# Role of Poly(ADP-ribose) glycohydrolase (PARG) in oxidative stress induced cell death in *D. discoideum*

## 4.1. INTRODUCTION

Poly(ADP-ribosyl)ation process involves a posttranslational modification of target proteins catalyzed by the poly(ADP-ribose) polymerase (PARP) enzyme with $NAD^+$ as the substrate, resulting in formation of long-branched polymers of ADP-ribose. The poly(ADP-ribose) units (PAR) significantly affect several important biological functions, including DNA repair and transcription. PAR attached to acceptor proteins is hydrolyzed rapidly and it is well-established that PAR has a very short half-life (~1 min), being promptly degraded by the constitutively active poly(ADP-ribose) glycohydrolase (PARG). PARG is a 110-kDa protein ubiquitously expressed in mammalian cells with endo- and exoglycosidic activities, cleaving PAR into free ADP-ribose units (Winstall *et al.*, 1999; Davidovich *et al.*, 2001).

Little is known, however, about the role of PARG in cell homeostasis, although different experiments indicate that the enzyme is involved in development (Hanai *et al.*, 2004), differentiation (Di Meglio *et al.*, 2003) and cell death (Affar *et al.*, 2001; Ying *et al.*, 2001). In contrast to PARP-1, there are fewer studies on PARG. PARG activity has been detected in both the cytoplasm and nucleus (Lin *et al.*, 1997; Ohashi *et al.*, 2003). Despite of being less abundant than PARP-1, PARG is the major enzyme involved in degradation of poly ADP-ribose (PAR) and is therefore a crucial determinant of PAR homeostasis, which has been proposed to be implicated in DNA repair and other cellular processes (D'Amours *et al.*, 1999; Davidovich *et al.*, 2001).

Significance of PAR homeostasis has been inferred from the finding that heterologous expression of PARP-1 in the yeast *Saccharomyces cerevisiae*, which lacks PARP and PARG, normally has no poly(ADP ribose) is lethal (Bernardi *et al.*, 1997; Collinge *et al.*, 1994; Kaiser *et al.*, 1992). In addition, overexpression of PARP-1 in mammalian cells (Van *et al.*, 1997) reduces cell survival after DNA damage. PARG inhibitors such as nobotanin B and gallotannin provide significant, but less complete protection against NMDA-induced neuronal death and can substantially reduce cell death

caused by $H_2O_2$ exposure (Ying *et al.*, 2001). PARG-deficient adult flies show neurodegeneration, reduced locomotor activity and a short lifespan, suggesting the involvement of PARG in normal neuronal cell metabolism and aging (Hanai *et al.*, 2004). Disruption of the *parg* gene by targeting exon 4 in the germline of mice, leading to complete suppression of functional PARG, causes early embryonic lethality (Koh *et al.*, 2004) due to PAR accumulation.

All these data highlight the significance of poly(ADP-ribose) homeostatsis *in vivo*. Depending on the therapeutic goal, enhancement or reduction in cell death could be accomplished by modulating PAR levels; one way to do this would be the inhibition of PARG, as it is the key enzyme involved in the degradation of PAR. Keeping with this life/death duality of PAR, the evidence presented indicates that under certain conditions PARG inhibition could be cytoprotective and under other conditions it could be cytotoxic.

Given the importance of PARG in PAR metabolism and to gain insight into the biological functions of PARG, we have investigated the effect of PARG inhibition on the PARP mediated oxidative stress induced cell death in *D. discoideum.*

## 4.2. RESULTS

### 4.2.1. Dose dependent effect of gallotannin on *D. discoideum* cell death, growth and development

For optimization of the concentration of gallotannin that does not exhibit toxicity, a dose dependent effect on cell death was monitored (Fig. 4.1). *D. discoideum* cells were treated with different doses of gallotannin for 12 hours and then cell death was studied by trypan blue staining. 50 μM gallotannin exhibited 5% cell death while 1% cell death was observed with 15 μM. 50 μM gallotannin exhibited toxicity to the cells and there was a delay of 24 hours in lag phase. 15 μM gallotannin does not have any effect on growth (Fig. 4.2) and development (Fig. 4.3). 15 μM concentration of gallotannin was selected for PARG inhibition experiments.

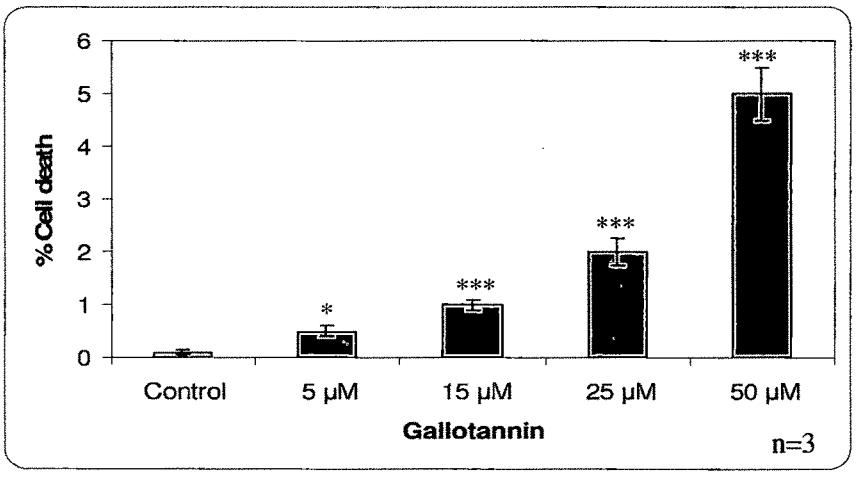



**Figure 4.1: Effect of gallotannin on *D. discoideum* cell death.** An increase in cell death was seen with increasing concentration of gallotannin by trypan blue staining after 12 hours treatment. *** p value <0.001, * p value <0.05 compared to control.

