PARP in staurosporine induced cell death in *D. discoideum*

5.1. Introduction

Staurosporine (STS) is a bacterial alkaloid obtained from *Streptomyces staurosporeus*. It possesses inhibitory activity against fungi and yeast. It is a cell permeable inhibitor of protein kinases. STS is widely used in cell death studies, and it induces cell death in many mammalian cell types by both caspase dependent and independent apoptotic pathways. STS induced caspase independent cell death mimics mitochondrial death pathway in which Bax and Bak play key roles. It causes limited lysosomal destabilization and thereby releases several cathepsins - B, D and L into the cytosol; amongst this cathepsin D, an aspartate protease is able to act at neutral pH. This is because the active conformation of cathepsin D is stabilised by binding with target cytosolic proteins. Cytosolic cathepsin D via Bax activation causes the release of AIF from mitochondria. Once released in to cytosol, AIF translocates to nucleus where it brings about large scale DNA fragmentation and ultimately paraptotic cell death (Bidere *et al.*, 2003).

The cell death pathway initiated by PARP activation is mediated by AIF. PARP activators, including the DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine, hydrogen peroxide (H_2O_2) and NMDA induce the translocation of AIF to nucleus, along with other mitochondrial changes and ultimately cell death (Rajawat, 2010; Yu *et al.*, 2002, 2003). As mentioned earlier STS induced cell death is well understood to be mediated by AIF, however the role of PARP and its contribution in this AIF mediated cell death has not been well explored. This study is an attempt to explore the role of PARP in STS induced AIF mediated cell death.

5.2. Results

5.2.1. Induction of cell death by staurosporine

Dose dependent effect of staurosporine (STS) on cell death was studied. It can be observed (Fig. 5.1) that as the concentration of STS increases from 100 to 500 nM % live decrease. This trend continues till 8 hours with significant decrease in cell number at 2

hours with 500 nM STS. Thus, for further experiments a lower dose of 400 nM was selected.

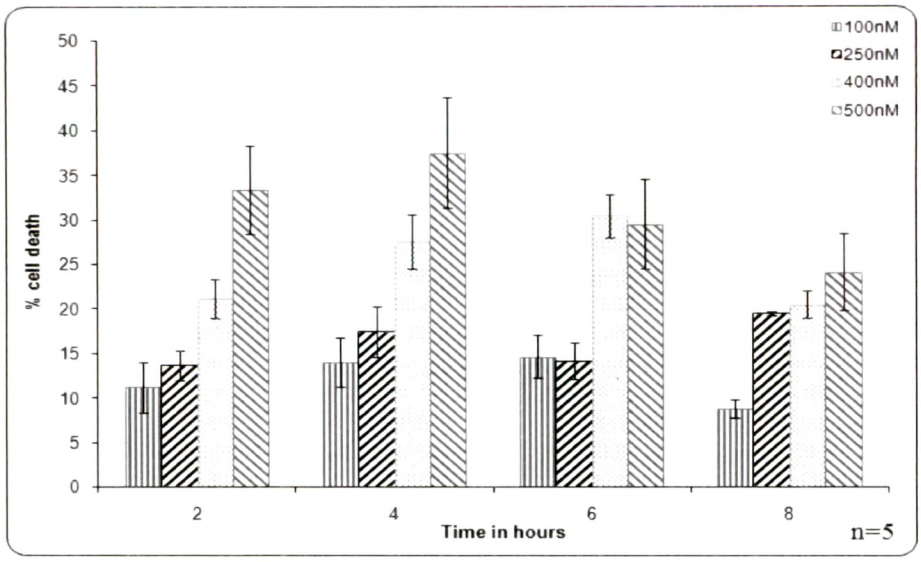


Figure 5.1: Dose and time dependent cell death induced by STS in *D. discoideum* cells as monitored by trypan blue exclusion method. Results are the mean ± SE of five independent experiments.

