Role of Poly {ADP-Ribose} Polymerase (PARP) during starvation induced cell death in *D. discoideum*

4.1. Introduction

ATP is required for many vital processes. Nutrient deprivation conditions where ATP levels are very low cause rapid metabolic collapse by way of disruption of various processes ranging from biochemical to physiological phenomena like dissociation of both ankyrin and fodrin from the actin cytoskeleton of a renal epithelial cell line- LLC-PK1 (Molitoris *et al.*, 1996). There is a hierarchy of responses of different energy consuming reactions to changes in ATP levels; pathways of macromolecules biosynthesis are most sensitive followed by sodium cycling and calcium cycling across the plasma membrane (Buttgereit and Brand, 1995). Chaos of ions boosts up the degrading activities of the cell and thereby causes cell death. Researchers reported that fall in the intracellular content of ATP is the immediate cause of tubular cell injury and death following ischemia (Siegel *et al.*, 1983; Stromski *et al.*, 1986; Venkatachalam *et al.*, 1988; Weinberg, 1991). Also studies with Madin-Darby canine kidney cells show that partial ATP depletion could lead to cleavage of PARP and apoptosis (Feldenberg *et al.*, 1999). Lieberthal *et al* (1998) demonstrate that acute depletion of ATP to the levels incompatible with maintenance of basal metabolism and activity of membrane transport pumps lead to necrosis, a process known to be "passive", whereas modest ATP depletion instead leads to apoptosis in mouse proximal tubular cells. Thus depleted ATP like other injurious cell death stimuli can lead to different cell death mechanisms depending upon the type of cells and the levels of ATP.

Cell death due to nutrient deprivation may be caused by oxidative stress. DNA damage, a signal for PARP activation, is an early outcome of oxidative stress. Till date ATP depletion has been considered as a consequence of PARP activation in DNA damage induced cell death. However, role of PARP in starvation induced cell death is unknown. Hence in this study role of PARP during starvation induced cell death has been addressed along with that of downstream effector enzymes like cathepsin D and calpain using specific inhibitors in *D. discoideum*.

4.2. Results

4.2.1 Characterization of starvation induced cell death

Cells were subjected to starvation using 1X SB (Sorenson's buffer) and cell death was monitored by trypan blue exclusion method every 2 hours till 24 hours and the results are shown in Figure 4.1. As can be seen from the figure there was significant decrease in the number of live cells in starving conditions compared to those in HL5 medium.

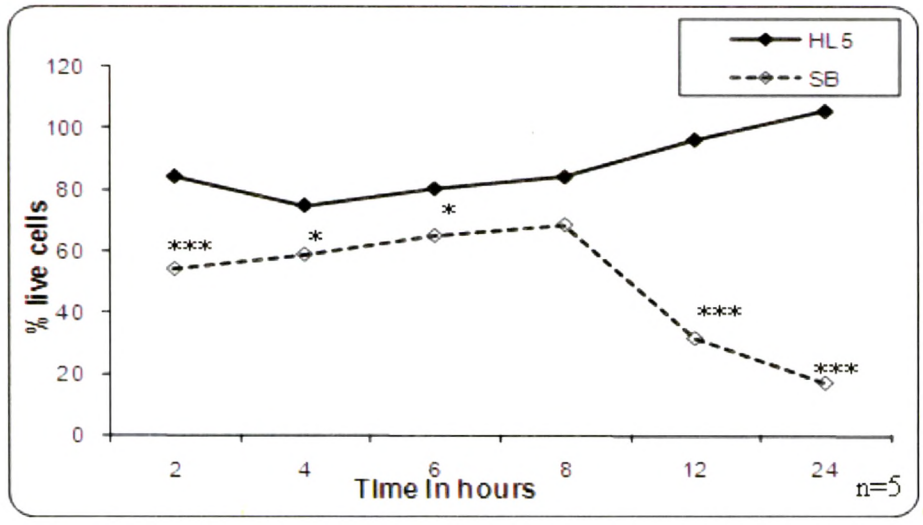


Figure 4.1: Starvation induced time dependent cell death as monitored by trypan blue exclusion method. Results are the mean ± SE of five independent experiments. * p value <0.05; *** p value <0.0001 as compared to respective HL5.

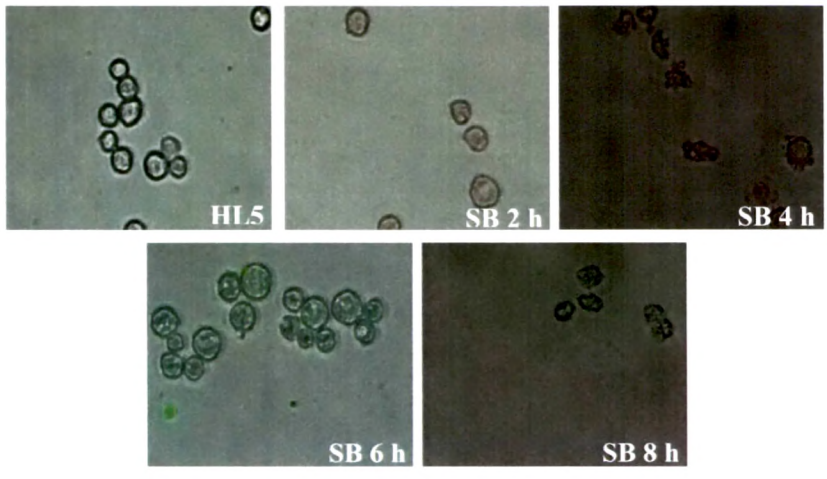


Figure 4.2: Annexin V staining of starving *D. discoideum* cells. Cell death is seen in two phases. In the first phase cells showed PS and PI staining together within 2 - 4 hours of starvation. However, in the later phase, PS exposure was seen at 6 hours while PI staining was seen at 8 hours. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

It was observed that under starvation *D. discoideum* cells underwent cell death in two phases. The mode of cell death was determined using PS-PI dual staining. As can be seen in Figure 4.2, in the early phase starved cells underwent necrotic cell death as PS exposure (Annexin V-FITC positive) as well as PI staining were observed simultaneously 2- 4 hours after starvation induction whereas, the later phase of cell death was paraptotic as PS exposure was observed 6 hours after starvation induction without PI staining. Nevertheless, PI staining was seen at 8 hours, indicating that the plasma membrane integrity was lost at 8 hours.

4.2.2. Levels of ATP and NAD^+ during starvation

PARP uses NAD^+ as the substrate; hence to elucidate the role of PARP during starvation in *D. discoideum* NAD^+ levels were monitored using enzyme cycling method and the results are shown in Figure 4.3. Pyridine levels of starving cells were maintained even after 1 hour of starvation induction. Reduced ATP levels indicate starvation condition (Fig. 4.4). Reduction in cellular ATP as induced by starvation may lead to a decline in functioning of ATP-dependent ion pumps on the plasma membrane. Perturbation of intracellular ion homeostasis can also result in mitochondrial dysfunction and diminish ATP production further deteriorating the cellular energy status (Zong & Thompson, 2006). Hence, mitochondrial membrane potential changes were studied in starving cells.

