Role of poly (ADP-ribose) polymerase (PARP) and effect of glutathione and cAMP in oxidative stress induced changes in *D. discoideum* growth and development

6.1. INTRODUCTION

Dictyostelium discoideum, a unicellular eukaryote exhibits multicellularity upon nutrient starvation and thus provides a simple but excellent model system to study various signal transduction pathways (Mir *et al.*, 2007), which can later be confirmed with complex eukaryotic systems. It is known that ROS influence *D. discoideum* development (Bloomfield and Pears, 2003; Garcia *et al.*, 2000, Garcia *et al.*, 2003). *D. discoideum* the unicellular stage is known to be highly resistant to DNA damaging agents and oxidative stress (Welker *et al.*, 1978; Katoch and Begum, 2003). However, the response of *D. discoideum* development to oxidative stress is not well understood. Recent studies showed that superoxide plays a vital role in the aggregation process of *D. discoideum* cells (Bloomfield and Pears, 2003), as inhibition of superoxide dependent signaling events affects the transition from unicellular to multicellular phase. Pre-starvation factor could be stimulating superoxide production in developing cells during starvation. In addition, during development *D. discoideum* cells produce nitric oxide, which was also postulated to act as a signaling molecule (Tao *et al.*, 1997). The response to oxidative stress seems to work in a similar manner to starvation response which involves growth arrest, induction of cAMP synthesis and PKA (Protein Kinase A) activation by YakA (Taminato *et al.*, 2002). Changes in the levels of ROS have been associated with differentiation and development in several systems.

ROS nevertheless, also have deleterious effects and are known to cause DNA damage (Du *et al.*, 2003) which in turn results in the activation of poly(ADP-ribose) polymerase (PARP). It catalyzes the transfer of ADP-ribose moieties to acceptor proteins by utilizing NAD^+ as the substrate and helps in DNA repair (Burkley, 2001; D'Amours *et al.*, 1999). PARP also monitors the status of DNA before entry into mitosis (Oliver *et al.*, 1998; Hoger *et al.*, 1999) and hence has been implicated in checkpoint control. Cells are arrested at different stages of the cell cycle depending upon the extent of PARP activation (Horton *et al.*, 2005) under stress conditions. Thus in higher eukaryotic cells

PARP contributes to cell homeostasis under mild stress conditions, and conversely during conditions of moderate/severe cellular stress PARP over activation leads to cell death, which results in several disease conditions (Virag and Szabo, 2002). Pharmacological inhibition of PARP during moderate/severe cellular stress is beneficial (Palomba *et al.*, 1996; Virag *et al.*, 1998), however the consequences of such inhibition on the genomic integrity are not yet understood.

D. discoideum is reported to have nine potential PARP genes (Otto *et al.*, 2005) unlike other unicellular eukaryote *S. cerevisiae* which does not possess PARP (Perkins *et al.*, 2001). Hence we have selected *D. discoideum* as a model system to study the role of PARP in its development under oxidative stress conditions.

Upon sensing starvation, growth phase genes are switched off and growth-differentiation transition genes are switched on. Several factors are released during starvation; prestarvation factor (PSF) and conditioned medium factor (CMF) are amongst the earliest secreted signals. These induce downstream factors to initiate aggregation and cAMP is one of them. *D. discoideum* cells communicate by cAMP which is produced and relayed by the developing cells at an aggregation center (Firtel, 1995). cAMP binds to nearby cells and activates adenylyl cyclase, resulting in a rapid increase in intracellular and secreted cAMP (Mann and Firtel, 1987). When cells form an aggregate, cAMP concentration is thought to rise to the micromolar range (Abe and Yanagisawa, 1983). This aggregation process is controlled by various signals which regulate the differential expression of genes whose products are necessary for chemotaxis towards cAMP (Konijin *et al.*, 1968). cAMP in early development induces the expression of cAMP receptor (*car* 1) and *discoidin* and in later development, it induces prestalk and prespore specific genes (Mann and Firtel, 1987; Mir *et al.*, 2007). The adenylyl cyclase gene *aca*A is one of the first genes expressed upon starvation. ACA produces extracellular cAMP that induces chemotaxis and aggregation in neighboring cells (Hideshi *et al.*, 1998). Intercellular signaling by secreted cAMP then induces the expression of another set of genes for further stages of development. Therefore the components that mediate the induction of adenylyl cyclase have the central role in the growth-differentiation transition in *D. discoideum.*

We have studied the dose dependent effect of hydroxylamine (*in situ* H_2O_2 generation) on *D. discoideum* development and also the role of PARP during oxidative stress induced effects on development. Our present study is the first report on the activation of PARP under oxidative stress in *D. discoideum* and our results suggest that *D. discoideum* is an excellent model system to investigate the long term effects of PARP inhibitors for two successive generations. As development is initiated by cAMP signaling we have further made an attempt to study the effect of addition of cAMP and glutathione on oxidative stress induced changes in development.

6.2. RESULTS

6.2.1. *D. discoideum* growth under oxidative stress

To monitor the effect of hydroxylamine (HA) on *D. discoideum* cell cycle, growth curve was carried out. The concentrations of HA used for development studies were 1.0, 2.5 and 4.0 mM based upon the cell death studies carried out in Chapter 3.

The growth curve showed a dose dependent increase in the lag phase from 36 to 60 hours, 36 to 72 hours and 36 to 96 hours at LD_{15} (1 mM), LD_{50} (2.5 mM) and LD_{90} (4 mM) respectively. Further the log phase was shortened to 48 hours, 48 hours and 36 hours at LD_{15}, LD_{50} and LD_{90} followed by faster attainment of stationary phase (Fig. 6.1), suggesting that HA caused cell cycle arrest leading to increased lag phase.