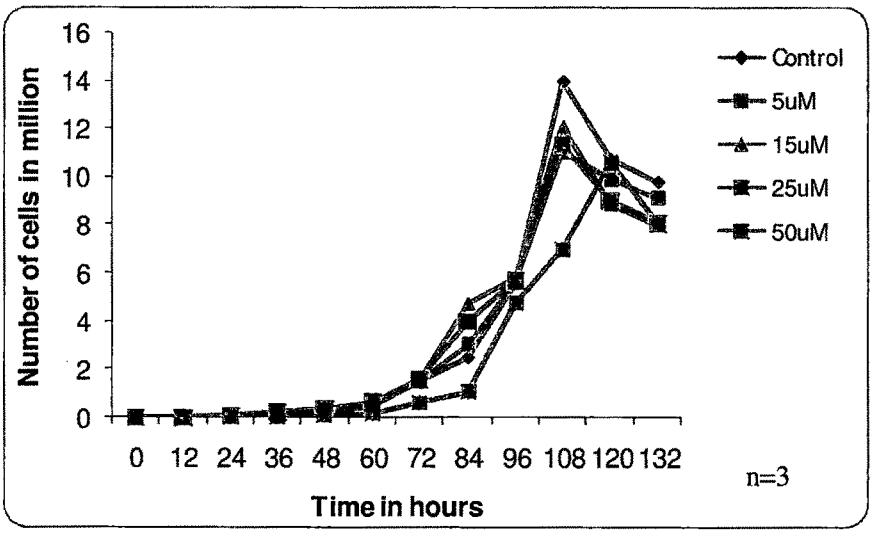



**Figure 4.2: Effect of gallotannin on growth of *D.discoideum*.** No effect was observed upto 25 µM gallotannin. 50 µM gallotannin delayed the lag phase. Results are the mean of three independent experiments.

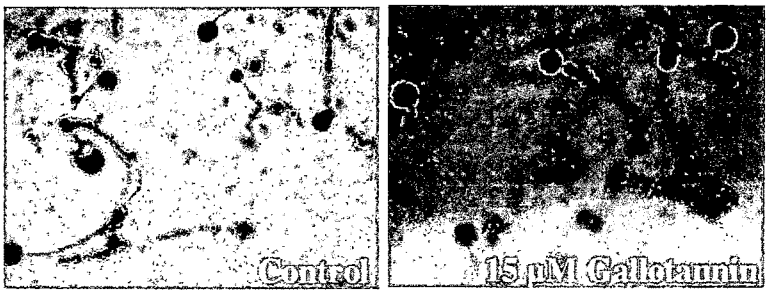



**Figure 4.3: Effect of gallotannin on development.** Fruiting bodies were formed up to 24 hours with 15 μM gallotannin. Photographs were taken with 4X objective.

### 4.2.2. Effect of PARG inhibition on PARP activation under oxidative stress in *D. discoideum*

1 mM HA and 0.03 mM $H_2O_2$ showed a peak in the PARP activity at 10 minutes and the activity declined by 15 minutes while 2.5 mM HA and 0.05 mM $H_2O_2$ exhibited peak within 5 minutes.

A

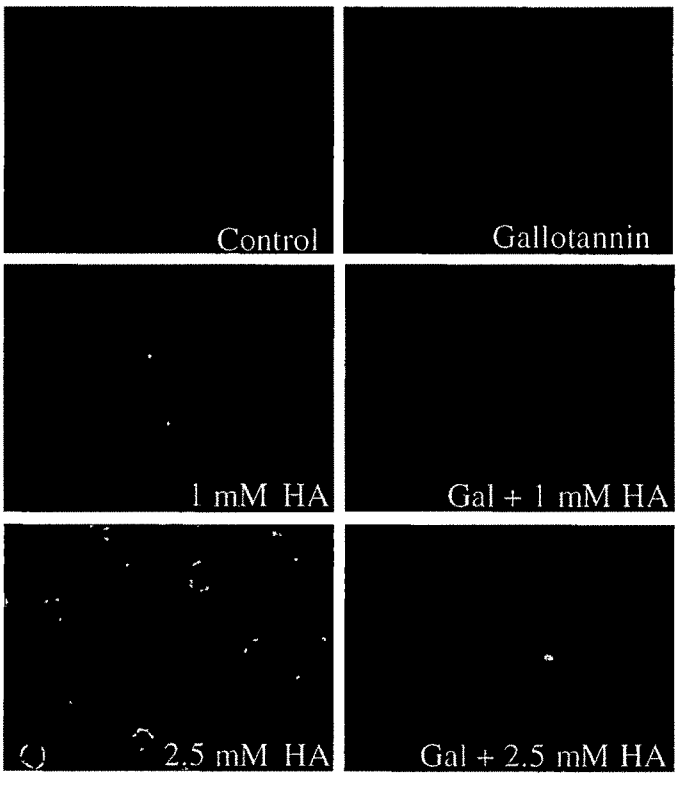
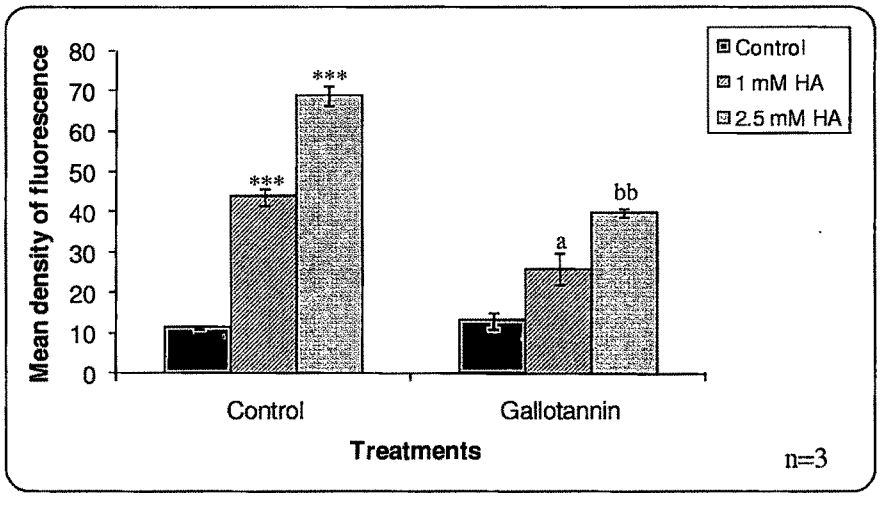



