Response of *Dictyostelium discoideum* to UV -C and involvement of PARP

6.1. Introduction

D. discoideum is a unicellular organism that feeds on bacteria, and divides, under favorable environmental conditions, by binary fission every 10-12 hours. During starvation, cells become chemotactically sensitive to cAMP pulses and initiate the developmental program to form a multicellular fruiting body structure, consisting of spores within a sorocarp, and a stalk. Spores germinate to form unicellular amoebae under favorable conditions. *D. discoideum* being a eukaryote that stands at the transition point of unicellularity and multicellularity is an exceptional model system to study various signal transduction pathways (Mir *et al.*, 2007) that can later be extrapolated to mammalian systems. The unicellular stage is known to be highly resistant to DNA damaging agents and oxidative stress (Welker and Deering, 1978; Katoch and Begum, 2003).

We have reported the long term consequences of such inhibition under oxidative stress (Rajawat *et al.*, 2007). In this study the role of PARP in UV-C induced growth and developmental changes in *D. discoideum* has been addressed by inhibiting its activity with benzamide and also the long-term effects of PARP inhibition for two successive generations has been studied.

6.2. Results

6.2.1. Cell death and PARP activation induced by UV-C radiation

D. discoideum cells when subjected to different doses of UV-C, dose dependent death could be seen as observed by trypan blue staining after 24 hours of stress (Fig. 6.1). PARP was assayed at various time points (2 and 5 minutes) post UV-C stress and the results are shown in Figure 6.2a. 10.4 J/m^2 and 13 J/m^2 UV-C treated cells showed highest PARP activity at 2 minutes which then declined, and reached to basal level after 5 minutes. Benzamide pretreated cells did not show activation of PARP after 2 minutes UV-C treatment (Fig. 6.2b).

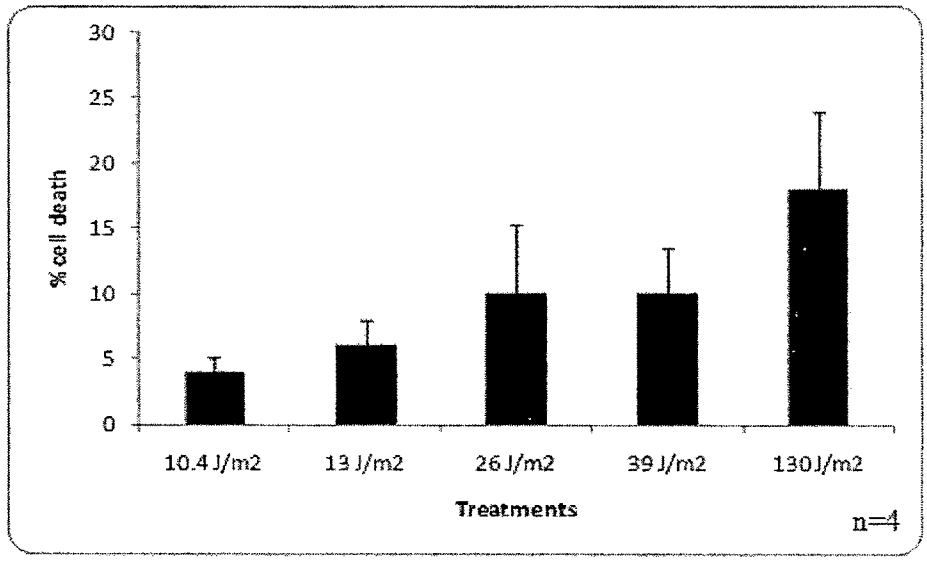

Figure 6.1: UV-C induced dose dependent cell death as monitored by trypan blue exclusion method. Results are the mean ± SE of four independent experiments.