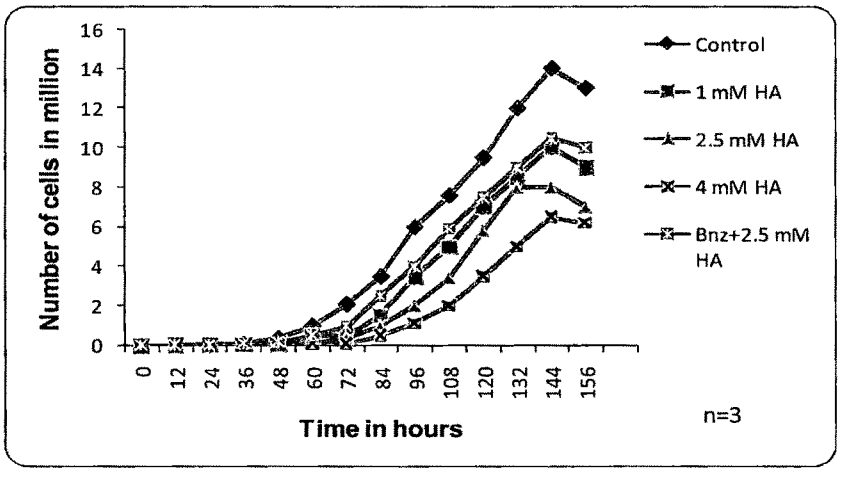

Figure 6.1: Effect of oxidative stress induced growth changes in *D. discoideum*. Under oxidative stress the growth curve showed a dose dependent increase in the lag phase. The log phase is shortened followed by faster attainment of stationary phase. Results are mean of 3 independent experiments performed in duplicates.

6.2.2. *D. discoideum* development under oxidative stress

To study the effect of oxidative stress on differentiation, developmental studies were performed. Dose dependent effect of HA on *D. discoideum* development was studied by exposing the cells to different concentrations of HA (1.0, 2.5, 4.0 mM) for 1 hour and then allowed to develop. As can be seen from Table 6.1 and Fig. 6.2, development was delayed in a dose dependent manner at the loose aggregation (LA) stage by 2 hours and 12 hours at LD_{15} and LD_{50} of HA respectively as compared to control cells. At 18 hours of development 40% loose aggregates were seen in 2.5 mM HA compared to control. The percent involvement of cells in development was slightly increased with time. Nevertheless, LD_{90} of HA showed no development till one week suggesting that development was arrested at loose aggregation stage. HA treated *D. discoideum* cells exhibited dose dependent decrease in the number and size of fruiting bodies as compared to control cells (Fig. 6.2). The most significant effect of HA seemed to occur at loose aggregation stage. Therefore the expression analysis was monitored for genes involved during aggregation. As judged by RT-PCR analysis expression of adenylyl cyclase A (*aca*A) and cAMP receptor 1 (*car*1) were affected during oxidative stress. The developmental expression pattern of *aca*A, *cAR*1 and *hsp*D are shown in fig. 6.3. At 9 hours expression of *aca*A and *car*1 was found to decrease in a dose dependent manner at 2.5 and 4 mM HA doses, while *hsp*D expression was comparable to control.

Table 6.1: HA induced developmental changes in *D. discoideum*

	LA (hr)	TA (hr)	SF (hr)	FBF (hr)	% CD	% FB
HA mM						
0.0	**6**	**12**	**18**	**24**	**1**	**100**
1.0	8	14	20	26	15	100
2.5	18	24	30	36	50	30
4.0	ND	ND	ND	ND	90	ND

LA: Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation; CD: cell death; FB: Fruiting body; ND: No development till one week.

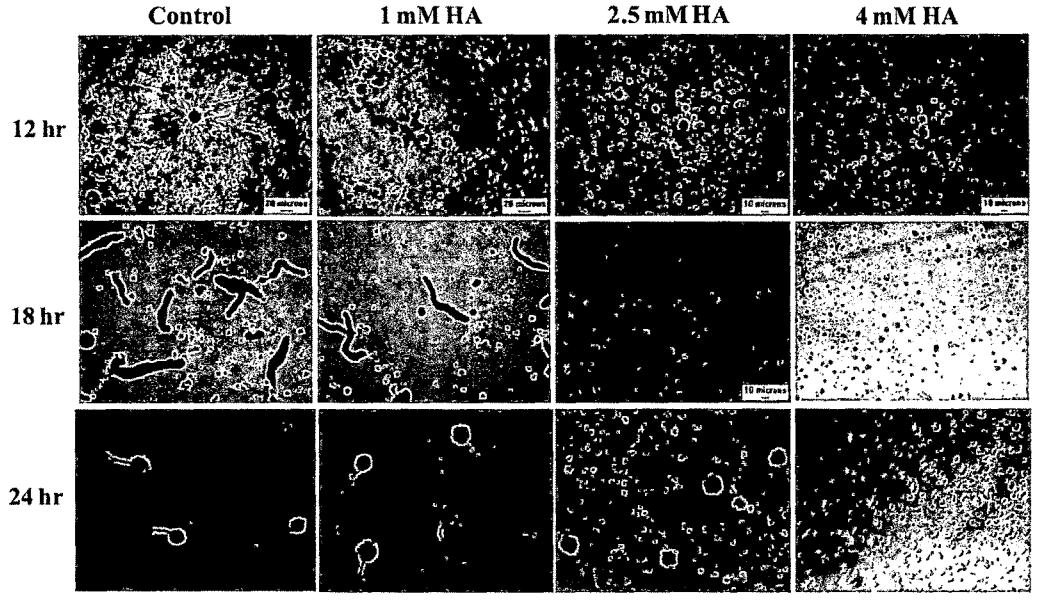

Figure 6.2: Development of *D. discoideum* cells under oxidative stress. *D. discoideum* cells after HA treatments was allowed to develop on nutrient free agar medium and were observed at every 6 hours. Developmental stages of control cells, 1 mM, 2.5 mM and 4 mM HA treated cells at 12, 18 and 24 hours. Results are mean of 3 independent experiments performed in duplicates. Scale bar, 10 and 20μm. Photographs were taken with 4X objective.