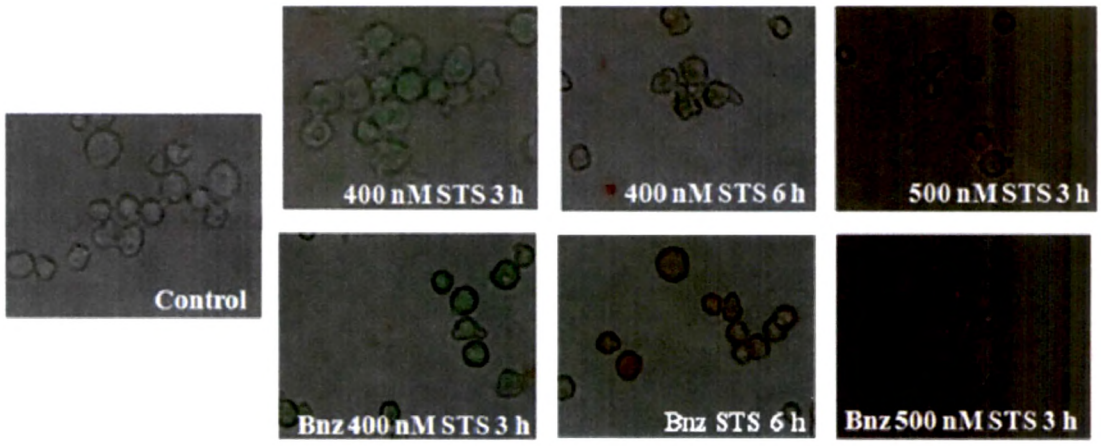


Figure 5.2: Annexin V staining of STS treated *D. discoideum* cells. PS exposure is seen at 3 hours while PI staining at 6 hours with 400 nM STS. 500 nM STS was found to be necrotic as both AnnexinV-FITC and PI staining were observed at 3 hours. Benzamide did not affect STS induced cell death. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

In 400 nM STS treated cells, PS exposure was obtained at 3 hours while PI staining at 6 hours (Fig 5.2). Benzamide pretreated cells showed PS-PI staining at 3 & 6 hours respectively.

5.2.2. Mitochondrial changes induced by STS

400 nM STS led to significant changes in MMP indicated by less accumulation of the dye at 3 hours (Figs. 5.3 a and b). Cells treated with 500 nM STS exhibited increased MMP 1 hour after stress which later declined by 3 hours (Figs. 5.3 a and b). Benzamide had no effect (Figs. 5.3 a and c) on STS induced MMP changes.

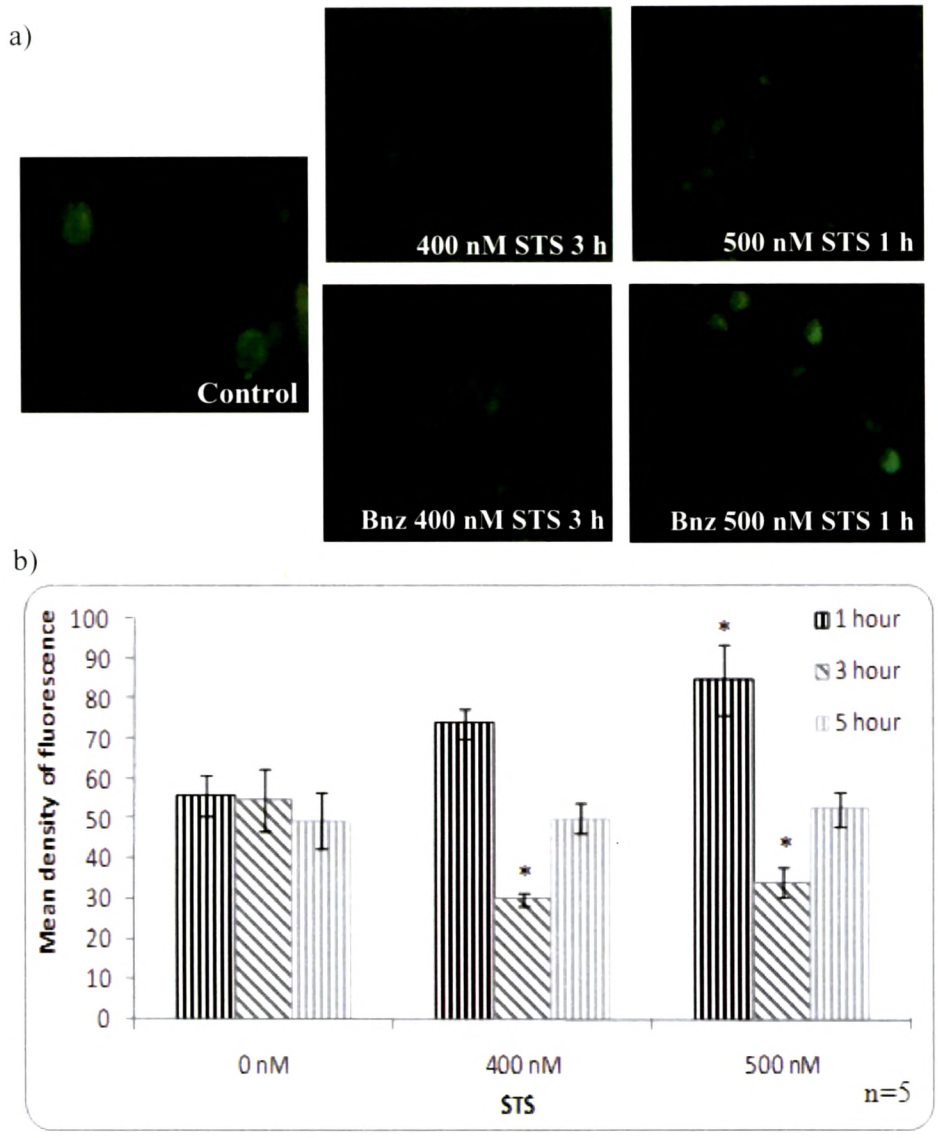


Figure 5.3a and b: Mitochondrial membrane potential changes induced by STS. Fluorescence intensity was found to change with STS. Benzamide did not affect STS induced MMP changes. a) Photographs were taken with 60X objective. b) Densitometric analysis of time dependent changes in mitochondrial membrane potential after STS. Significant increase at 1 hour with necrotic dose was observed. MMP decreased significantly in both paraptotic and necrotic cell 3 hours after STS treatment. Results are the mean of five independent experiments ± SE. *p value <0.05 compared to respective time point of 0 nM STS.