**Figure 4.4: PARP activity in presence of PARG inhibitor gallotannin. (A)** Peak PARP activity which was observed at 10 minutes and 5 minutes post HA stress was partially prevented by 15 μM gallotannin.

15 μM gallotannin pretreatment significantly reduced the activation of PARP which was observed at 10 minutes of 1 mM HA and 0.03 mM $H_2O_2$ treatment and 5 minutes of 2.5 mM HA and 0.05 mM $H_2O_2$ stress (Fig. 4.4 A & B). Hence, the 15 μM gallotannin concentration is found to be adequate for indirect inhibition of PARP in this study.

B




C

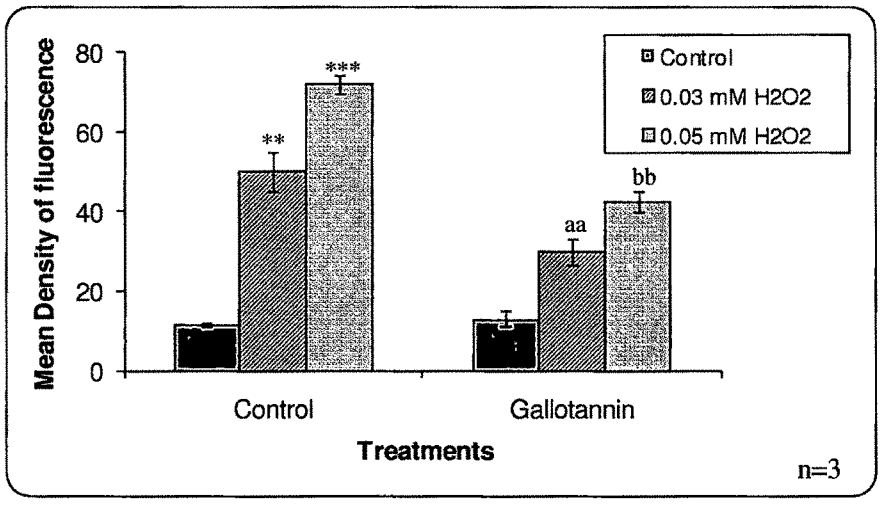



**Figure 4.4: PARP activity in presence of PARG inhibitor gallotannin. (B)** Graphical representation of PARP fluorescence during HA stress. (C) Gallotannin prevented PARP activation during cumene $H_2O_2$ stress. *** p value <0.001 compared to control; ** p value <0.01 compared to control; [a] p value <0.05 compared to 1 mM HA and 0.03 mM $H_2O_2$; [aa, bb] p value <0.01 compared to respective treatments.

### 4.2.3. Assessment of cell death by AnnexinV-FITC/PI dual staining

Effect of gallotannin was also monitored on oxidative stress induced cell death by trypan blue staining and it was found that 15 μM gallotannin partially intercepted the cell death induced by 1 mM and 2.5 mM HA (Fig. 4.5).