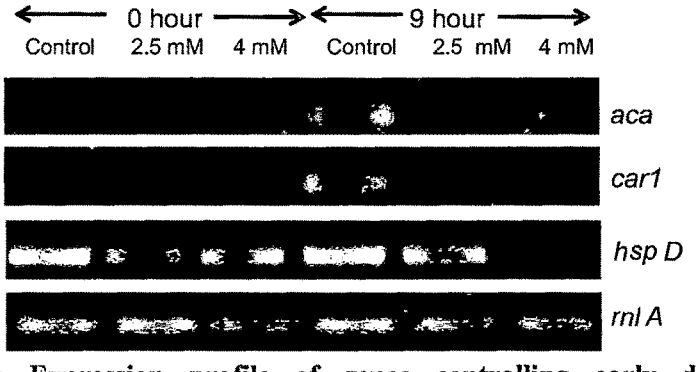

Figure 6.3: Expression profile of genes controlling early development in *D. discoideum*. Expression of cAMP receptor- *cAR*1 and adenylyl cyclase A- *aca*A were found to be decreased in HA treated cells. *hsp*D expression was unchanged. *rnl*A (mitochondrial rRNA IG7) was used as an internal control.

6.2.3. Effect of glutathione on oxidative stress induced *D. discoideum* development

Dose dependent effect of glutathione was observed on development of *D. discoideum* and 2 mM and higher doses of GSH showed arrested development at the aggregation

stage (previous lab studies). Hence for our further studies 1mM GSH was used to monitor the effect of glutathione on oxidative stress induced changes in development.

6.2.4. Continuous presence of glutathione on *D. discoideum* development

Cells were pre exposed with reduced GSH (1 mM) for 12 hours and then allowed to develop with addition of GSH on PBA to explore the role of ROS during *D. discoideum* development. We found 6 hours delay in development in GSH treated *D. discoideum* cells compared to control (Table 6.2).

Table 6.2: Effect of GSH on *D. discoideum* development. Continuous exposure of 1mM GSH delayed aggregation by 6 hours.

	LA (hr)	TA (hr)	SF (hr)	FBF (hr)	% FB
Control	**6**	**12**	**18**	**24**	**100**
GSH (1 mM)	12	16	22	28	90

LA: Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation; FB: Fruiting body.

6.2.5. Partial rescue of oxidative stress induced changes in growth by GSH

2.5 mM HA induced delay of lag phase was partially restored i.e. by 14 hours ($p<0.01$) in GSH pretreated cells, followed by a 12 hours delay ($p<0.01$) in the attainment of the stationary phase, as compared to HA stressed *D. discoideum* cells (Fig. 6.4).

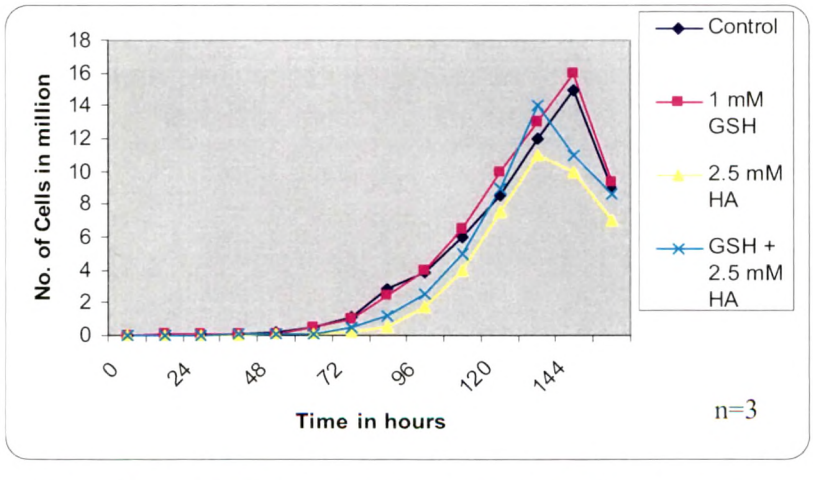

Figure 6.4: Effect of GSH on HA induced changes on *D. discoideum* growth. 1 mM GSH treatment rescued the delay in lag phase and log phase of 2.5 mM HA treated cells.

6.2.6. Rescue of oxidative stress induced developmental changes by GSH

Higher doses of HA either delayed or blocked the development (Fig. 6.2). The effect of GSH on oxidative stress (HA) induced developmental changes in *D. discoideum* was investigated. Our results (Table 6.2) suggest that GSH could partially restore HA induced developmental delay i.e. GSH rescued the HA induced delay at LD_{50} by 6 hours.

Table 6.3: Effect of GSH on HA induced delayed development of *D. discoideum*. 1 mM GSH partially restored the delay by 6 hours with 2.5 mM HA and development was observed with 4 mM HA by 42 hours.