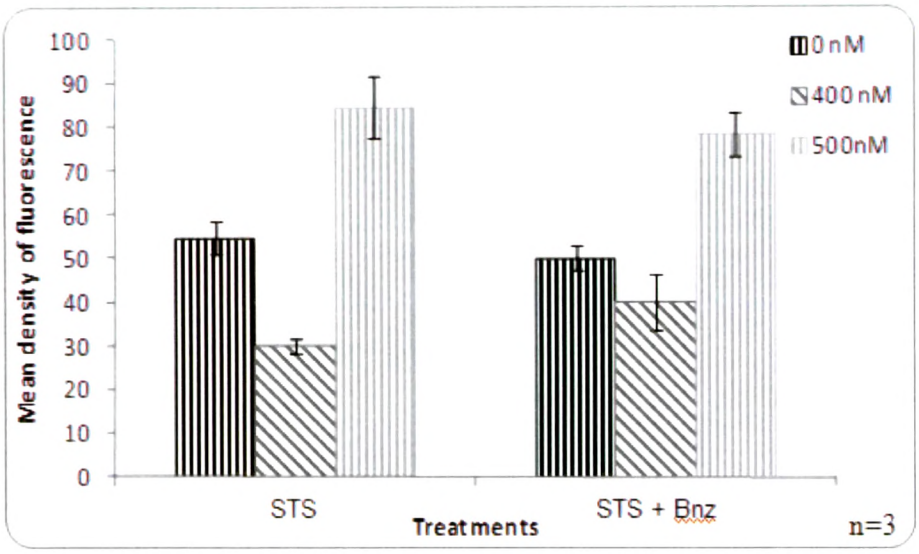


Figure 5.3 c: Mitochondrial membrane potential changes induced by STS were not rescued by benzamide. MMP change with 400 nM STS at 3 hours and 500 nM STS at 1 hour were not affected by benzamide. Data (mean ± S.E.) are from three independent experiments.

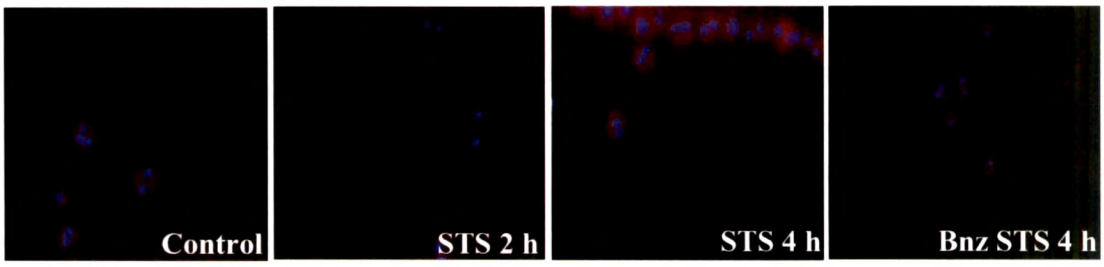


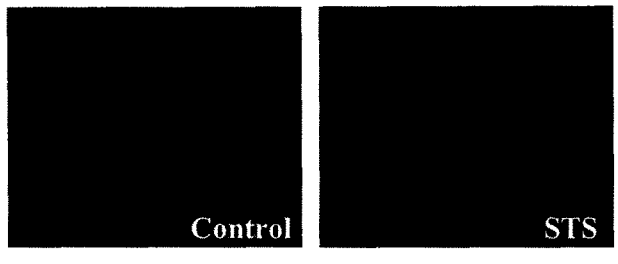
Figure 5.4: Fluorescence microscopy for the mitochondria-nuclear translocation of AIF at different time points after STS treatment. Nucleus of control cells stained blue due to DAPI indicating AIF is localized in mitochondria. AIF translocation to nucleus was observed at 4 hours of STS treatment as seen by cells showing pink fluorescence. Pretreatment with pepstatin A prevented translocation of AIF to nucleus while benzamide failed to do so. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

These effects of STS stress and benzamide on changes in MMP were also reflected their effect on AIF release. During STS induced cell death AIF release was observed at different time points with paraptotic and necrotic doses. With 400 nM STS translocation was seen at 4 hours (Fig. 5.4). Inhibition of PARP activity by benzamide did not intercept this AIF release during paraptosis.