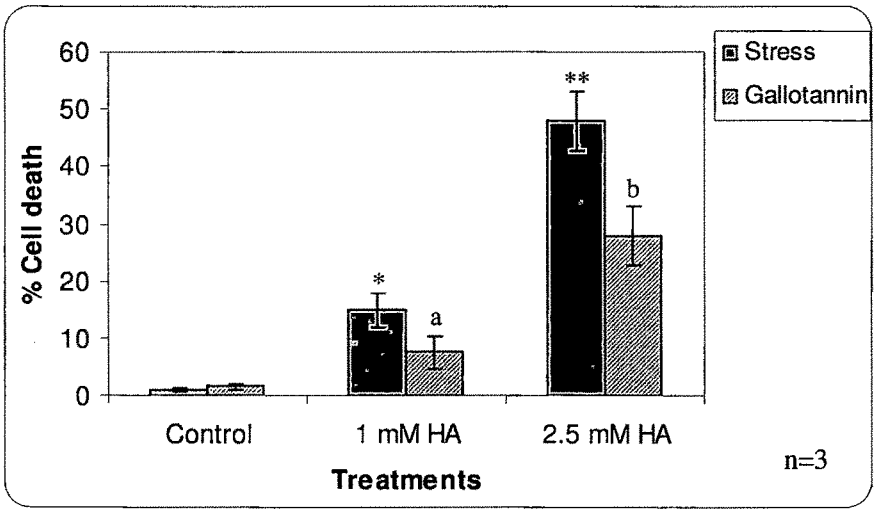



**Figure 4.5: PARP mediated cell death is reduced by PARG inhibitor gallotannin in *D. discoideum*.** Trypan blue exclusion assay shows that 15 μM gallotannin partially rescued the *D. discoideum* cells from HA induced cell death. ** p value <0.01 compared to control; * p value <0.05 compared to control; [a] and [b], p values <0.05 compared to 1 mM HA and 2.5 mM HA respectively.

Based on Annexin V-PI dual staining results 2.5 mM HA treated cells exhibited the uptake of both the stains simultaneously at 3 hours indicating that the cells underwent necrotic cell death. 1 mM HA treated cells were PS positive at 5 hours and both PS-PI positive at 12 hours. PARG inhibition delayed paraptotic cell death (1 mM HA) by preventing PS exposure at 5 hours and membrane integrity was maintained even after 12 hours and also necrotic cell death (2.5 mM HA) was switched to paraptotic type where PS exposure was seen at 3 hours and both PS-PI staining at 6 hours post stress (Fig. 4.6).

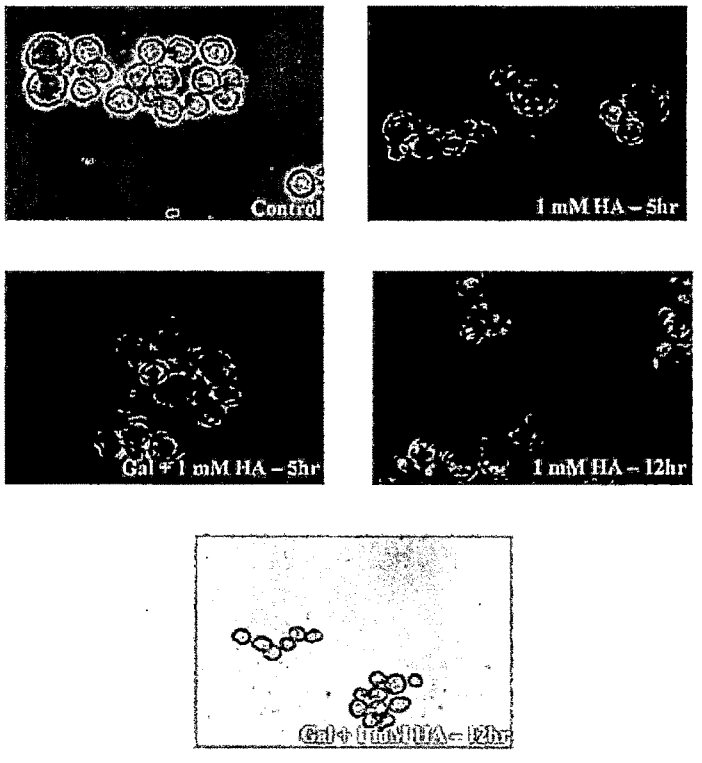



**Figure 4.6: Effect of PARG inhibition on PS-PI staining during oxidative stress induced cell death.** PS exposure which is observed at 5 hours during oxidative stress is delayed up to 12 hours upon gallotannin pretreatment. Photographs were taken with 60X objective.

### 4.2.4. Effect of PARG inhibition on oxidative stress induced MMP changes

Gallotannin prevents MMP changes in cells exposed to 1 mM HA and 0.03 mM $H_2O_2$ at 3 hours but, it has showed no effect on MMP changes at 2.5 mM HA dose and 0.05 mM $H_2O_2$ dose. Also further rescue in other cell death parameters were not observed at necrotic dose. This could be due to initial spurge of PARP activity immediately after oxidative stress and later it was inhibited due to PARG inhibition. Thus the initial PAR polymers formed were sufficient to induce downstream signal. As can be seen from fig. 4.7, gallotannin treatment alone could not bring significant MMP changes in *D. discoideum* cells.