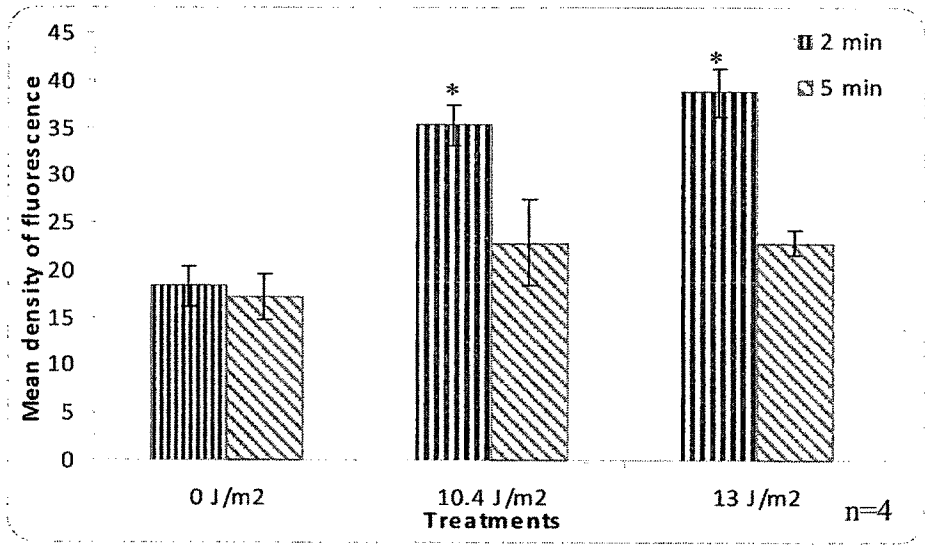

Figure 6.2a: Densitometric analysis of PARP activity. PARP activity was measured at two different time points post UV-C irradiation. Peak activity was seen at 2 minutes post 10.4 J/m^2 and 13 J/m^2 UV-C exposure. Data (mean ± S.E.) are from four independent experiments. * p value <0.05 compared to respective control.

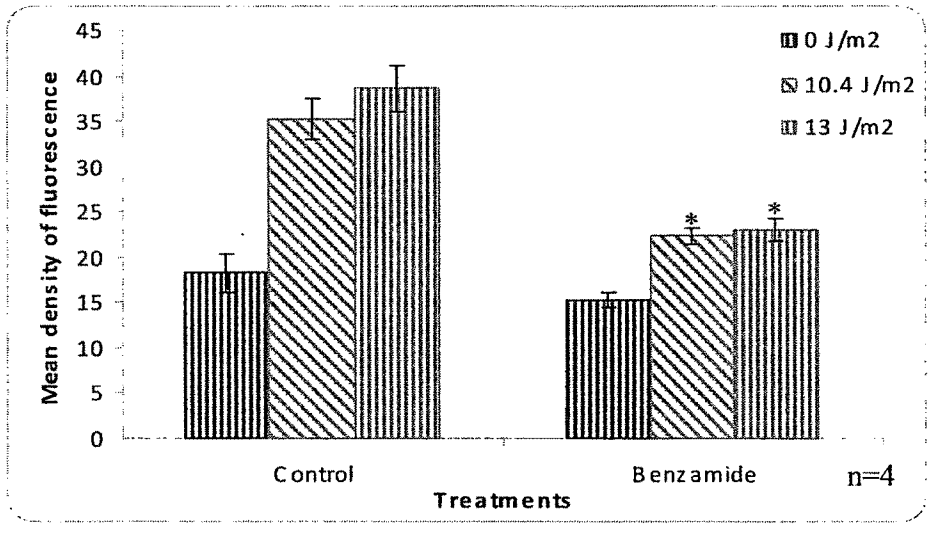

Figure 6.2b: Peak PARP activity induced by UV-C irradiation was intercepted by benzamide. Benzamide inhibited PARP activity at 2 minutes post 10.4 J/m^2 and 13 J/m^2 irradiation respectively. Data (mean ± S.E.) are from four independent experiments. * p value <0.05 as compared to respective UV-C irradiated control.