5.2.3. PARP acitivity and NAD levels during STS induced cell death

To study the kinetics of PARP activation PARP activity was assayed at various time points (5, 10 and 15 minutes) post STS treatment and the results are shown in Figures 5.5 a and b. Increase in PARP activity could not be observed after STS treatment as compared to control cells.

a)



b)

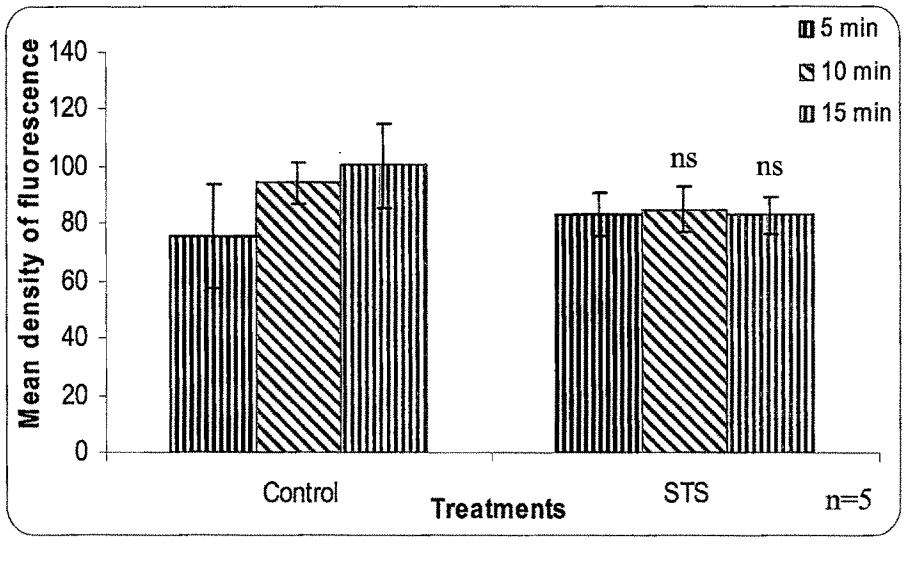


Figure 5.5a and b: PARP activity assay by indirect immunofluorescence. PARP activity was measured at different time points post STS stress. Under control conditions basal PARP activity was observed. No significant change in PAR immunoreactivity was seen under STS stress. a) Photographs were taken with 60X objective. b) Densitometric analysis of PARP activity. No significant increase was observed in PARylation. Data (mean ± S.E.) are from five independent experiments.

Consequent to PARP activation cellular NAD^+ pools deplete. NAD^+ levels were assayed 2 hours after STS treatment and the results are shown in Figs. 5.6. NAD^+ levels in STS treated cells were unchanged compared to control cells. These results support the PARP assay results.

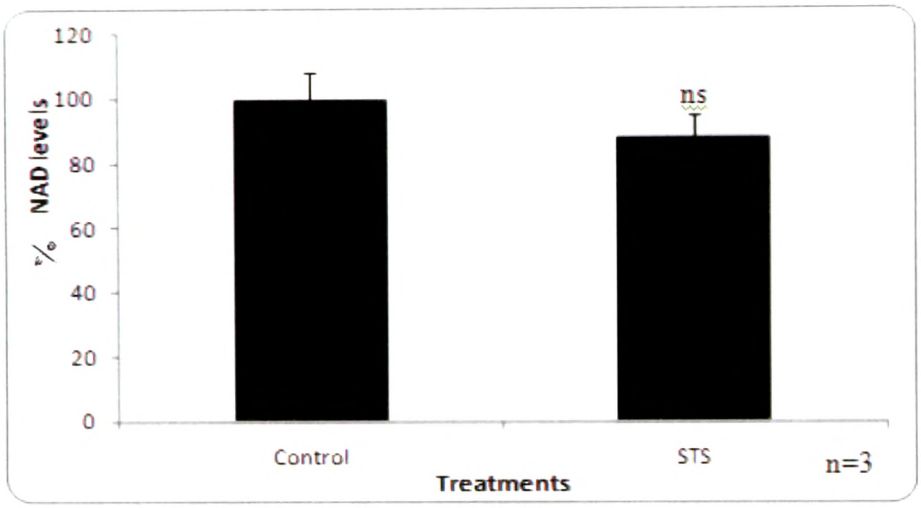


Figure 5.6: STS treatment does not affect NAD^+ content of *D. discoideum* cells. Data (mean ± S.E.) are from three independent experiments. No significant difference was observed in NAD^+ levels of STS treated cells and control cells.