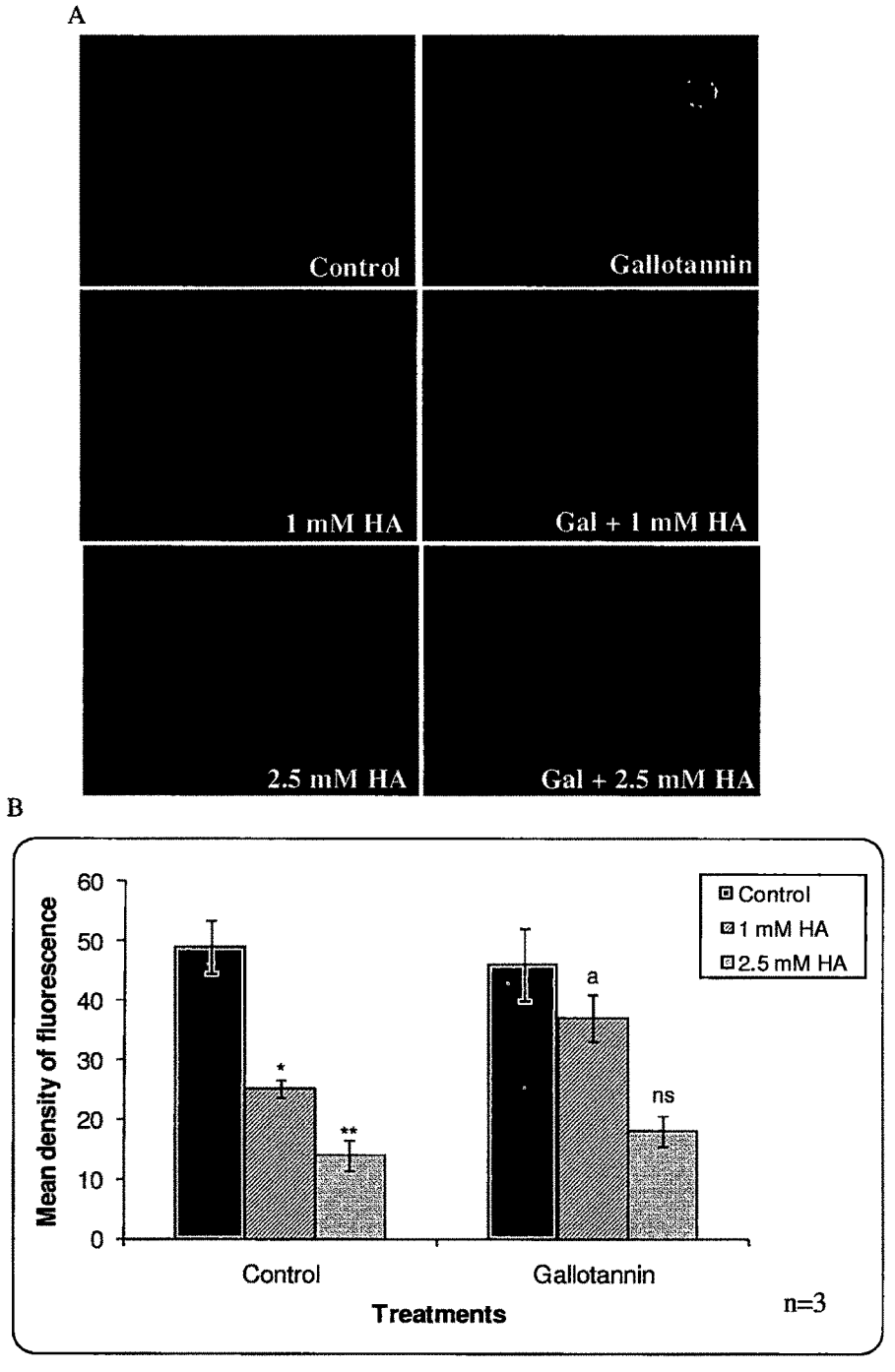



**Figure 4.7: MMP changes during oxidative stress with and without 15 µM gallotannin pretreatment.** (A) Photographs were taken with 60X objective. (B) MMP changes were partially restored at paraptotic dose but non significant change was observed with necrotic dose. * p value <0.05 compared to control; ** p value <0.01 compared to control; [a] p value <0.05 compared to 1 mM HA and non significant (ns) for necrotic doses.

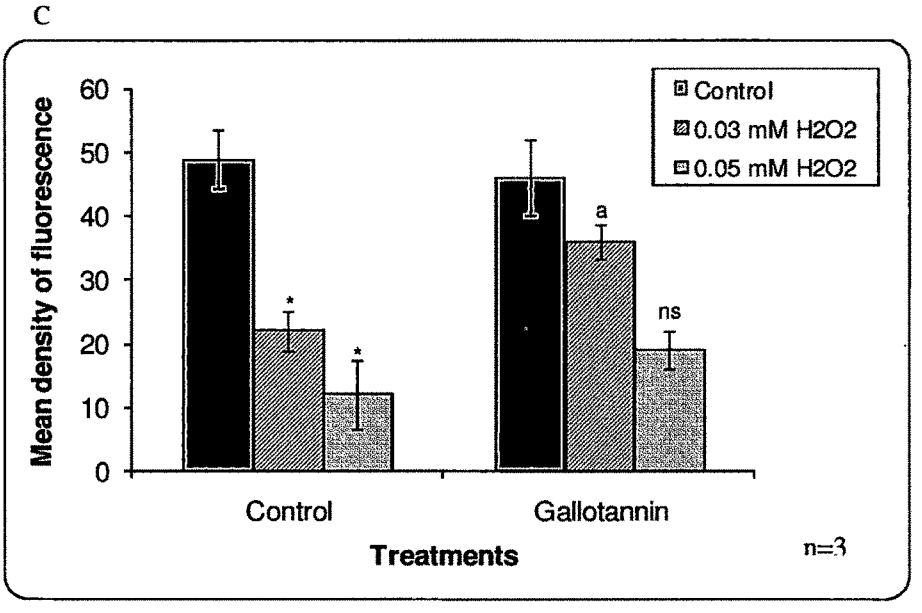



**Figure 4.7: MMP changes during oxidative stress with and without 15 µM gallotannin pretreatment.** (C) MMP changes were partially restored at paraptotic dose but non significant change was observed with necrotic dose. * p value <0.05 compared to control; ** p value <0.01 compared to control; [a] p value <0.05 compared to 0.03 mM $H_2O_2$ respectively and non significant (ns) for necrotic doses.