6.2.2. *D. discoideum* growth under UV-C stress

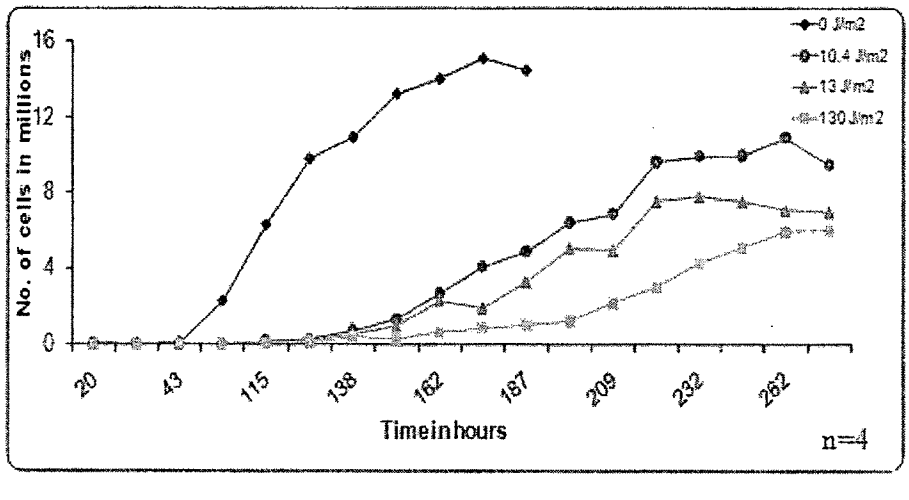

Figure 6.3: Effect of UV-C induced growth changes in *D. discoideum*. Under UV-C stress the growth curve showed a dose dependent increase in the lag phase. The stationary phase is achieved at lower cell density. Results are mean of 4 independent experiments.

As can be seen from Figure 6.3 there was a dose dependent increase in the lag phase with UV-C dose i.e. with increase in UV-C dose there was an increase in the lag phase and consequently a late entry in to the log phase. Also the stationary phase is achieved at a

lower density compared to control cells. Pretreatment of cells with benzamide showed rescue in UV-C induced changes in the growth (Fig. 6.4).

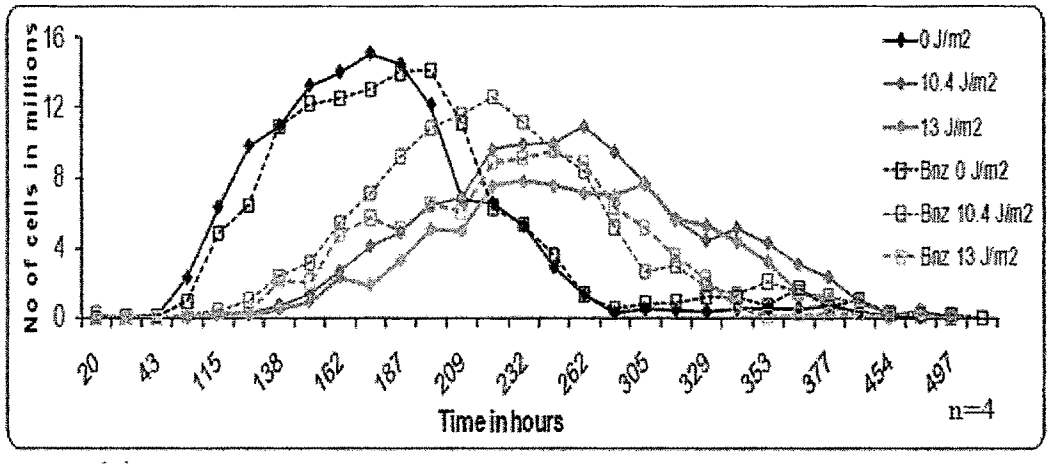

Figure 6.4: Effect of PARP inhibition on UV-C induced growth changes in *D. discoideum*. Benzamide could rescue the UV-C induced changes in growth curve. The stationary phase was achieved at higher cell density in benzamide pretreated cells as compared to respective control. Results are mean of 4 independent experiments.

6.2.3. *D. discoideum* development under UV-C stress

The effect of UV-C on development also reflected dose dependency as seen in cell death experiment (Fig. 6.1) however, the effect was more drastic. 10.4 J/m^2 UV-C irradiated cells could develop, however, cells exposed to 13 J/m^2, 65 J/m^2 and 130 J/m^2 UV-C failed to enter the development (Fig. 6.5). Also 10.4 J/m^2 UV-C treated cells displayed delayed development compared to control cells. Also the delay in development induced by 10.4 J/m^2 was rescued when the cells were pretreated with benzamide (Fig.6.5).Developing bodies, formed after UV-C stress were smaller and lesser in number. Cells subjected to UV-C stress showed more loose body formation compared to control (Fig. 6.5). This observation led us to assess the expression profile of development related genes such as *countin*, *countin*2, *discoidin*I, *gp*24 and *gp*80 were assessed. These genes are associated with the regulation of the aggregate size (Okuwa *et al.*, 2001; Barondes *et al.*, 1985; Kamboj *et al.*, 1990). Expression profile of *yak*A and *car*1 were also studied as these genes are important for growth to differentiation transition and cAMP mediated signaling respectively (Souza *et al.*, 1998; Mir *et al.*, 2007). As shown in Figure 6.6, UV-C affected the levels of countin, yakA and car1 mRNA. Benzamide pretreatment prevented the changes in the expression of *countin* however *yakA* and *car1* expression levels could not be restored. Benzamide also increased the levels of gp80 mRNA as compared to control cells.