5.2.4. Effects of STS on DNA

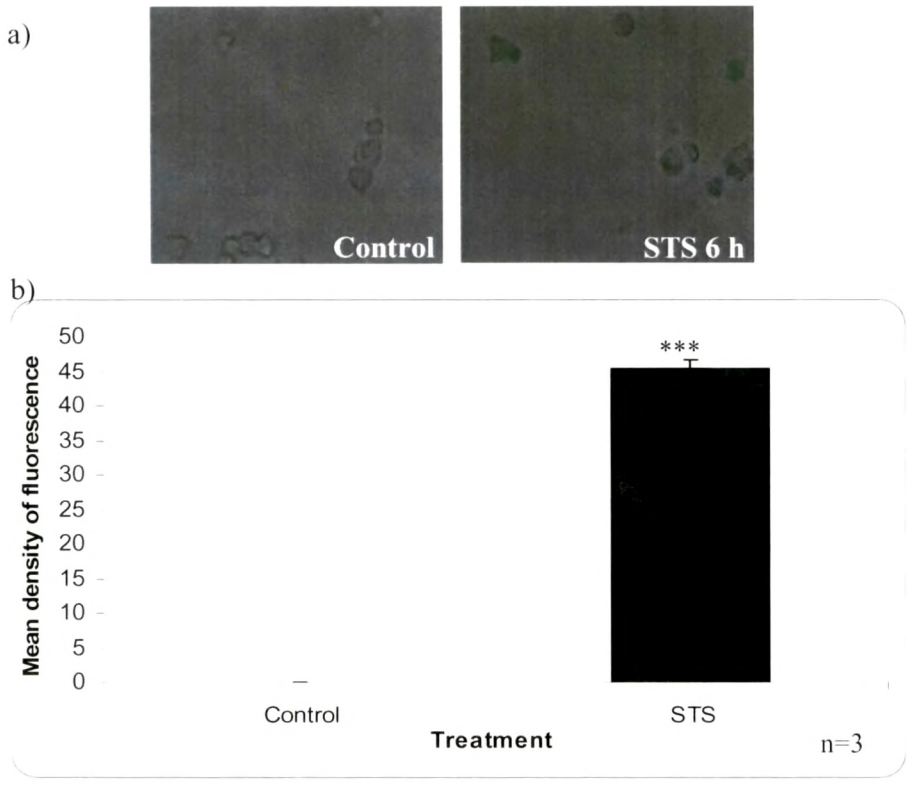


Figure 5.7 a and b: DNA fragmentation was monitored under STS stress using TUNEL assay. STS led to significant DNA damage. a) Photographs were taken with 60X objective. b) Densitometric analysis. Results are the mean of three independent experiments ± SE. *** p value <0.001 compared to control.

DNA fragmentation could be detected by TUNEL assay and a significant increase in TUNEL positive cells was seen (Figs. 5.7 a and b) at 6 hours after 400 nM STS treatment suggesting that *D. discoideum* cells undergo large scale DNA fragmentation. However, 500 nM STS treated cells exhibited DNA smear on agarose gel electrophoresis (Fig. 5.8) supporting the cell death to be of necrotic type. Thus these results reinforce that *D. discoideum* exhibits PARP independent paraptosis and necrosis with low and high doses of STS respectively.

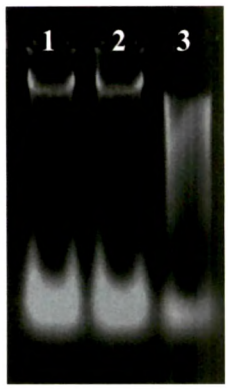


Figure 5.8: Monitoring DNA Fragmentation by agarose gel electrophoresis.

Lane 1: DNA from control cell

Lane 2: DNA from 400nM STS treated cells

Lane 3: DNA from 500nM STS treated cells

5.2.5. Effect of cathepsin D inhibition on STS induced cell death

Caspase independent cell death induced by STS is known to be inhibited by pepstatin A (Bidere *et al.*, 2003; Moubarak *et al.*, 2007). Pepstatin A is an inhibitor of acid proteases (aspartyl peptidases) *viz.*, cathepsin D. It functions as a transition state analogue. It binds to the active site of the enzyme and prevents its binding with cytosolic proteins like Bax.

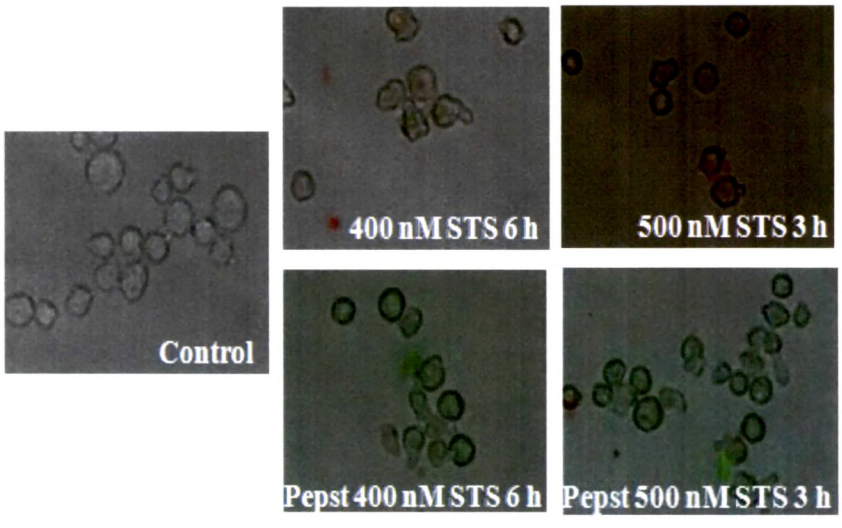


Figure 5.9 : Annexin V staining of STS treated *D. discoideum* cells. PS exposure is seen at 3 hours while PI staining at 6 hours with 400 nM STS. 500 nM STS was found to be necrotic as both AnnexinV-FITC and PI staining were observed at 3 hours. Benzamide did not affect STS induced cell death. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