### 4.2.5. Effect of PARG inhibitor gallotannin on AIF translocation during HA induced cell death

During oxidative stress (1 mM HA) *D. discoideum* cells exhibited pink color (formed due to overlapping of blue color of DAPI with red color of AIF) indicating translocation to the nucleus at 6 hours. While 15 µM gallotannin pretreated cells exhibited partial prevention of AIF translocation to nucleus at 1 mM HA (Fig. 4.8).


**Figure 4.8: AIF translocation in 1 mM HA induced cell death pretreated with gallotannin.** AIF translocated at 6 hours post 1 mM HA treatment is partially prevented by PARG inhibition. Photographs were taken with 60X objective.

### 4.2.6. Effect of gallotannin on oxidative stress induced changes in $NAD^+$ levels

The $NAD^+$ in cells treated with oxidative stress was estimated by enzyme cycling assay. The amount of $NAD^+$ rapidly decreased by ~65% and 80% of control levels upon 1 mM and 2.5 mM HA treatments respectively (Fig. 4.9 & 4.10).

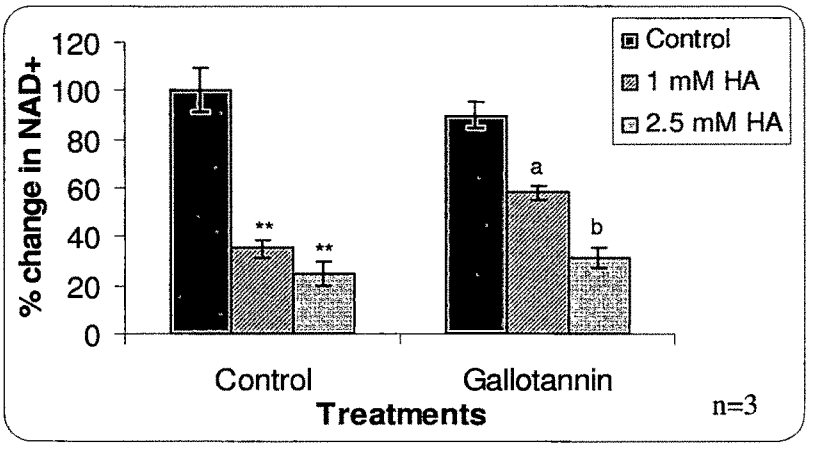



**Figure 4.9: HA induced $NAD^+$ depletion was prevented by gallotannin.** PARG inhibition partially rescued the depletion in $NAD^+$ levels post 30 minutes oxidative stress. ** p value <0.01 compared to control; [a] and [b], p value <0.05 compared to the respective HA stress.

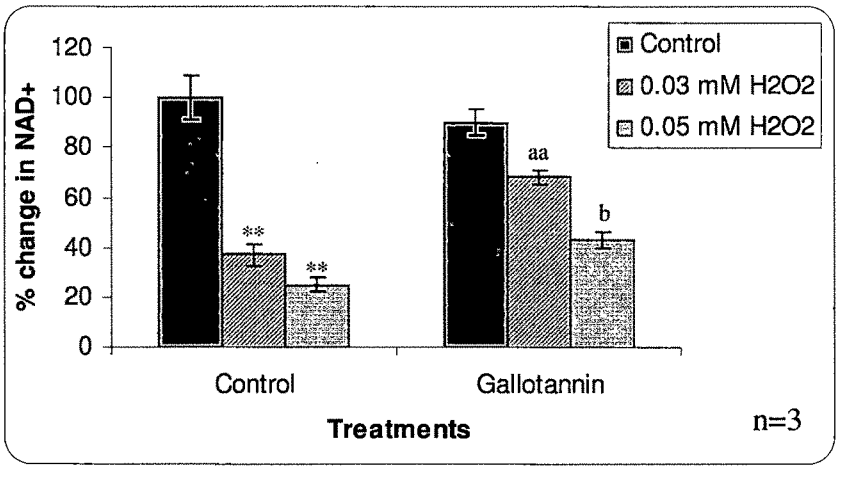



**Figure 4.10: Oxidative stress induced $NAD^+$ depletion was prevented by gallotannin.** PARG inhibition significantly prevented the depletion in $NAD^+$ levels after 30 minutes post 0.03 mM and 0.05 mM cumene $H_2O_2$ stress. ** p value <0.01 compared to control; [aa] p value <0.01 compared to 0.03 mM cumene $H_2O_2$; [b] p value <0.05 compared to 0.05 mM cumene $H_2O_2$ dose.

Similar results were also obtained with cumene $H_2O_2$ stress. $NAD^+$ content in the cells exposed to 0.03 and 0.05 mM cumene $H_2O_2$ were drastically reduced to ~35% and 25% of control. $NAD^+$ levels were restored to ~40% upon PARG inhibition.
We found that incubation of *D. discoideum* cells with 15 μM gallotannin prior to oxidative stress significantly prevented the depletion of $NAD^+$ levels.

