 4.6.1-4.6.7). Our results showed a dose dependent effect of oxidative stress on MMP changes. Change in MMP is taken as an indicator of changes in the mitochondrial physiology which could be associated with the release

of various pro apoptotic proteins from mitochondria into the cytosol. With Cd^{2+} change in MMP was observed at 3 hours (Fig. 4.6.1). Increase in MMP observed in the initial phase could be due to shift in respiration from state 3 to state 4. Similar results were reported with Hela cells upon exposure to MNNG (N-Methyl-N'-Nitro-N-Nitrosoguanidine). These cells exhibited an increase in MMP initially which was attributed to state 4 respiration due to ADP deficiency (Cipriani *et al.*, 2005). A decrease in state 3 respiration may be attributed to oxidative damage of the inner mitochondrial membrane or the mitochondrial complexes, which inhibits the electron transport and may increase membrane leakiness producing the enhanced state 4 respiration (Rego *et al.*, 2001).

Changes in the mitochondrial membrane potential are associated with release of apoptosis-related killer proteins into the cytoplasm that are usually present in the inter-membrane space (IMS) of the healthy cell (Newmeyer and Ferguson-Miller, 2003). AIF, one such protein, plays an important role in inducing nuclear chromatin condensation as well as large-scale DNA fragmentation (~50 kbp). *D. discoideum* exhibits caspase independent cell death and also AIF (Apoptosis-inducing factor) mediated cell death is proposed to be predominating (Arnoult *et al.*, 2000). Interestingly, as monitored by western blot NAD^+ supplementation led to reduction in AIF release from mitochondria during oxidative stress induced cell death. Reduced mitochondrial AIF levels were found in stressed cells compared to control cells as confirmed by densitometric analysis (unpublished lab results). This result further confirms the existence of AIF dependent paraptotic pathway in this organism.

However PARP activation and mitochondrial changes did occur but the link between them is yet to be addressed. To find out the signal which is involved during Nucleur-Mitochondrial cross talk we detected the NAD^+ levels. Our results showed 62% and 78% reduction in NAD^+ level in paraptotic and necrotic doses of H_2O_2 (Fig. 4.5.4). NAD^+ depletion caused by PARP overactivation triggers changes in MMP following AIF release from mitochondria to cytosol and then nucleus. Additionally, NAD^+ supplementation showed maximum rescue in cell death. As can be seen NAD^+, benzamide, and gallotannin pretreated *D. discoideum* cells could prevent NAD^+

depletion followed by MMP changes induced by oxidative stress. In other words, NAD^+ depletion is a turning point during entire cell death cascade, suggesting NAD^+ to be the **"Currency coin"** during nuclear-mitochondrial cross talk; thus preventing NAD^+ depletion could block the downstream events leading to cell death. Furthermore, NAD^+ supplementation prevent release of AIF from mitochondria, which is key molecule for cell death execution (unpublished lab results).

Also as stated earlier PARP is important for various physiological processes, hence its inhibition would not be an appropriate approach for treatment of PARP associated diseases. Our results strongly support that replenishment of NAD^+, despite it being the central metabolite in a number of biological pathways, would serve the best as a pharmacological agent against diseases related to oxidative stress induced PARP mediated cell death. Thus this study sheds light on the current use of NAD^+, benzamide, gallotannin and ALLN as pharmacological agents for treatment of diseases associated with oxidative stress induced PARP mediated cell death.

Heterologous expression of *Sf*-1 caspase using pA15-cas (constitutive promoter) in *D. discoideum*

D. discoideum lacks caspases while it interestingly possesses PARP and paracaspase providing a tempting model organism for dissecting the evolutionary conserved cell death pathway. However paracaspase is not required for *D. discoideum* developmental cell death (Uren *et al.*, 2000). We tried to heterologously express caspase-3 (*Sf*-1 cas) in *D. discoideum* using constitutive expression vectors (Fig 4.7.1). The purpose was to study the effect of caspase on *D. discoideum* growth and development using constitutive hetelogous expression (pA15 cas). pA15-cas transfected *D. discoideum* cells did not survive however, pA15-GFP (vector control) transfected *D. discoideum* cells grew normally and also showed development (Fig. 4.7.2). Hence stage specific expression of caspase may be attempted in future. The "induced proximity model" is predicted on the empirical observation that the zymogen forms of unprocessed caspases are not entirely inactive but rather possess weak protease activity. When brought into close contact through protein interactions, the zymogens can trans-

process each other, producing the fully active proteases (Wang *et al.,* 2005). In support with this report, our caspase transfectants *D. discoideum* cells might have induced cell death via mechanism similar to induced proximity model of caspase activity.

Heterologous expression of caspase-3/CED-3 in yeast showed that substrates for caspase-3 are present in *S. cerevesiae* and may participate in the normal cell growth and division processes (Michael *et al.,* 1998). Evidence suggest that overproduction of the single metacaspase YCA1 resulted in autocatalytic processing and rendered cells more sensitive to exogenous or aging-related oxidative stress, as determined by reduced clonogenicity (Madeo *et al.*, 2002). A yeast strain with a disrupted YCA1 gene (Δyca1) was also shown to be three-fold less sensitive to H_2O_2, and ~5% of the cells escaped from aging-related cell death (Madeo *et al.*, 2002). Interestingly, extracts of H_2O_2-treated YCA1-overproducing yeast were highly active toward the synthetic caspase substrates Val-Glu-Ile-Asp-AMC and Ile-Glu-Thr-Asp-AMC. Thus, YCA1 metacaspase behaved as a bona fide caspase; in other words metacaspases constitute a new group of cysteine proteases homologous to caspases. (Madeo *et al.*, 2002). Heterologous expression of *Trypanosoma brucei* metacaspase TbMCA4 in the budding yeast *Saccharomyces cerevisiae* resulted in growth inhibition, mitochondrial dysfunction and clonal death (Alexander *et al.*, 2002).

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