	LA (hr)	**TA (hr)**	**SF (hr)**	**FBF (hr)**	**% CD**		**LA (hr)**	**TA (hr)**	**SF (hr)**	**FBF (hr)**	**% FB**	**% CD**
HA (mM)						**GSH (1mM) +HA (mM)**						
0.0	**6**	**12**	**18**	**24**	**1.0**	0.0	**6**	**12**	**18**	**24**	100	**1.0**
1.0	6	12	18	24	15	1.0	6	12	18	24	100	10
2.5 (LD_{50})	18	24	30	36	50	2.5	12	18	24	30	50	20
4.0 (LD_{90})	ND	ND	ND	ND	90	4.0	24	30	36	42	20	50

LA: Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation; CD: cell death; FB: Fruiting body; ND: No development till one week.

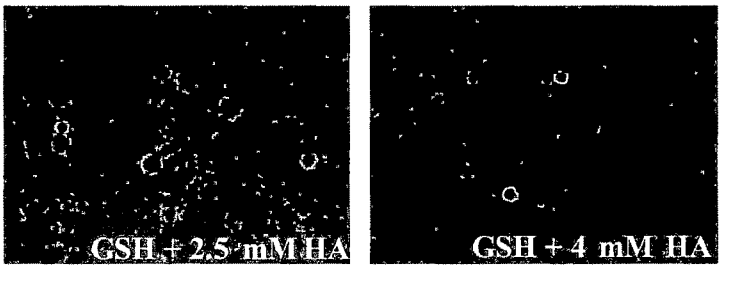

Figure 6.5: Effect of 1 mM GSH pretreatment on oxidative stress induced delay in development. 4 mM HA induced blocked development was restored by 1 mM GSH. Data are representative of three independent experiments. Photographs were taken with 4X objective.

While cells subjected to LD_{90} of HA did show arrested development; interestingly GSH pretreated cells exposed to LD_{90} of HA exhibited development delayed by 24 hours (Table 6.3). 4 mM HA upon pretreatment with GSH formed fruiting bodies within 48 hours. HA (LD_{50}) treated *D. discoideum* cells showed smaller fruits, which were restored by antioxidant pretreatment (Fig. 6.5).

6.2.7. PARP inhibition during oxidative stress induced growth changes in *D. discoideum*

PARP inhibition conferred protection against 2.5 mM HA induced delay in growth. The lag phase in benzamide pretreated cells was reduced from 60 to 50 hours, followed by 36 hours longer log phase (Fig. 6.1).

6.2.8. Role of PARP during *D. discoideum* development

The role of PARP in *D. discoideum* development was investigated by its inhibition with benzamide. Benzamide (1.0, 2.0 and 3.0 mM) did not show any effect on development. However, benzamide at 4 mM dose delayed the transition from tight aggregate (TA) to slug by 3-4 hours (Table 6.4). This suggests the likely role o PARP during development. Interestingly, 3.0 and 4.0 mM benzamide treated *D. discoideum* cells showed abnormal fruiting body with bigger size fruits.

Table 6.4: Effect of PARP inhibitor, benzamide on *D. discoideum* development. Table shows the effect of benzamide on *D. discoideum* development. Low doses of benzamide have no effect on development of *D. discoideum*.

Benz (mM)	LA (hr)	TA (hr)	SF (hr)	FBF (hr)	% CD	% FB
0.0	**6**	**12**	**18**	**24**	**1**	100
1.0	6	12	18	24	2	100
2.0	6	12	18	24	2	100
3.0	6	12	18	24	4	95
4.0	6	12	22	28	10	95

LA: Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation, CD: cell death; ND: No development till one week.

6.2.9. PARP involvement during oxidative stress induced developmental changes in *D. discoideum*

To determine the role of PARP on oxidative stress induced developmental changes, *D. discoideum* cells were exposed to benzamide (1 mM for 24 hours) prior to HA (LD_{15}, LD_{50} and LD_{90}) treatment, and subjected to development and the results are shown in Table 6.5. Benzamide pretreated cells upon exposure to high dose of HA (2.5 mM) exhibited development where the delay at the loose aggregation stage was restored from 18 to 12 hours (Table 6.5). The percentage of loose aggregates formed were also increased; while in case of LD_{90} delayed development could be observed in the presence of benzamide compared to developmental arrest of 4 mM HA treated cells. Fruiting bodies formed were very small with poor stalks and smaller fruits and also the fruits were few in number (Fig. 6.6).

Table 6.5: Effect of PARP inhibitor, benzamide on HA induced developmental changes in *D. discoideum*. The Table shows developmental stages of *D. discoideum* at different time intervals. 2.5 X 10^6 cells were treated with 2.5 mM and 4 mM HA for 1 hour, plated on non nutrient agar and observed at different time points. Pretreatment with benzamide exhibited partial rescue at both the doses of HA.

	LA (hr)	**TA (hr)**	**SF (hr)**	**FBF (hr)**	**% CD**	**% FB**		**LA (hr)**	**TA (hr)**	**SF (hr)**	**FBF (hr)**	**% CD**	**% FB**
HA mM							**Benz 1mM + HA**						
0.0	**6**	**12**	**18**	**24**	**1**	**100**	**0.0**	**6**	**12**	**18**	**24**	**1**	**100**
1.0	8	14	20	26	15	100	1.0	6	12	18	24	5	100
2.5	18	24	30	36	50	30	2.5	12	17	23	29	20	70
4.0	ND	ND	ND	ND	90	ND	4.0	18	24	30	36	40	20

LA: Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation, CD: cell death; ND: No development till one week.