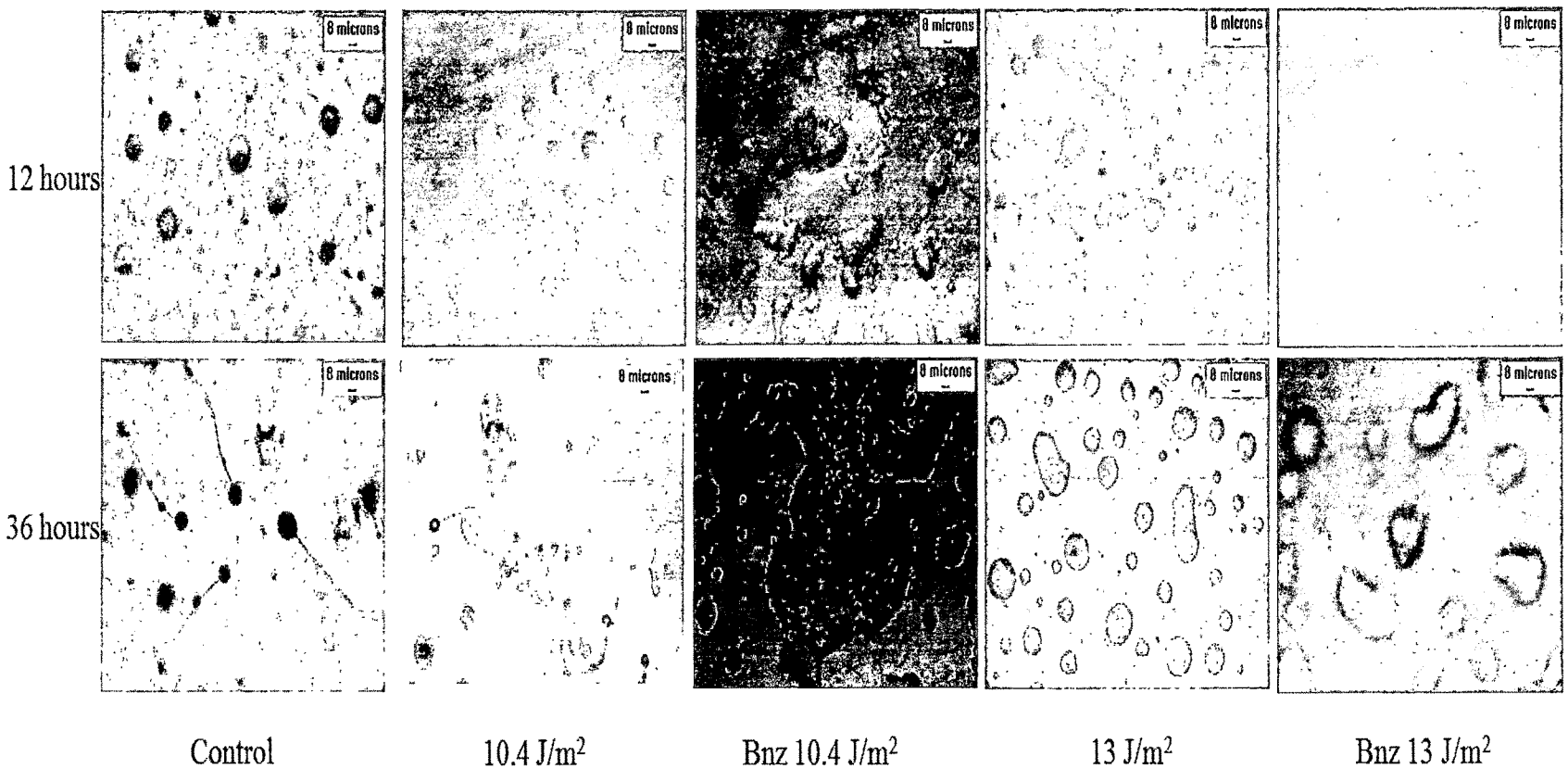

Figure 6.5: Development of *D. discoideum* cells under UV-C stress. *D. discoideum* cells after UV-C treatment were allowed to develop on nutrient free agar medium and were observed at various time intervals. Benzamide pretreatment could restore the delay induced by 10.4 J/m^2 but could not lead the 13 J/m^2 UV-C treated cells to enter development. Photographs were taken with 4X objective.