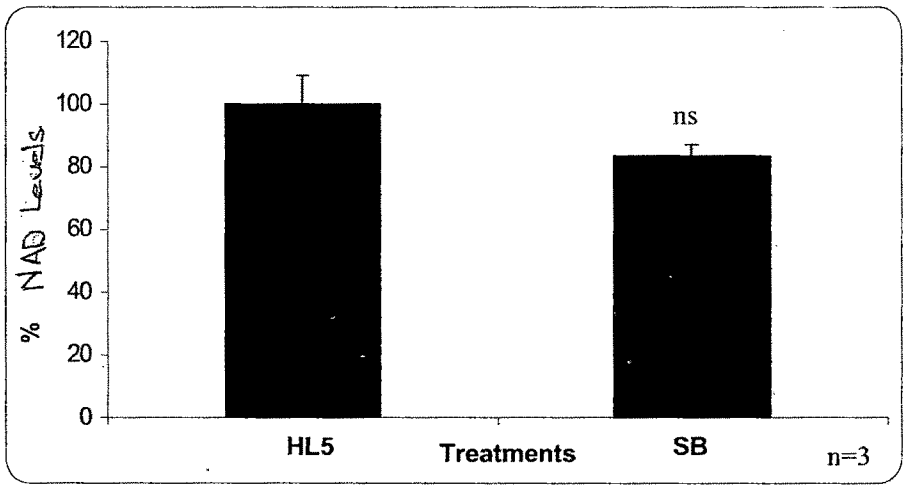


Figure 4.3: Starvation does not affect NAD^+ content of *D. discoideum* cells in an hour. Results are the mean of three independent experiments ± SE. Change in NAD levels was not significant in starving cells as compared to control cells.

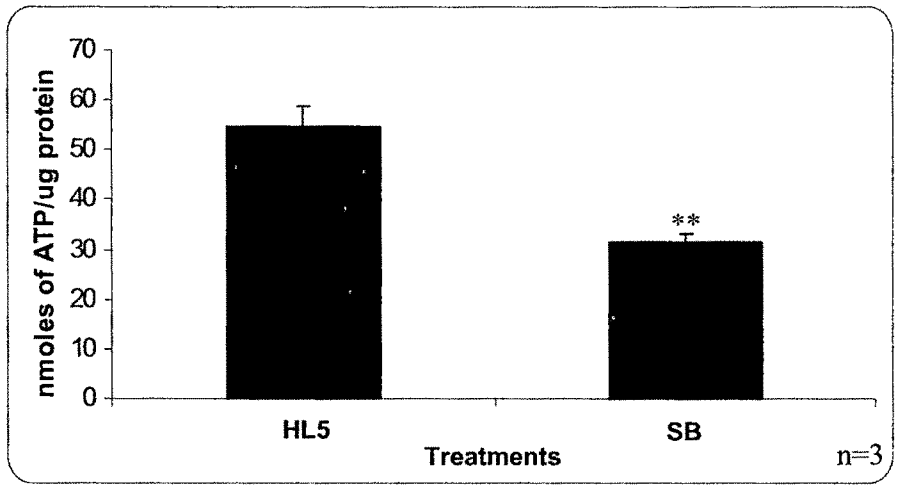


Figure 4.4: Starvation depletes ATP content of *D. discoideum* cells. Results are the mean of three independent experiments ± SE. ** p value <0.01 compared to control.

4.2.3. MMP changes during starvation

DiOC6 was used to evaluate changes in mitochondrial membrane potential (MMP) and the results are shown in Figure 4.5. MMP in starving cells showed significant decrease at 2 - 4 hours, whereas 6 hours after starvation MMP increased significantly in starving cells compared to control.

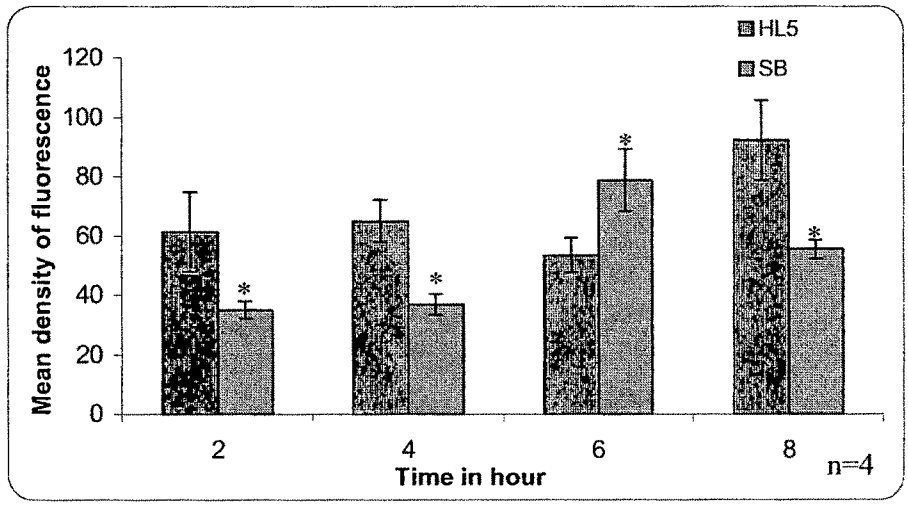


Figure 4.5: Densitometric analysis of time dependent changes in mitochondrial membrane potential during starvation. Significant decrease was found within 2 - 4 hours of starvation induction. MMP increased significantly 6 hours after starvation induction. Results are the mean of four independent experiments ± SE. * p value <0.05 compared to control.

4.2.4. ROS generation during starvation

ROS are known to be generated during starvation. Hence ROS levels were monitored in starving cells with and without pretreatment with an antioxidant such as glutathione (GSH). The amount of ROS produced was measured fluorimetrically with the DCFDA dye. Starving cells showed increased levels of intracellular ROS at 3 hours which were decreased in GSH pretreated cells (Fig. 4.6).

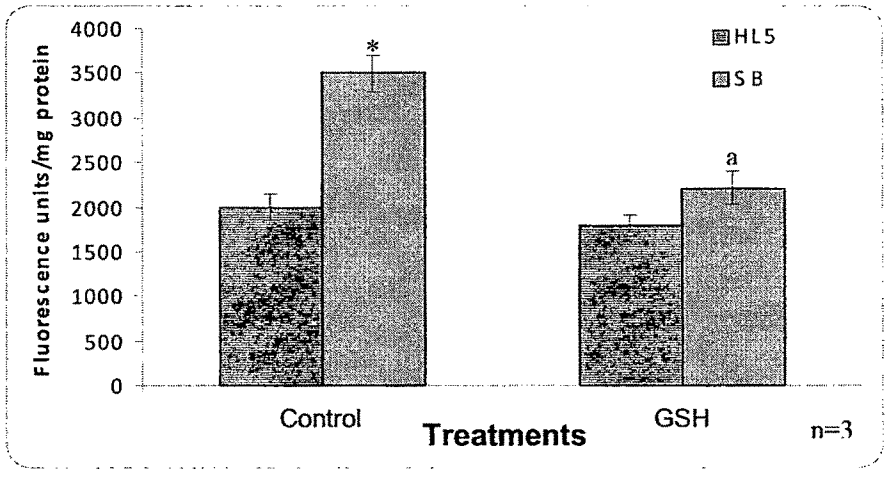


Figure 4.6: ROS is generated during starvation. Starving cells showed significant increase in the ROS levels as compared to control cells at 3 hours. GSH pretreatment showed significant reduction in the reactive oxygen species. Results are the mean ± SE of three independent experiments. * p value <0.05 as compared to respective HL5 and [a] p value <0.05 as compared to respective SB.