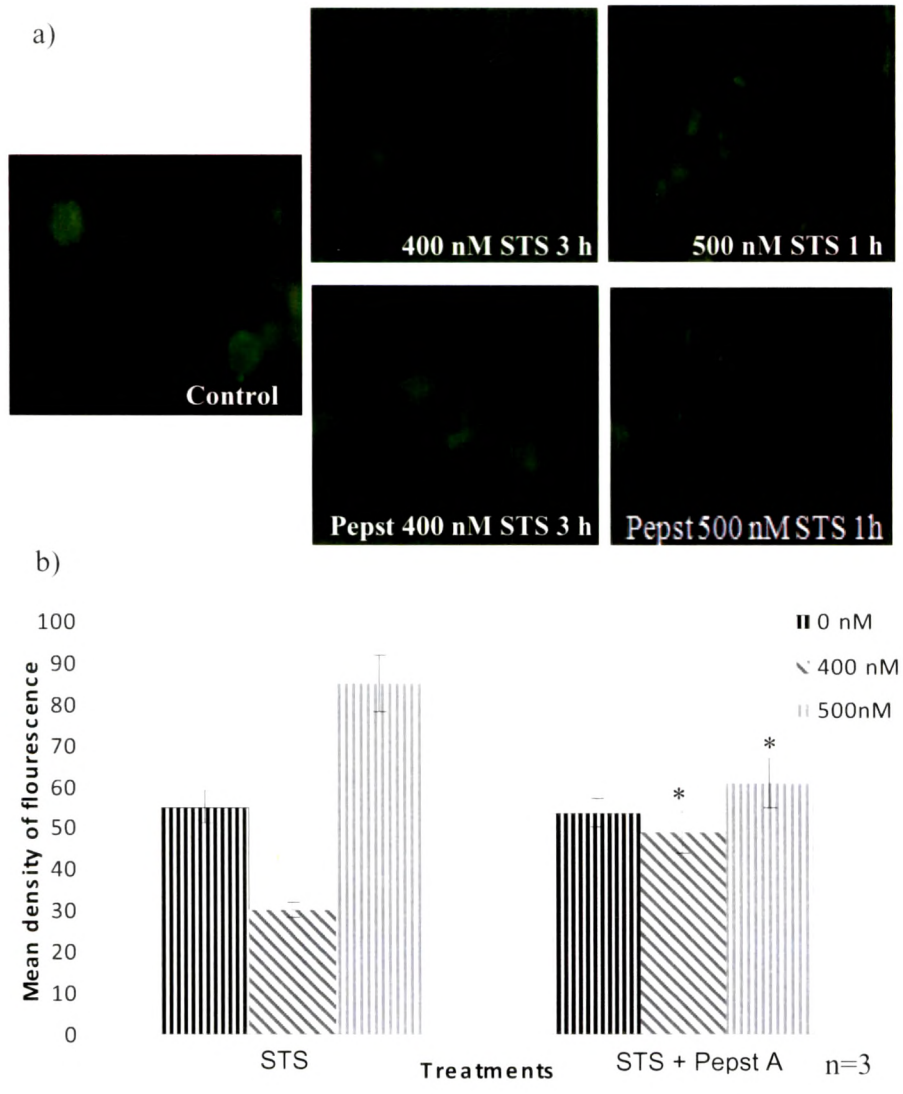


Figure 5.10a and b: Mitochondrial membrane potential changes induced by STS. MMP change with 400 nM STS at 3 hours and 500 nM STS at 1 hour were rescued partially by pepstatin A pre-treatment. a) Photographs were taken with 60X objective. b) Densitometric analysis of fluorescence. Data (mean ± S.E.) are from three independent experiments. * p value <0.05 compared to respective dose of STS without pepstatin A pre-treatment.

Pepstatin A pretreatment could prevent the changes in MMP induced by STS (Figs. 5.9 a and b). Also pepstatin A prevented nuclear translocation of AIF 4 hours after 400 nM STS treatment (Fig. 5.10). Indicating that cathepsin D activity is mediates STS induced cell death.