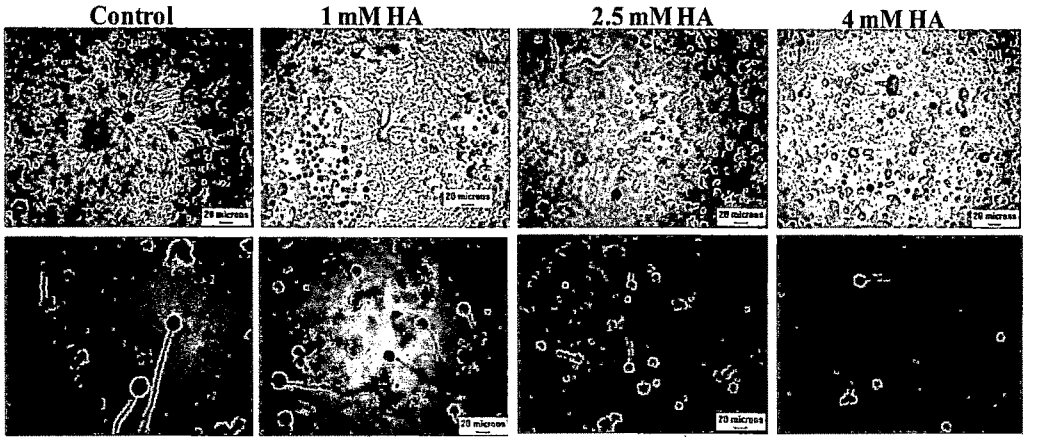

Figure 6.6: Effect of PARP inhibition during oxidative stress induced developmental changes in *D. discoideum*. Cells were preincubated with 1 mM benzamide for 24 hours, treated with HA and washed and plated at a density of 2 $X10^5$ cells/cm^2. Benzamide pretreatment partially restored the delayed development of 2.5 mM HA and rescued the developmental arrest of 4 mM HA treated cells. Upper panel indicates development at 6 hours. Fruiting body formation at different time intervals of the development of benzamide pre exposed HA treated cells are shown in lower panel. The size of fruiting body was smaller compared to that of control. Scale bar, 20um. Data is mean of 3 independent experiments performed in duplicates.

6.2.10. PARP inhibition restored spore revival delayed due to oxidative stress

To investigate the germination efficiency of spores and the fate of the germinated amoebae, spore revival experiment was attempted. Control and only benzamide treated spores revived within 108-120 hours, while the spores formed under 2.5 mM HA stress showed a significant delay i.e., ~ 56 hours ($p<0.001$) in spore revival. There was a partial rescue in the developmental delay i.e., ~ 32 hours ($p<0.012$) in the presence of benzamide. Spores formed from benzamide pre exposed and 4 mM HA treated cells revived after 60 hours compared to control (Fig. 6.7). To avoid ambiguity in the number of spores added in each flask, spores were picked up from at least four different areas and it was ensured that single spore was inoculated per ml of medium. Our results were also confirmed by counting the number of cells germinated from each spore microscopically and it was found to be the same. To DNA damage was not observed in second generation cells (Fig. 6.8)

Spore revival when reached to log phase, the cells were plated on PBA plates for development and 2.5 mM and 4 mM HA treated cells exhibited normal development.

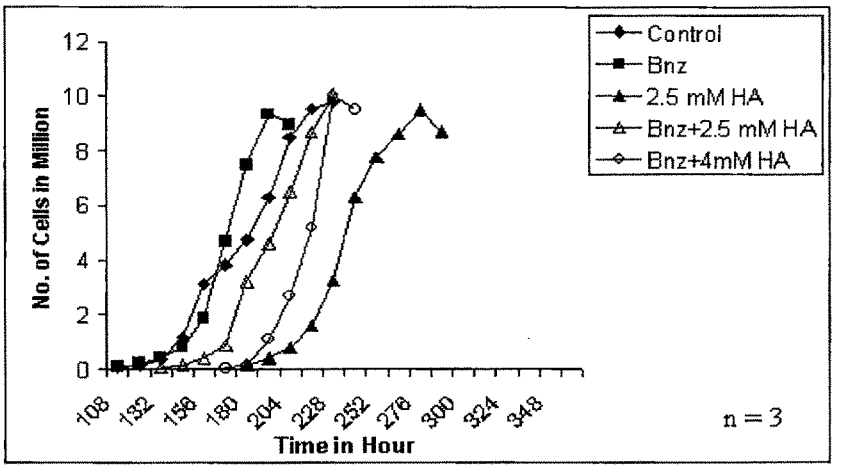

Figure 6.7: Effect of PARP inhibition on the fate of spores that were developed under oxidative stress. Spores of control cells revived within 108 hours whereas spores formed under oxidative stress (2.5 mM HA) exhibited 56 hours delay in spore revival, which was partially rescued by benzamide pretreatment. Spores formed from benzamide preexposed HA stressed (2.5 and 4 mM HA) cells revived earlier than only 2.5 mM HA stressed cells; 4mM HA treated cells showed no development and hence no spores. Data is mean of 3 independent experiments performed in duplicates.

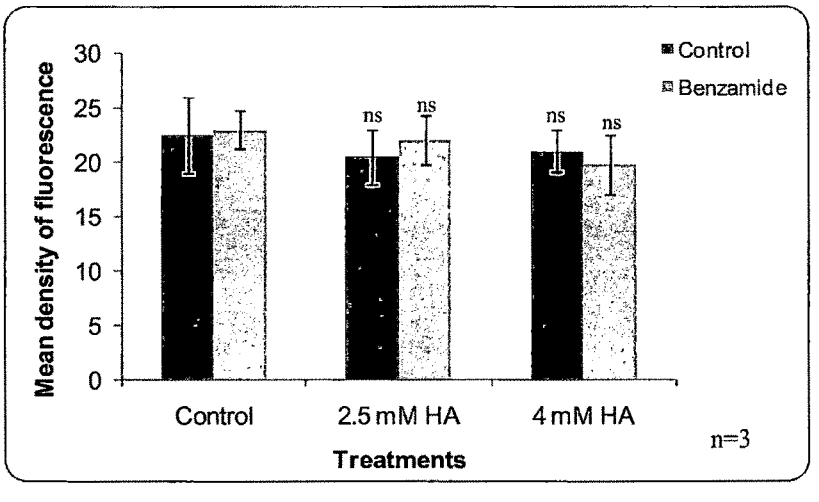

Figure 6.8: DNA damage monitored in second generation *D. discoideum* cells. No significant damage was observed in second generation cells.