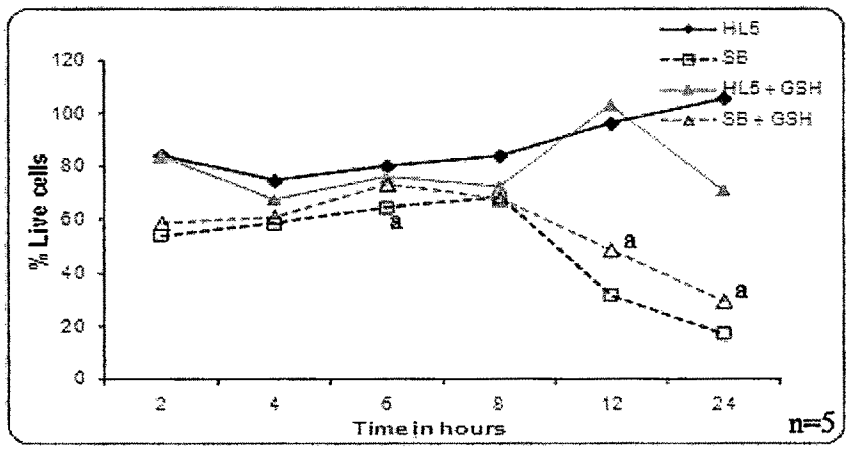


Figure 4.7: Time dependent effect of glutathione on starvation induced cell death as monitored by trypan blue exclusion method. GSH pretreatment rescued cell death after 4 hours. Results are the mean ± SE of five independent experiments. [a] p value <0.05 as compared to respective SB.

Protective effect of GSH was reflected in starvation induced cell death as monitored by trypan blue exclusion method. % live cells increased significantly after 4 hours in GSH pretreated cells compared to starved cells (Fig. 4.7).

4.2.5. DNA damage by immunofluorescence

Histone H2A is known to be phosphorylated during oxidative stress (Minami *et al.*, 2005). Hence presence of H2AX is an indicator of oxidative stress induced DNA damage. To further confirm ROS mediated DNA damage was monitored using immunofluorescence technique. Starving cells showed intense fluorescence corresponding to H2AX 3 hours after starvation induction compared to control cells (Fig. 4.8) indicating that starvation induced ROS leads to DNA damage as early as 3 hours.

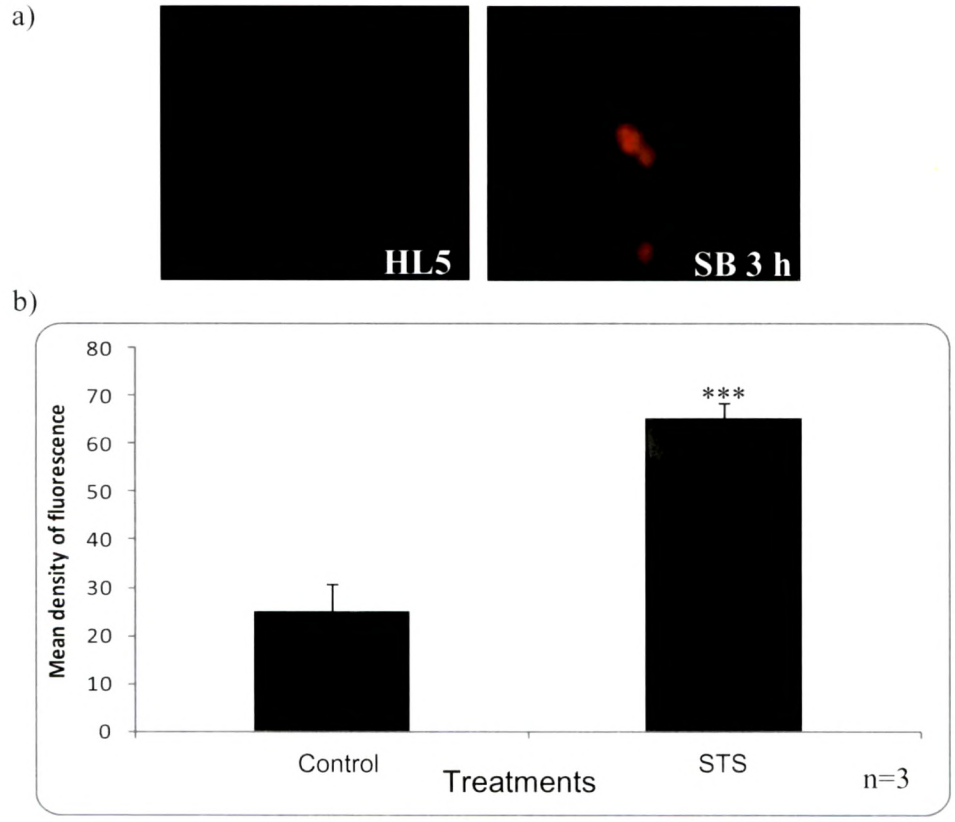


Figure 4.8: DNA damage during starvation. DNA damage was observed 3 hours post starvation induction by immunofluorescence using antibody against H2AX. a) Photographs were taken with 60X objective. b) Data (mean ± S.E.) are from four independent experiments. *** p value <0.01 as compared to respective SB.

4.2.6. Effect of PARP inhibition on starvation induced cell death

To study the role of PARP in starvation induced cell death, cells were preincubated with 1 mM benzamide 12 hours prior to starvation induction. Results of cell death as monitored by trypan blue exclusion method are shown in Figure 4.9. As can be seen from the Figure 4.9, benzamide pretreatment gave significant rescue from cell death at 6 and 8 hours after starvation induction. As can be seen from the Figure 4.10 at 6 hours of starvation, the cells exhibited PS exposure; however, benzamide pretreated cells were still PS negative.

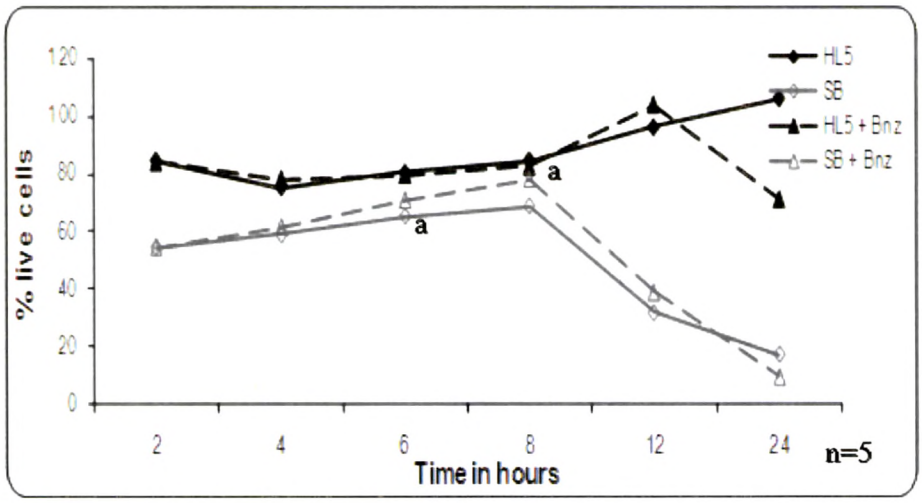


Figure 4.9: Time dependent effect of PARP inhibition by benzamide on starvation induced cell death by trypan blue exclusion method. Results are the mean ± SE of five independent experiments. [a] p value <0.05 as compared to respective SB.