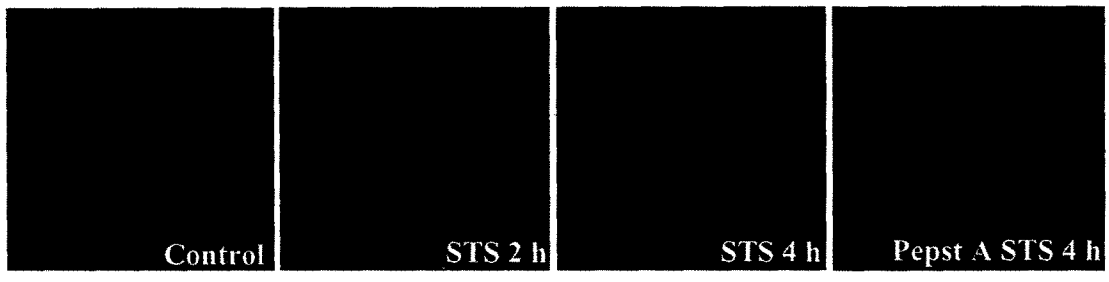


Figure 5.11: Fluorescence microscopy for the mitochondria-nuclear translocation of AIF at different time points after STS treatment. Nucleus of control cells stained blue due to DAPI indicating AIF is localized in mitochondria. AIF translocation to nucleus was observed at 4 hours of STS treatment as seen by cells showing pink fluorescence. Pretreatment with pepstatin A prevented translocation of AIF to nucleus. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

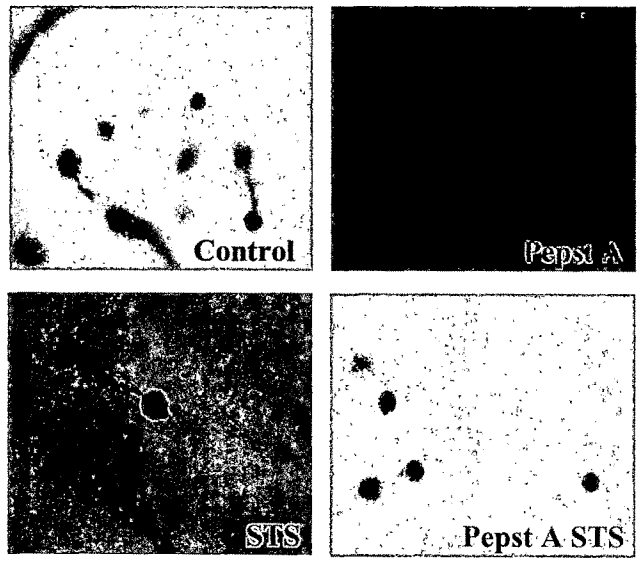


Figure 5.12: *D. discoideum* development affected by STS

The effect of staurosporine on the development of *D. discoideum* cells in the presence and absence of cathepsin D activity was studied. It was observed that the number of developing bodies was less in STS treated cells however, the development occurred within the normal time period i.e. fruiting bodies were seen at 24 hours. Cathepsin D inhibition could restore the number of developing bodies to normal in STS treated *D. discoideum* cells (Fig. 5.11). This could be due to the reduction in the number of dying cells thereby an increase in the effective number of cells taking part in the developmental process.

5.3. Discussion

Staurosporine is widely used as an inducer of apoptosis in many mammalian cell types (Kruman *et al.*, 1998). It induces both the types of cell death- caspase dependent and caspase independent (Zhang *et al.*, 2004). Caspase independent cell death is executed with the help of lysosomal destabilisation causing release of cathepsins D in cytosol. The key player in this cascade is Apoptosis Inducing Factor (AIF) which is released due to limited permeablization of outer mitochondrial membrane. Events following AIF release include chromatin condensation and large scale DNA fragmentation leading to nonapoptotic cell death (Bidere *et al.*, 2003).

5.3.1. Staurosporine induced cell death in *D. discoideum* cells is mediated *via* AIF

Human corneal cells show PI staining post 12 hours of STS treatment (0.2μM) (Thuret *et al.*, 2003). *D. discoideum* cells show PS exposure at 3 hours and PI staining at 6 hours post 400 nM STS treatment (Fig. 5.2). These cells undergo MMP changes 3 hours (Figs. 5.3 a and b) while exhibit AIF release 4 hours past STS treatment (Fig. 5.4). Melanoma cells show mitochondrial membrane potential changes at 6 hours (Zhang *et al.*, 2004). AIF release takes place in melanoma cells at 16 hours post STS treatment (Zhang *et al.*, 2004). AIF release at 4 hours is followed by large scale DNA fragmentation as seen at 6 hours after 400 nM STS treatment (Figs. 5.7 a and b) which is in accordance with the observations made by Bidere *et al* (2003).

500 nM STS treated *D. discoideum* cells exhibit PS-PI simultaneously at 3 hours (Fig. 5.2). MMP changes with this necrotic dose commence within 1 hour of STS treatment. These cells exhibited a smeared DNA (Fig. 5.8) further indicating the cell death type to be of necrosis.