6.2.11. Effect of cAMP on oxidative stress induced *D. discoideum* development

To explore whether the inefficiency of *D. discoideum* cells to secrete cAMP during oxidative stress caused the delay in development, it would be of interest to observe the effect of cAMP addition during development. 1 μM cAMP was added exogenously to HA stressed *D. discoideum* cells and incubated for 2.5 hours in 1X SB and then subjected to development. As can be seen from Table 6.6, 1 μM cAMP addition could partially restore oxidative stress induced developmental delay by 6 hours at LD_{50} of HA, however, exogenous cAMP could not rescue LD_{90} HA induced arrested development. A moderate increase in size of the fruiting body was observed in cAMP treated cells compared to those of HA stressed *D. discoideum* cells (Fig. 6.9). 1 μM cAMP addition also resulted in an increase in the number of fruiting body ~ 5-10 % at LD_{15} and LD_{50} of HA. 10 μM cAMP does not have any significant effect compared to 1 μM cAMP on developmental changes. Interestingly, 10 μM cAMP could restore the number of fruiting bodies at LD_{50} of HA by 20% (Table 6.6).

Table 6.6: Effect of cAMP on HA induced developmental changes in *D. discoideum*.
10 μM cAMP did not rescue blocked development with 4 mM HA.

	LA (hr)	**TA (hr)**	**SF (hr)**	**FBF (hr)**	**% FB**		**LA (hr)**	**TA (hr)**	**SF (hr)**	**FBF (hr)**	**% FB**
HA (mM)						HA + cAMP (1μM)					
0	**6**	**12**	**18**	**24**	**100**	**0.0**	**6**	**12**	**18**	**24**	**100**
1.0	6	12	18	24	80	1.0	6	12	22	28	90
2.5	18	24	30	36	50	2.5	12	18	24	30	60
4.0	ND	ND	ND	ND	ND	4.0	ND	ND	ND	ND	ND
2.5	18	24	30	36	50	HA + cAMP (10μM)	12	18	24	30	70

LA: Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation; FB: Fruiting body; ND: No development till one week.

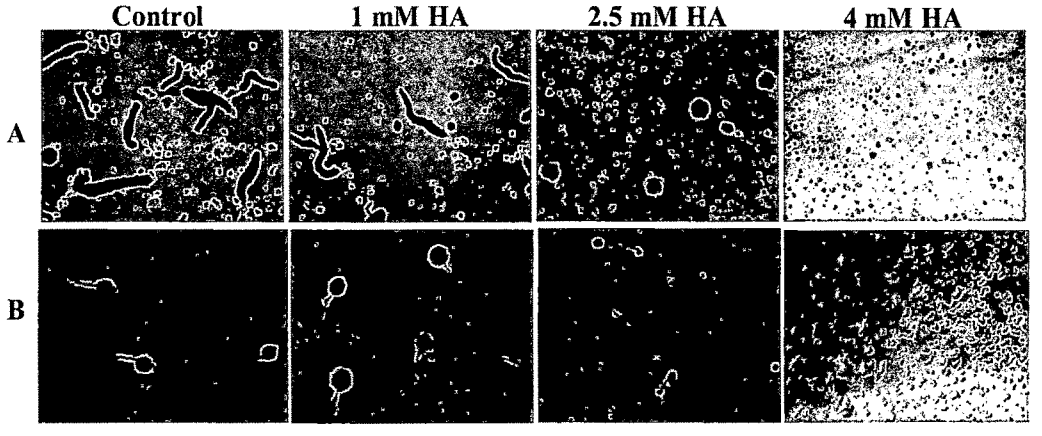

Figure 6.9: HA treated *D. discoideum* cells exhibit partial rescue in development upon exogenous addition of cAMP. (A) shows development at 18 hours of HA treated cells preincubated with cAMP (B) shows development at 30 hours of HA treated cells preincubated with cAMP where partial rescue by cAMP in the development of 2.5 mM HA treated cells while no effect of cAMP was seen on 4 mM HA treated cells.

6.2.12. cAMP levels in oxidative stress induced *D. discoideum*

As exogenous addition of cAMP partially restored the development it would be of interest to measure the cAMP levels in *D. discoideum*. After exposure to oxidative stress (HA) cells were suspended in buffer and extracellular cAMP was monitored in buffer after 6 hours. However, no change in cAMP levels was observed with HA treated cells (Fig. 6.10).

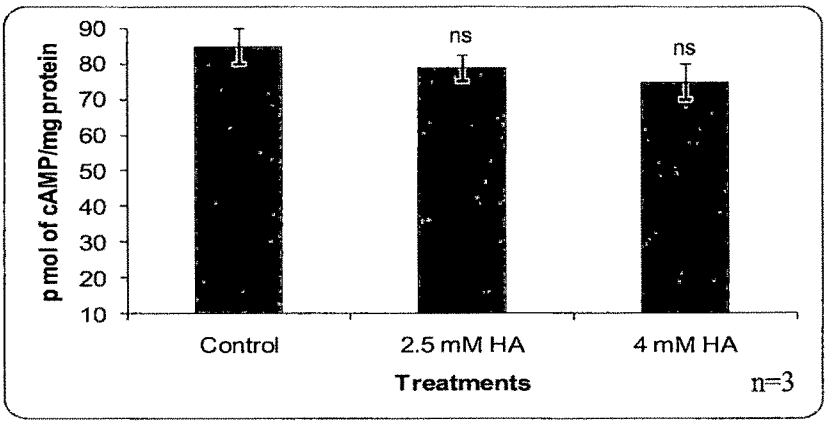

Figure 6.10: cAMP estimation in oxidative stress treated *D. discoideum*. cAMP levels were monitored in control cells and cells exposed to HA after 6 hours of starvation by ELISA method and no significant change were found during oxidative stress.