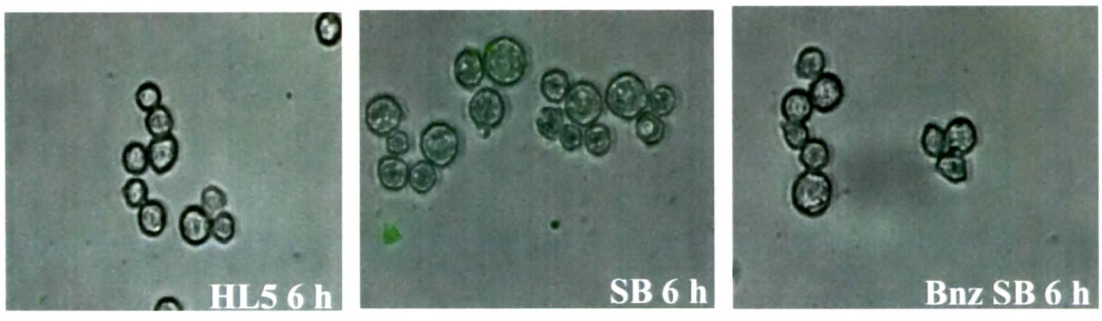


Figure 4.10: Annexin V staining of starving *D. discoideum* cells. Benzamide prevents the PS exposure seen during starvation at 6 hours. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

4.2.7. PARP inhibition affects NAD^+ depletion during starvation

1 hour after starvation cellular NAD^+ levels were not affected (Fig. 4.4). However, rescuing effect of PARP inhibition on starvation induced cell death indicates the involvement of PARP. PARP activation is known to lead to depletion of NAD^+ which was also found with starving *D. discoideum* cells. PARP inhibition partially restored depleted NAD^+ levels 4 hours after starvation (Fig 4.11) further supporting the trypan blue studies.

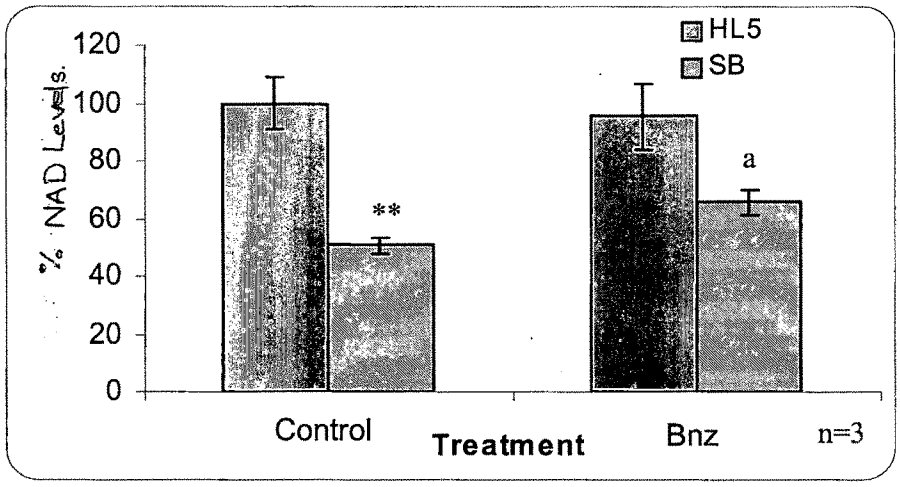


Figure 4.11: Starvation depletes NAD^+ content of *D. discoideum* cells by 4 hours in a PARP dependent manner. Results are the mean ± SE of three independent experiments. ** p value <0.01 compared to control; [a] p value <0.05 compared to respective SB.

4.2.8. MMP changes during starvation in context to PARP

Figure 4.12 a: Benzamide partially restores starvation induced MMP changes. Live cells accumulate the dye in mitochondria so fluorescence intensity was found to be high in control cells and decreased in starving cells. Benzamide partially restored the changes in MMP at 4 hours. Photographs were taken with 60X objective. Images are representative of four independent experiments.

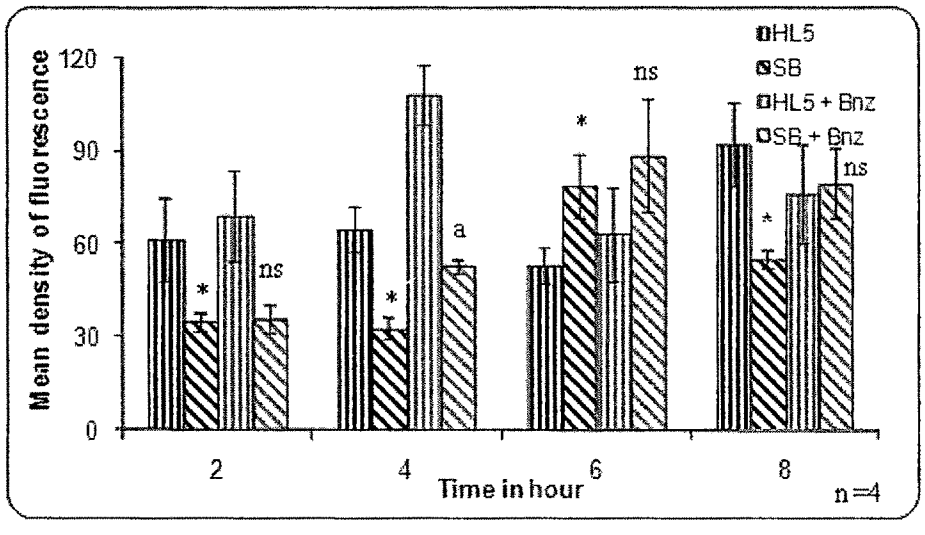


Figure 4.12 b: Starvation induced MMP changes were partially restored by benzamide. Benzamide partially restored the changes in MMP at 4 hours. Data (mean ± S.E.) are from four independent experiments. ns (non significant), * p value <0.01, compared to respective HL5; [a] p value <0.05 compared to respective SB.

As can be seen from Figures 4.12 a and b benzamide pretreatment could restore the starvation induced decrease in MMP at 4 hours.

4.2.9. Protease involvement in starvation induced cell death and development

To elucidate the molecular mechanism of starvation induced cell death protease inhibition studies were done. Inhibition of serine, cysteine, aspartic and metallo-proteases was done by cocktail of 4-{2-aminoethyl}benzenesulphonyl fluroride (AEBSF), pepstatin A, E-64 and 1,10-phenanthroline as a preliminary experiment. As can be seen from Figure 4.13 proteases involvement was observed in starvation induced cell death. Also the cells when preincubated with the protease inhibitors showed rescue in MMP changes at two time points namely 2 and 6 hours of starvation (Fig. 4.14 a and b).