5.3.2. Staurosporine induced cell death is independent of PARP activity

STS is well known to initiate a caspase independent cell death pathway which is mainly dependent on AIF release and consequent large scale DNA fragmentation (Daugas *et al.*, 2000). On the other hand, PARP which is known to get activated subsequent to DNA damage, gets cleaved in caspase dependent cell death. However, till date the role of PARP in the cascade of events led by staurosporine is not yet known. In this study attempt has been made to deal with this aspect of STS induced cell death. Results on PARP activation and benzamide inhibition suggest that PARP does not get activated after

STS (Figs. 5.5 a and b) and also PARP inhibition by benzamide does not rescue the MMP changes induced by STS (Figs. 5.3 a, c and 5.4). Unchanged NAD^+ levels in STS treated cells (Fig. 5.6) further support PARP independent cell death. These results point out that PARP might not be involved in STS induced cell death.

5.3.3. Staurosporine induced cell death in *D. discoideum* cells involves cathepsin D

Cathepsin D causes activation of Bax by proteolytic cleavage in the cytosol. Activated Bax leads to limited permeabilisation of the outer mitochondrial membrane. This in turn results in the release of AIF from the intermembrane space of mitochondria to the cytosol (Bidere *et al.*, 2003). Hence inhibition of cathepsin D delays cell death. Pepstatin A is an inhibitor of acid proteases (aspartyl peptidases) viz., cathepsin D. It functions as a transition state analogue. It binds to the active site of the enzyme and prevents its binding with cytosolic proteins like Bax etc. STS induced cell death in *D. discoideum* cells preincubated with pepstatin A is delayed as seen with PS-PI dual staining (Fig. 5.9). Effect of cathepsin D inhibition on the mitochondrial changes in terms of MMP (Figs. 5.10 a and b) and AIF release (Fig. 5.11) underlies the rescuing effect of pepstatin A on STS induced cell death.

Cathepsin D is reported to mediate its effect in STS induced cell death via Bax activation. Lam *et al* (2007) report absence of Bcl family of proteins in *D. discoideum* via *in silico* investigations. These facts make the above stated results intriguing raising the possibility of an alternative mechanism for the execution of STS induced cell death in *D .discoideum*.

5.3.4. Developmental changes under STS stress is dependent on cathepsin D activity

The effect of STS on the development of *D. discoideum* cells in the presence and absence of PARP and cathepsin D activity was studied. The number of developing bodies was less in STS treated cells however, the development occurred within the normal time period (24 hours). Cathepsin D inhibition could restore the number of developing bodies to normal in STS treated *D. discoideum* cells (Figs. 5.12).

Based on the results a pathway for kinetics of STS induced cell death (Fig. 5.13) is proposed.

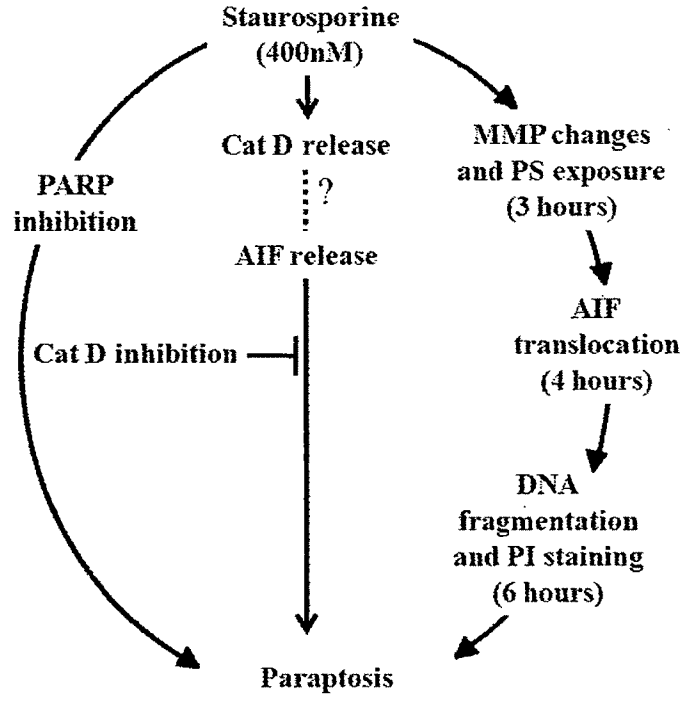


Figure 5.13: Mechanism of Staurosporine induced cell death in *D. discoideum*. PARP inhibition does not affect STS induced paraptosis. Cathepsin D leads to mitochondrial changes and AIF release *via* an unknown mechanism.

The present study shows that like mammalian cell types cathepsin D and AIF play central role in STS induced cell death in *D. discoideum* as well, albeit *via* a mechanism that circumvents the involvement of Bcl family of proteins. However, PARP is not involved in this type of cell death. In other words this study emphasises the point that PARP is dispensable for paraptotic cell death which is mediated via AIF.

5.4. References

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