6.2.13. Nitric oxide levels during oxidative stress in *D. discoideum*:

During development *D. discoideum* cells produce nitric oxide, which was also postulated to act as a signaling molecule (Tao *et al.*, 1997). It has been reported that PARP activation is known to induce nitric oxide generation and bring about downstream effects (Virag and Szabo, 2002). To dissect out this possibility nitric oxide (NO) levels were estimated in supernatant of HA stressed cells after 6 hours of starvation, however no significant difference could be seen with different doses of HA compared to control (Table 6.7).

Table 6.7: Effect of oxidative stress on nitric oxide levels in *D. discoideum* cells. No change was observed in nitric oxide levels detected in supernatant of HA stressed *D. discoideum* cells.

Treatment	nmoles of NO per mg protein
Control	0.074
1 mM HA	0.077
2.5 mM HA	0.081
4 mM HA	0.075

6.3. DISCUSSION

Effect of oxidative stress on development

Among the eukaryotic organisms, the cellular slime mold *D. discoideum* is an excellent model system for studying cell death and developmental aspects (Escalante and Vicente, 2000). The ability of living cells to cope with various stresses is very crucial for maintaining the proper development. Reactive oxygen species are generally viewed as cytotoxic molecules. However ROS at lower concentrations have physiological functions and serve as second messengers in different signal transduction pathways (Firtel, 1991), while at higher concentrations exhibit DNA damage (Cooke *et al.*, 2003) among other cytotoxic effects. The redox status of the cell is a major regulator of a broad range of physiological and cellular functions.

Our results suggest that 2.5 mM HA delayed the development due to cell cycle arrest while at 4 mM HA dose due to 90% cell death, cell density was not sufficient for aggregation leading to complete developmental arrest (Table 6.1 & Fig. 6.2). Expression profiling of a few genes was done to assess the effect of HA on gene expression (Fig. 6.3). cAMP regulated genes, *car*1 and *aca*A were affected during oxidative stress which could be one possible reason for the delay in development.

Reduction of superoxide by pharmacological or genetic means leads to inhibition of aggregation (Blusch *et al.*, 1995). Cellular antioxidant system also affects the concentration of reactive oxygen species, and antioxidants are known to have crucial roles in the differentiation of *D. discoideum* (Kim *et al.*, 2005; Choi *et al.*, 2006). GSH is indispensable for the growth of *D. discoideum* and is related to cell type differentiation. *Glutathione synthase* null cells cannot differentiate into prespore cells and GSH is essential for differentiation into prespore cells (Kim *et al.*, 2005). It has been reported that glutathione (GSH) is essential for development of *Dictyostelium* (Kim *et al.*, 2005) and an increase in GSH levels leads to the inhibition of culmination (Choi *et al.*, 2006).

In our study, when the cells were preexposed to GSH (1 mM for 12 hours) and subjected to development, 6 hours delay was observed at the loose aggregation stage (Table 6.2). H_2O_2 may or may not be involved directly but this could be possibly due to conversion of superoxide to H_2O_2, generating superoxide scarcity resulting in delayed development. Thus, our results suggest that moderate levels of ROS are possibly acting as signaling molecules during *D. discoideum* development, whereas high levels of ROS (*in situ* generated H_2O_2 upon HA treatment) are toxic for development. Antioxidant pretreatment rescued *D. discoideum* cells from HA induced cell death and growth defects (Fig. 6.4), suggesting that HA is toxic to the cells *via* H_2O_2 generation. HA induced developmental delay at 2.5 mM dose while 4 mM dose blocked the development completely up to one week (Fig. 6.2). Oxidative stress induced developmental changes were partially rescued by antioxidant treatments. Upon GSH pretreatment 4 mM HA exhibited fruiting body by 48 hours (Table 6.3 and Fig. 6.5). Thus oxidative stress arrests the cell cycle and blocked the development while GSH could scavenge the ROS and thus partially restored the delayed/arrested growth and development.

Effect of PARP inhibition

PARP is known to play an important role under oxidative stress (Bakondi *et al.*, 2002), however, there is no report on the role of PARP in *D. discoideum* development. We have investigated the role of PARP in *D. discoideum* development by inhibiting its activity with the known PARP inhibitor, benzamide and studied its effects on normal development and oxidative stress induced developmental changes. Our results show that *D. discoideum* exhibits basal PARP activity and its inhibition by benzamide (1-3 mM) did not affect the development. However, benzamide (4 mM) treated *D. discoideum* cells were unable to differentiate properly (Table 6.4) and exhibited delayed development, especially at the differentiation stage of pre-stalk and pre-spore formation. These results suggest that lower doses of benzamide have no deleterious effects on *D. discoideum* development.