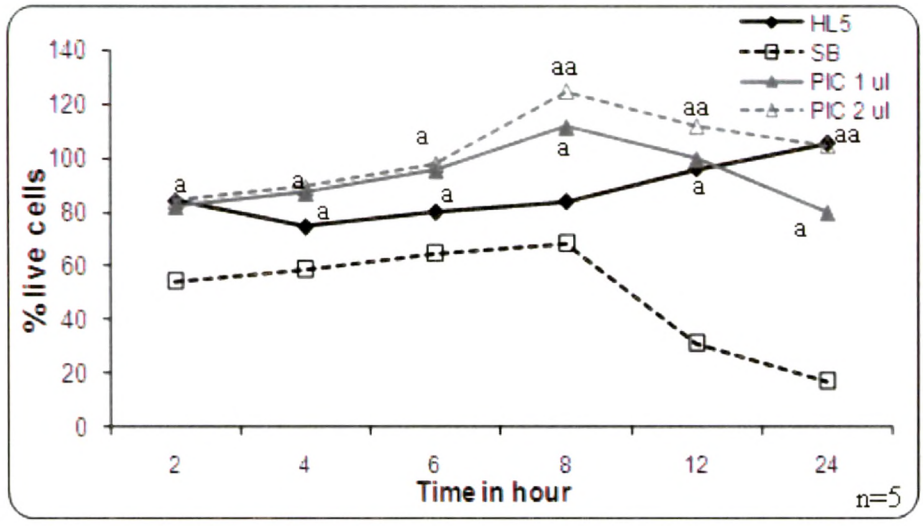


Figure 4.13: Time dependent effect of protease inhibitors on starvation induced cell death as monitored by trypan blue exclusion method. Results are the mean ± SE of five independent experiments. pretreatment with PIC rescued starvation induced cell death [a] p value <0.05 and [aa] p value <0.01 as compared to respective SB.

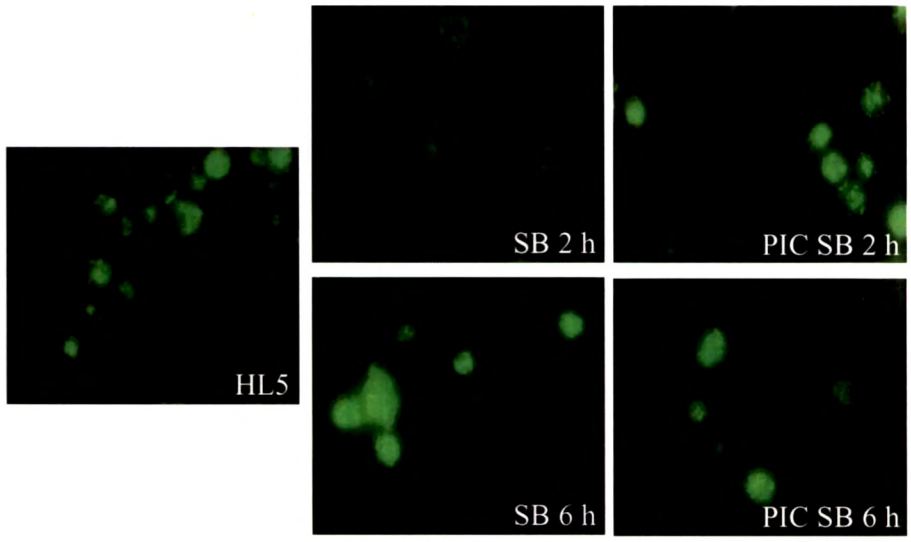


Figure 4.14 a: Starvation induced MMP changes were partially restored by protease inhibitors. Protease inhibitor cocktail partially restored the MMP at 2 and 6 hours. Photographs were taken with 60X objective and are representative of three independent experiments.

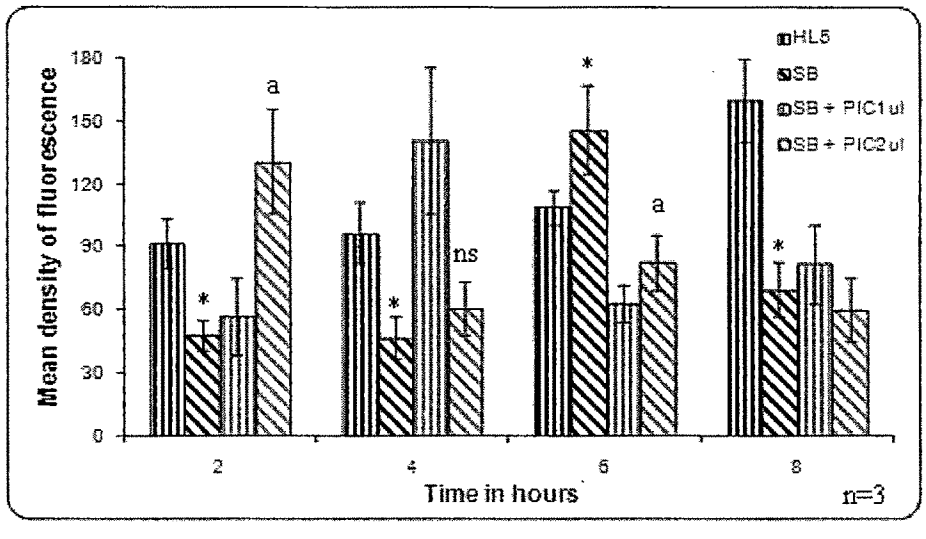


Figure 4.14 b: Starvation induced MMP changes were partially restored by protease inhibitors. Protease inhibitor cocktail partially restored the MMP at 2 and 6 hours. Data (mean ± S.E.) are from three independent experiments. ns (non significant), * p value <0.01, compared to respective HL5; [a] p value <0.05 compared to respective SB.

Probing the involved proteases further studies were done with pepstatin A and ALLN, cathepsin D and calpain inhibitors respectively. Cathepsin D is a lysosomal protease that is active at the cytosolic pH on the other hand there are reports that ATP depletion could lead to a rise in the cytosolic Ca^{2+} levels. Ca^{2+} overload contributes to activation of calpains, cysteine proteases (Yamashima, 2004; Lum *et al.*, 2005). These two proteases could be involved in starvation induced cell death.

Cathepsin D inhibition showed a significant rescue in cell death at 2, 4 and 12 hours of starvation (Fig. 4.15). As shown in Figure 4.16, cells at 2 hours of starvation were PI positive whereas at the same time point pepstatin A pretreated cells exhibited only PS exposure but no PI staining. Also at 4 hours starving cells were PI positive, whereas only ~50% of pepstatin A pretreated cells were both PS-PI positive and rest of them were only PS positive. These effects of pepstatin A on cell death were also reflected in its effect on MMP changes which were prevented significantly at 6 hours after starvation induction (Fig. 4.17 a & b).

With trypan blue exclusion assay it was observed that calpain inhibition with ALLN significantly reduced the cell death occurring at 2 and 12 hours after starvation induction (Fig. 4.18). Figure 4.19 (a and b) shows the effect of calpain inhibition on the mitochondrial membrane potential changes induced by starvation. Starving cells exhibited

decrease in MMP at 4 hours whereas cells treated with calpain inhibitor (ALLN) could prevent this reduction in MMP.