## 4.3. DISCUSSION

PARP-1 activation has been established as a major component of both oxidative and excitotoxic neuronal death (Ying *et al.*, 2001). PARG plays an important role in PARP mediated cell death by regulating PAR turn over. PAR catabolism in the nucleus could be regulated, at least in part, by a nuclear-cytosolic shuttling of PARG. PARG shuttling to and from the nucleus is probably a very efficient and rapid process that would make it very difficult for the actual detection of PARG translocation. The presence of both nuclear export signal (NES) and nuclear localization signal (NLS) in the PARG protein would allow a dynamic shuttling of PARG throughout the cell where PARP activity is found (Lin *et al.*, 1997; Shimokawa *et al.*, 1999). PARP-1, PARG, and XRCC1 interplay to regulate apoptotic cell death induced by supralethal N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) doses (Keil *et al.*, 2006). Cellular PAR levels are the net result of the competing processes of PAR formation, primarily by PARP-1, and PAR degradation, by PARG (Burns *et al.*, 2009). Reduced catabolism of PAR also alters selective inflammatory gene expression profile. Pharmacological inhibition of PARG leads to nuclear accumulation of PAR, which in turn triggers the expression of iNOS and COX-2 in cultured macrophages (Rapizzi *et al.*, 2004).

PAR catabolism is accelerated during genotoxic stress condition which is largely attributed to PARG (Cortes *et al.*, 2004). The protection afforded by PARG silencing against $H_2O_2$-induced apoptosis was similar in its extent to that provided by 3-Amino benzamide (3-AB) up to 100 μM $H_2O_2$ (Blenn *et al.*, 2006). Selective down regulation of nuclear PARG prevents cell death after exposure to $H_2O_2$ (Burns *et al.*, 2009). The PARG antisense-treated cells when exposed to genotoxic agent, MNNG showed a

delayed rate of nuclear PAR degradation, reduced nuclear condensation, and reduced cell death (Burns *et al.*, 2009).

To study the mechanism by which PARG could influence PARP mediated cell death, we inhibited PARG with one of its potent inhibitor gallotannin. Gallotannin dose was standardized by trypan blue staining and 15 μM was used for further experiments (Fig. 4.1). PARG inhibition showed no effect on normal growth (Fig. 4.2) and development (Fig. 4.3). PARP activity which was observed at 10 minutes and 5 minutes of HA and cumene $H_2O_2$ stress was partially prevented by 15 μM gallotannin (Fig. 4.4). It is shown to be a potent rescuer of cell death under 1 mM HA (Fig. 4.5) where it prevents MMP changes at 3 hours (Fig. 4.7) and prevention of PS exposure at 5 hours (Fig. 4.6). Nevertheless under 2.5 mM HA, 15 μM gallotannin pretreated cells showed no rescue in MMP changes (Figs. 4.7 A, B & C). Also 15 μM gallotannin pretreated cells exhibited partial prevention of AIF translocation to nucleus with 1 mM HA exposure at 6 hours (Fig. 4.8). Cellular $NAD^+$ levels were depleted during HA and cumene $H_2O_2$ treatments. PARG inhibition resulted in lower $NAD^+$ consumption during oxidative stress induced cell death (Figs. 4.9 & 4.10). These results can also be correlated with our previous results where the role of PARP in oxidative stress induced cell death has been established with the use of PARP inhibitor, benzamide.

Several studies have reported that PARG inhibition is harmful to cells. Ying *et al.* (2001) suggest that PARP normally functions in DNA repair and its genetic inhibition can also have detrimental effects. Ablation of PARG enzymatic activity by gene disruption in the mouse causes massive PAR accumulation and embryonic lethality (Koh *et al.*, 2004). PARG-deficient adult flies show neuro-degeneration, reduced locomotor activity and a short lifespan, suggesting the involvement of PARG in normal neuronal cell metabolism and aging (Hanai *et al.*, 2004).

Enhancement or reduction in cell death could be accomplished by modulating PAR levels. Effects of PARG inhibitors unlike PARP inhibitors, do not prevent initial PAR formation after DNA damage, nevertheless PARG inhibitors may have less deleterious effects on DNA repair. Blocking PAR degradation by PARG inhibitors would 'trap' DNA damage-dependent PARP in the auto-(ADPribosyl)ated inactive state

thus slowing the turn over of poly (ADP-ribose) and in turn slowing the consumption of $NAD^+$. Thus it can be concluded that PARG inhibitors could in principle maintain PARP in an inactive state by preventing removal of PAR groups.

Our findings support the hypothesis suggested by Ying *et al.* (2001) that PARG could influence PARP-mediated cell death and PARG activity is protective in function during oxidative stress and PARG inhibitor gallotannin when used at 15 μM concentration could effectively rescue oxidative stress induced paraptotic cell death in *D. discoideum*. PARG inhibition prevents cell death by slowing the turn over of PAR and thus preventing $NAD^+$ depletion. Reduced PARP activity might also contribute to cell death prevention in presence of PARG inhibitor. Thus, paraptotic cell death induced by oxidative stress can be intercepted by PARG inhibition, suggesting that PARP-mediated cell death requires the concomitant action of PARG. The evidence presented below indicates that under certain conditions PARG inhibition could be cytoprotective and under other conditions it could be cytotoxic. Still extensive work is required to identify potent molecules to specifically inhibit PARG and to study its molecular mechanism. Further knockout of PARG would throw more light on the cellular function of PARG.

## 4.4. REFERENCES

Affar E.B., Germain M., Winstall E., Vodenicharov M., Shah R.G., Salvesen G.S. and Poirier G.G. Caspase-3-mediated processing of poly- (ADP-ribose) glycohydrolase during apoptosis. (2001) *J. Biol. Chem.* 276, 2935–2942.