We have documented that HA induced oxidative stress activates PARP within five minutes and its role during oxidative stress is further confirmed by the use of low concentrations of benzamide (1 mM). Cells preincubated with benzamide prevented the peak activity observed during oxidative stress. Under oxidative stress partial inhibition of PARP activity led to altered growth suggesting that oxidative stress could be leading to cell cycle arrest (Shapira *et al.*, 2004) and PARP inhibition possibly overcomes this arrest (Fig. 6.1). PARP inhibition also rescued the oxidative stress induced delay in development (Table 6.5) though the size of fruiting body is smaller compared to that of control (Fig. 6.6). Thus our results not only suggest the presence of PARP in *D. discoideum* but also it's over activation under moderate to severe oxidative stress. Our present study is the first report on the role of PARP in *D. discoideum* development (Rajawat *et al.*, 2007).

PARP inhibitors are potential powerful cell protective agents that block cell death in response to oxidative stress and hence are used as therapeutic molecules to control oxidative stress related diseases (Virag and Szabo, 2002). However, the consequences of the blockade of cell death by PARP inhibitors in the long term cell survival are not entirely clear. In this context, we have studied the effect of PARP inhibition under oxidative stress on the next two generations by reviving the spores and monitoring growth and the doubling time. It was found that in normal cells PARP inhibition (1mM

benzamide) has no effect on spore germination. However, cells that are exposed to oxidative stress (2.5 mM HA) and allowed to develop, the spores remained dormant for longer time compared to control spores as the spores took more time (56 hours) to revive compared to control spores. Conversely, cells that are exposed to oxidative stress (2.5mM and 4 mM HA) with PARP inhibition and allowed to develop, the spores showed faster revival (32 hours and 60 hours) compared to cells exposed to oxidative stress alone (2.5 mM HA) as shown in fig. 6.7.

To observe the effect of oxidative stress and PARP inhibition on successive generation further development and DNA damage was monitored. Interestingly the amoebae of second generation thus formed due to spore revival (2.5 and 4 mM HA) exhibited normal development, suggesting that second generation cells have overcome the effect of oxidative stress. DNA damage was not detected in the second generation of *D. discoideum* cells (Fig. 6.8) suggesting that cells have repaired the damage completely.

Oxidative stress and cAMP

Multicellular development in *D. discoideum* is initiated upon starvation and is controlled by a number of extracellular signaling molecules including cAMP (Gomer *et al.*, 1991; Kimmel and Firtel, 1991; Schaap *et al.*, 1991). These molecules regulate the formation of the multicellular structure through chemotactic aggregation in response to extracellular cAMP and subsequent cell differentiation, differential gene expression, cell patterning and morphogenesis (Mehdy *et al.*, 1983; Devreotes, 1983; Siegert and Weijer, 1983; Berks and Kay, 1983; Kimmel and Firtel, 1991; Williams, 1991; Firtel, 1991; Traynor *et al.*, 2000). The cells with disrupted *acaA* did not secrete cAMP and were unable to chemotactically signal each other unless pulsed with extra cellular cAMP or genetically engineered to over express the catalytic subunit of the cAMP dependent protein kinase A (PKA) (Wang and Kuspa, 1997). Based on these reports, we proposed that HA might affect the initial cAMP secretion, and this was confirmed by monitoring the effect of exogenous addition of cAMP to HA stressed cells undergoing development (Fig. 6.9) as well as by monitoring the extracellular cAMP levels (Fig. 6.10). Interestingly, partial rescue did occur in HA induced delayed development upon cAMP addition. Exogenous cAMP treatment to the cells after HA treatment resulted in 6 hour restoration of HA (LD_{50}) induced delayed development (Table 6.6). However, 1 μM

cAMP treatment has no effect on the developmental delay at higher doses of HA (4 mM). Similar effect was observed with 10 μM cAMP. Surprisingly, expression of *aca*A was reduced but cAMP levels were unaffected with HA while addition of cAMP partially restored the development with 2.5 mM HA. As ACA and cAMP degrading enzyme PDE are expressed reciprocally (Bosgraaf and van Haastert, 2002), this could be the possible reason for the unchanged cAMP levels with HA stress.

Thus our results suggest that *D. discoideum* cells under HA stress could also exhibit cell cycle arrest as evident from the increased lag phase of the growth curve (Fig. 6.1) and hence showed delayed development. Hence cell cycle arrest leads to delay in development with 2.5 mM HA. cAMP addition thus partially restored the development with 2.5 mM HA but no effect was observed with 4 mM HA even with 10 μM cAMP. Apart from causing cell cycle arrest, PARP activation is known to induce nitric oxide generation and bring about downstream effects (Virag and Szabo, 2002). To dissect out this possibility we estimated the levels of nitric oxide (NO) in HA stressed cells, however no significant difference could be seen with different doses of HA (Table 6.7). Whereas UV-C induced changes in development at 60 seconds UV-C exposure blocked the development which was restored by cAMP addition. UV-C affects cAMP and NO levels in a dose dependent manner (unpublished data). UV-C has more prominent effect on cAMP signaling pathway compared to oxidative stress.

Results from our experiments show that HA induced oxidative stress leads to changes in the development of *D. discoideum* due to altered ROS levels and cAMP signaling. Alterations in cAMP signal transduction lead to changes in the chemotaxis and aggregation while PARP activation causes cell cycle arrest which may further influence a number of signaling pathways and thereby affects the development of *D. discoideum*. Thus we suggest that ROS levels regulate the differentiation and proper development of *D. discoideum*.

Thus, our results demonstrate that partial PARP inhibition under mild or severe oxidative stress did not affect repair of the damage incurred due to oxidative stress, as the amoebae formed upon spore germination exhibited normal growth and development for two successive generations. Our data support that PARP inhibition is beneficial under

oxidative stress and PARP inhibitors are potential powerful therapeutic molecules to control oxidative stress related diseases. This study also opens the possibility for identifying genes involved in *D. discoideum* spore dormancy under stress conditions. A part of this chapter has already been published (Rajawat *et al.*, 2007).

6.4. REFERENCES

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