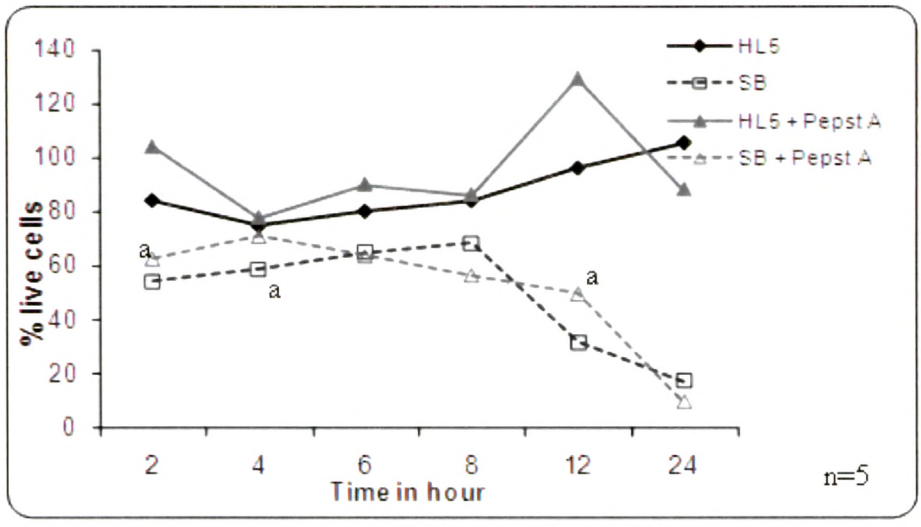


Figure 4.15: Time dependent effect of cathepsin D inhibition by Pepstatin A on starvation induced cell death as monitored by trypan blue exclusion method. Pepstatin A rescued cell death occurring at 2, 4 and 12 hours during starvation. Results are the mean ± SE of five independent experiments. [a] p value<0.05 as compared to respective SB.

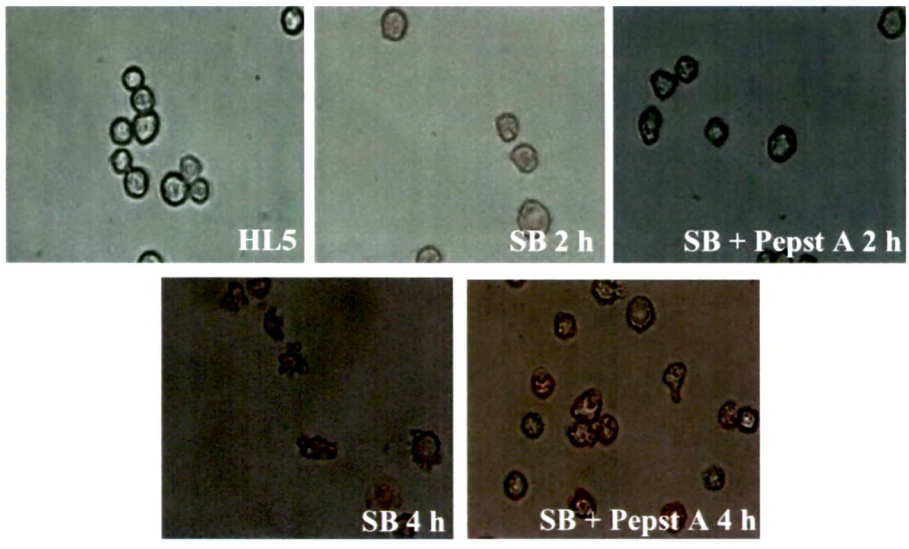
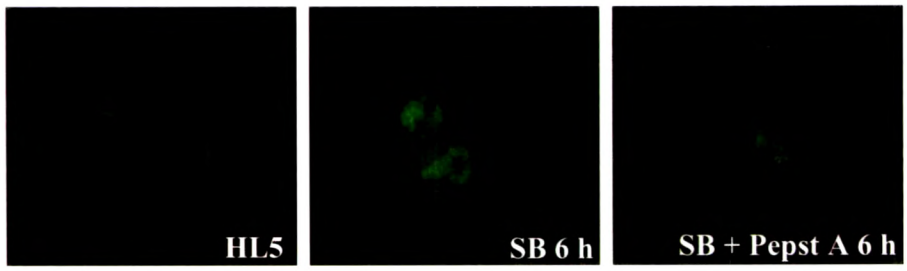


Figure 4.16: Annexin V-PI staining of starving *D. discoideum* cells in the presence of pepstatin A. Pepstatin A delays the PI staining and hence the loss of plasma membrane integrity. Starving cells in the presence of pepstatin A show only PS exposure at 2 hours and PI staining at 4 hours. Data are representative of three independent experiments. Photographs were taken with 60X objective.

a)



b)

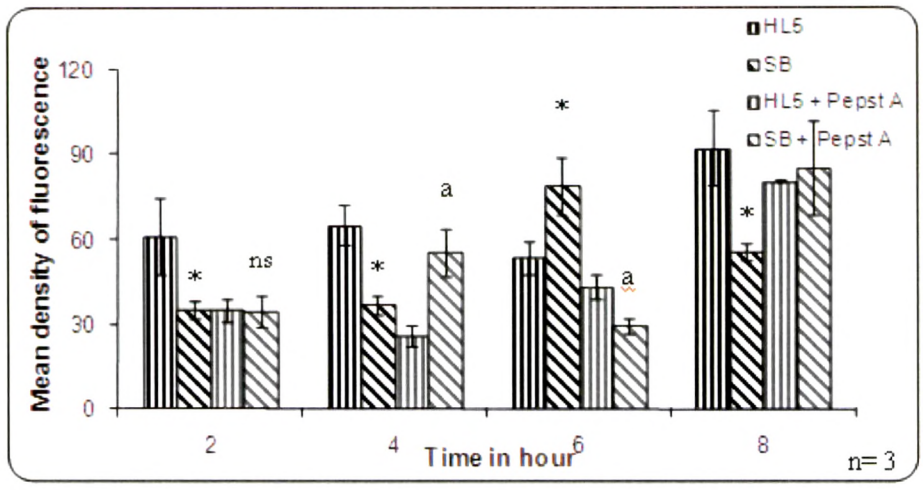


Figure 4.17: Starvation induced mitochondrial membrane potential changes were partially restored by cathepsin D inhibition. Pepstatin A partially restored the MMP at 6 hours. a) Photographs were taken with 60X objective. b) Data (mean ± S.E.) are from three independent experiments. ns (non significant), * p value <0.01, compared to respective HL5; [a] p value <0.01 compared to respective SB.