Bernardi R., Rossi L., Poirier G.G. and Scovassi A.I. Analysis of poly(ADP-ribose) glycohydrolase activity in nuclear extracts from mammalian cells. (1997) *Biochim. Biophys. Acta.* 1338, 60–68.

Blenn C., Althaus F.R. and Malanga M. Poly(ADP-ribose) glycohydrolase silencing protects against H2O2-induced cell death. (2006) *Biochem. J.* 396, 419–429.

Burns D.M., Ying W., Kauppinen T.M., Zhu K., Swanson R.A. Selective Down-Regulation of Nuclear Poly(ADP-Ribose) Glycohydrolase. (2009) *PLoS ONE* 4, e4896.

Collinge M.A. and Althaus F.R. Expression of human poly(ADPribose) polymerase in Saccharomyces cerevisiae. (1994) *Mol. Gen. Genet.* 245, 686–693.

Cortes U., Tong W.M., Coyle D.L., Meyer-Ficca M.L., Meyer R.G. et al. Depletion of the 110-kilodalton isoform of poly(ADP-ribose) glycohydrolase increases sensitivity to genotoxic and endotoxic stress in mice. (2004) *Mol. Cell Biol.* 24, 7163–7178.

D'Amours D.S., D'Silva I., Desnoyers and Poirier G.G. Poly(ADPribosyl)ation reactions in the regulation of nuclear functions. (1999) *Biochem. J.* 342, 249–268.

Davidovic L., Vodenicharov M., Affar E.B. and Poirier G.G. Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADPribose) metabolism. (2001) *Exp. Cell Res.* 268, 7–13.

Di Meglio S., Denegri M., Vallefuoco S., Tramontano F., Scovassi A.I. and Quesada P. Poly(ADPR) polymerase-1 and poly(ADPR) glycohydrolase level and distribution in differentiating rat germinal cells. (2003) *Mol. Cell. Biochem.* 248, 85–91.

Hanai S., Kanai M., Ohashi S., Okamoto K., Yamada M., Takahashi H. and Miwa M. Loss of poly(ADP-ribose) glycohydrolase causes progressive neurodegeneration in Drosophila melanogaster. (2004) *Proc. Natl. Acad. Sci. USA.* 101, 82–86.

Kaiser P., Auer B. and Schweiger M. Inhibition of cell proliferation in Saccharomyces cerevisiae by expression of human $NAD^+$ ADP-ribosyltransferase requires the DNA binding domain ("zinc fingers"). (1992) *Mol. Gen. Genet.* 232, 231–239.

Keil C., Gröbe T. and Oei S.L. MNNG-induced Cell Death Is Controlled by Interactions between PARP-1, Poly(ADP-ribose) Glycohydrolase, and XRCC1. (2006) *J. Biol. Chem.* 281, 34394-34405.

Koh D.W., Lawler A.M., Poitras M.F., Sasaki M., Wattler S., Nehls M.C., Stöger T., Poirier G.G, Dawson V.L, Dawson T.M. Failure to degrade poly(ADP-ribose) causes increased sensitivity to cytotoxicity and early embryonic lethality. (2004) *Proc. Natl. Acad. Sci. USA.* 101:17699-704.

Lin W., Ame J.C., Aboul-Ela N., Jacobson E.L. and Jacobson M. K. Isolation and characterization of the cDNA encoding bovine poly(ADPribose) glycohydrolase. (1997) *J. Biol. Chem.* 272, 11895–11901.

Ohashi S., Kanai M., Hanai S., Uchiumi F., Maruta H., Tanuma S. and Miwa M. Subcellular localization of poly(ADP-ribose) glycohydrolase in mammalian cells. (2003) *Biochem. Biophys. Res. Commun.* 307, 915–921.

Rapizzi E., Fossati S., Moroni F. and Chiarugi A. Inhibition of Poly(ADP-Ribose) Glycohydrolase by Gallotannin Selectively Up-Regulates Expression of Proinflammatory Genes. (2004) *Mol. Pharmacol.* 66, 890–898.

Shimokawa T., Masutani M., Nagasawa S. *et al.* Isolation and cloning of rat poly(ADP-ribose) glycohydrolase: presence of a potential nuclear export signal conserved in mammalian orthologs. (1999) *J Biochem. (Tokyo)* 126, 748-55.

Van Gool L., Meyer R., Tobiasch E., Ciepluch C., Jauniaux J.C., Mincheva A., Lichter P.G., Poirier G.G., Burkle A. and Kupper J.H. Overexpression of human poly(ADP-ribose) polymerase in transfected hamster cells leads to increased poly(ADP-ribosyl)ation and cellular sensitization to gamma irradiation. (1997) *Eur. J. Biochem.* 244, 15–20.

Winstall E., Affar E.B., Shah R.G., Bourassa S., Scovassi A.I., and Poirier G.G. Poly(ADP-ribose) glycohydrolase is present and active in mammalian cells as a 110-kDa protein. (1999) *Exp. Cell Res.* 246, 395–398.

Ying W., Sevigny M.B., Chen Y. and Swanson R.A. Poly(ADP-ribose) glycohydrolase mediates oxidative and excitotoxic neuronal death. (2001) *Proc. Natl. Acad. Sci. USA.* 98, 12227–12232.