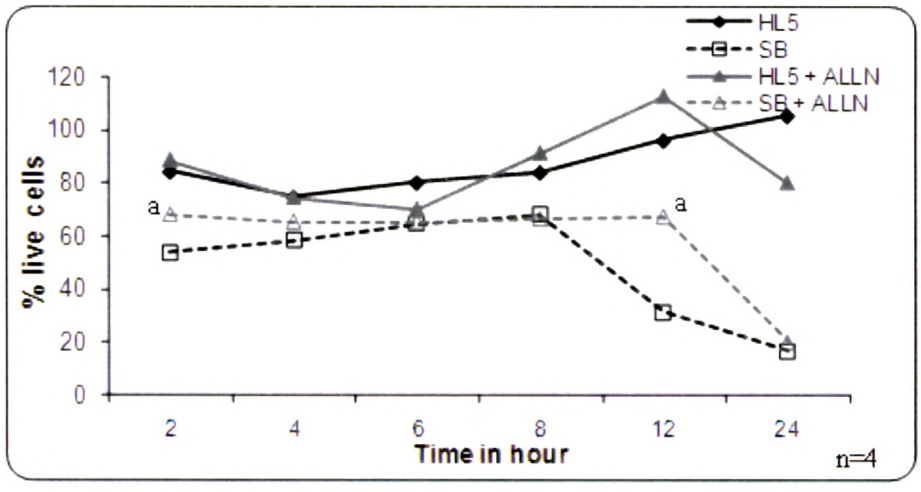
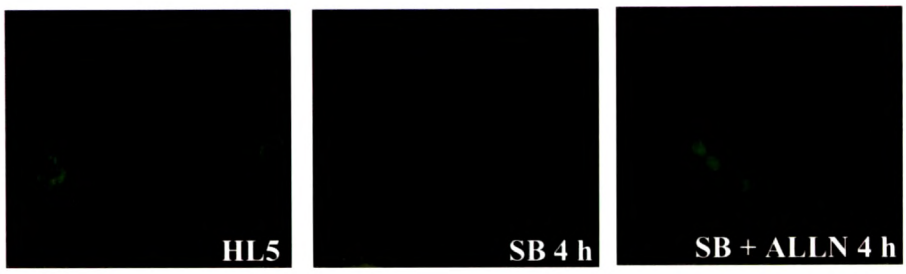


Figure 4.18: Time dependent effect of calpain inhibition by ALLN on starvation induced cell death as monitored by trypan blue exclusion method. ALLN significantly

rescued cell death occurring at 2 and 12 hours during starvation. Results are the mean ± SE of four independent experiments. [a] p value<0.05 as compared to respective SB.

a)



b)

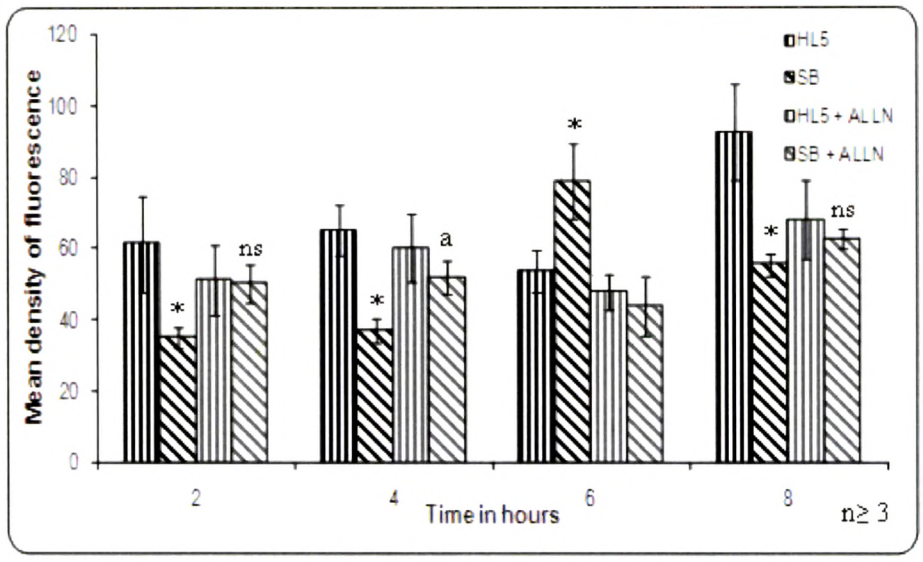


Figure 4.19: Starvation induced mitochondrial membrane potential changes were partially restored by calpain inhibition. ALLN partially restored the MMP at 4 hours. Data are representative of at least three independent experiments. a) Photographs were taken with 60X objective. b) ns (non significant), * p value <0.01, compared to respective HL5; [a] p value <0.05 compared to respective SB.

4.2.10. PARP inhibition and *D. discoideum* development

The role of PARP in development was also studied. It was observed that 1 mM benzamide, a dose sufficient to prevent the over activation of PARP during various other stresses, does not affect the development suggesting that *D. discoideum* development does not require PARP over activation.

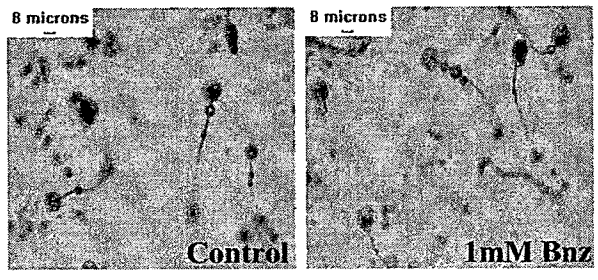


Figure 4.20: Development in *D. discoideum* cells in the presence of benzamide. Data are representative of six independent experiments. The photographs have been taken under 4X objective.

4.3. Discussion

Nutrient deprivation or growth factor withdrawal from *in vitro* cultured cells, leads to apoptotic cell death initiated by Bcl-2 family of proapoptotic proteins (Lindsten *et al.*, 2000; Rathmell *et al.*, 2000, 2003; Wei *et al.*, 2001). The cells die by necrosis when their intracellular supply of metabolic substrates depletes, unless nutrients and/or growth factors are restored to the culture medium (Lum *et al.*, 2005). Thus, nutrient deprivation could result in apoptosis as well as necrosis (Zong & Thompson, 2006). As a part of this study an attempt was made to study the role of PARP during starvation induced cell death in *D. discoideum*.

Studies with conditioned medium have shown that *D. discoideum* cells are capable of undergoing paraptotic cell death in vegetative form (Arnoult *et al.*, 2000). *D. discoideum* also exhibits paraptotic cell death during oxidative stress (Rajawat, 2010), UV-C irradiation and exposure to staurosporine. In this study we subjected *D. discoideum* cells to starvation by incubating the cells with SB instead of HL5 medium. These cells showed time dependent increase in cell death as shown in Figure 4.1. There was significant decrease in number of live cells in SB compared to the number of cells in HL5 medium.

4.3.1. *D. discoideum* cells show two modes of cell death under starvation

Extensive studies show that severe ATP depletion leads to necrosis; cells subjected to milder degree of ATP depletion undergo apoptosis (Lieberthal *et al.*, 1998). *D. discoideum* cells under starvation showed reduced ATP levels 1 hour after induction of

starvation (Fig. 4.3). The mode of death in starving cells was determined by PS-PI dual staining. It was observed that starving *D. discoideum* cells undergo cell death in two phases. In the early phase starved cells showed necrotic death whereas during later phase of starvation cells took up paraptotic mode of cell death (Fig. 4.2). As mentioned earlier necrosis results from a more severe stress condition compared to paraptosis which is a more modest and controlled form of cell death. Hence the results of PS-PI dual staining were intriguing as necrosis should have occurred later during starvation when the ATP depletion was sustained for longer duration and not in the early phase. At the same time the possibility of paraptosis being triggered earlier during starvation could not be negated. Also different cellular Ca^{2+} levels during different phases of cell cycle may influence the type of death taken up by the cell differently (Kahl and Means, 2003). In other words, calcium is a key regulator of mitochondrial function and acts at several levels to stimulate ATP synthesis. Hence the differences in the cellular Ca^{2+} levels could underlie the differences in the sensitivity of cells to depletion of ATP; hence different types of cell death may be observed within a cell culture.

Depleted levels of ATP perturb the ion homeostasis of the cells *via* its effect on Na^{+} and Ca^{2+} ion cycling (Buttgereit and Brand, 1995). Resulting Ca^{2+} overload can lead to enhanced generation of reactive oxygen species by affecting complexes I and IV and hence causing mitochondrial dysfunctioning (Brookes *et al.*, 2004; Zong & Thompson, 2006). Starving *D. discoideum* cells with depleted ATP levels also showed ROS generation which could be scavenged by GSH (Fig. 4.6). Starvation induced cell death could be reduced significantly by GSH (Fig. 4.7). ROS once generated could affect mitochondrial functioning and *vice versa*. Starvation leads to changes in MMP (at 2, 4 and 6 hours) at different time points (Fig. 4.5). Increase in MMP of starving cells at 6 hours could be due to shift of cellular respiration from state 3 to state 4 as observed in case of reduced cellular ATP in Hela cells (Cipriani *et al.*, 2005). These changes in MMP at different time points under starvation may be accounted by the two phases of cell death taken up by the starving cells.

4.3.2. Second phase of starvation induced cell death requires PARP

ROS apart from leading to other deleterious effects may also cause damage to DNA. Using immunofluorescence technique probing phosphorylation of histone it could be seen that starving *D. discoideum* cells have oxidatively damaged DNA (Fig. 4.8). PARP activation is one of the immediate responses to damaged DNA. To explore the role

of PARP in starvation induced cell death benzamide was used. PARP inhibition had no significant effect on cell death during the first 4 hours of starvation. However, inhibition of PARP was found to be protective for starving cells at 5-6 hours after starvation induction (Fig. 4.9). PS exposure and PI staining results substantiate this observation (Fig. 4.10). Also PARP inhibition rescues the change in MMP at 4 hours (Fig. 4.12 a & b). Starving cells do not show significant change in the levels of NAD^+ 1 hour after starvation (Fig. 4.4) however 4 hours after starvation the reduction in NAD^+ levels was found to be PARP dependent (Fig. 4.11). These results together imply that PARP may not be getting activated during initial hours of starvation however, it is important for the execution of starvation induced cell death occurring during late hours (5-6 hours).

4.3.3. Starvation induced development is independent of PARP activation

As 1 mM benzamide, a dose sufficient to prevent the over activation of PARP (Fig. 3.5), does not affect the development (Fig. 4.13) suggesting that *D. discoideum* development does not involve PARP over activation. However, *D. discoideum* development may require basal PARP activity which may be confirmed in PARP deficient strains (Rajawat, 2010).

4.3.4. Protease involvement in starvation induced cell death and development

Caspases are known to be absent in *D. discoideum.* Hence other proteases are involved in cell death of this organism. Preliminary studies (Figs. 4.14 and 4.15) with protease inhibitor cocktail showed that certain serine and cysteine proteases serve as surrogate caspases in *D. discoideum.* Also rescue in the MMP changes implicates that protease activation precedes mitochondrial changes during starvation.

Lysosomes are the cellular abode for zymogen forms of proteases that get activated only at acidic pH. However, cathepsin D is a lysosomal protease that is known to be active at the cytosolic pH and could serve as the protease involved in starvation induced cell death. Hence experiments were done using cathepsin D inhibitor pepstatin A. Cathepsin D inhibition showed a partial rescue in cell death (Fig. 4.16). This was also reflected in its effect on MMP changes which were prevented significantly at 4 and 6 hours of starvation (Figs. 4.18 a & b). Starving cells in the presence of pepstatin A showed only PS exposure at 2 hours and PI staining at 4 hours implying that inhibition of cathepsin D shifts the necrotic mode of cell death to paraptotic mode (Fig. 4.17). It has been reported that ATP depletion affects the ATP dependent Ca^{2+} pumps thereby leads to

a rise in the cytosolic Ca^{2+} levels. Calpains activated due to Ca^{2+} overload contribute to the lysosomal membrane permeability (LMP), which can lead to release of lysosomal enzymes including cathepsins and ultimately results in necrotic cell death (Yamashima, 2004; Lum *et al.*, 2005). Trypan blue studies (Fig. 4.19) and MMP studies (Fig. 4.20) of starving cells with calpain inhibitor, ALLN imply the role of calpains in starvation induced cell death. Hence, protection observed with protease inhibitors could be attributed to either calpains and/or lysosomal proteases, as caspases are absent in *D. discoideum* (Bouffay *et al.*, 2004). Based on our results following pathway is proposed for the role of PARP and the proteases during starvation induced cell death (Fig. 4.21).

Starvation in *D. discoideum* is well known to trigger a complex signaling ultimately leading to development of a fruiting body. This aspect has been discussed in more detail in chapter 1. Results presented in this study highlight that starvation in vegetative *D. discoideum* cells induces two phases of cell death i.e. initially necrosis and followed by paraptosis. Interestingly PARP is involved in the second phase of cell death i.e. paraptosis. Starvation induced cell death employs an array of enzymes, and lysosomes and mitochondria also contribute in different ways ultimately causing the starving cells to die.

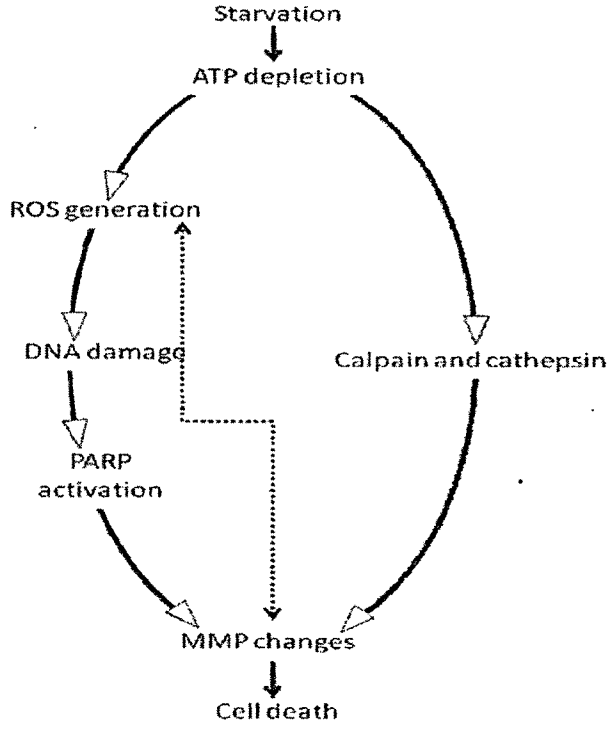


Figure 4.21: Proposed pathway for starvation induced cell death

4.1. References

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