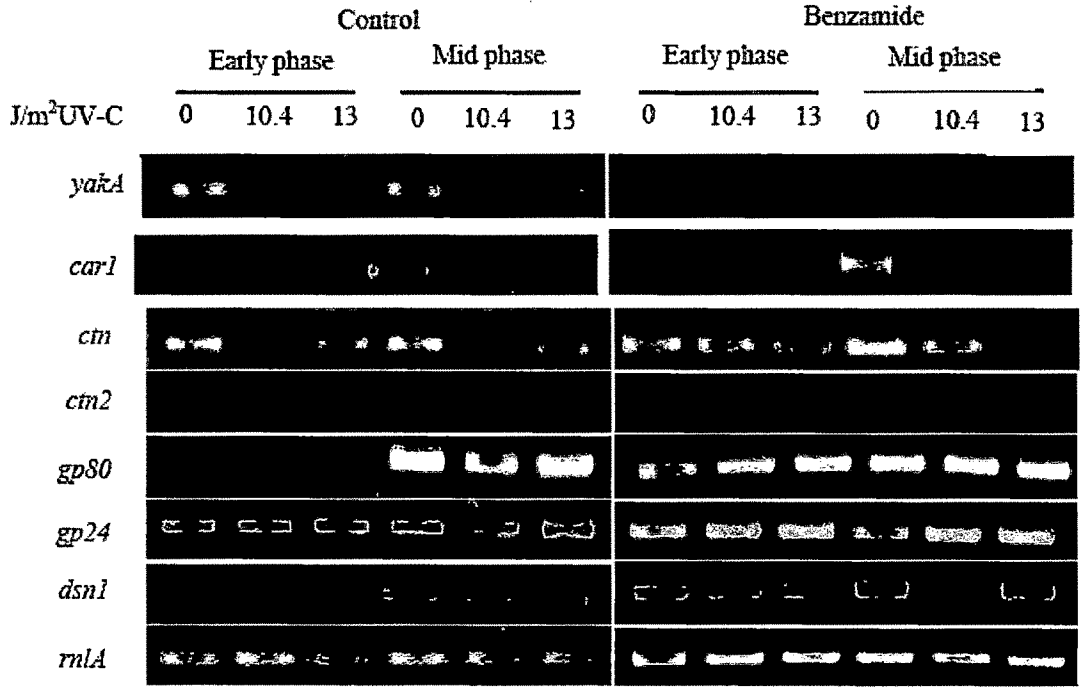

Figure 6.6: Irradiation with UV-C affects the mRNA expression of various genes involved in *D. discoideum* development. Expression of cell cycle arrest protein, YakA; cAMP receptor- cAR1 and protein involved in cell counting mechanism viz. countin were found to be decreased in UV-C treated cells as compared to the internal control rnlA (mitochondrial rRNA IG7). Benzamide pretreatment restored the expression levels of countin however did not affect yakA and cAR1 levels. Levels of countin2 (another protein of cell counting mechanism), gp80 (cellular streaming regulation) mRNA were found to be increased in benzamide pretreated cells whereas discoidin1, a protein that helps cells to adhere to the substratum and gp24 (intercellular contacts and aggregation) were not affected. mRNA levels of *yakA* and *car1* were assayed at 0 and 9 hour while rest genes were assayed at 6 and 10 hour of development induction.

6.2.4. Effect of UV-C and PARP inhibition second generation of *D.discoideum* cells

Spores developed after 10.4 J/m^2 UV-C irradiation showed a delay of ~27 hours in germination (111 hours ± 6.0) compared to control cells (81 hours ± 6.658) (Fig. 6.7). These spores also showed longer lag phase compared to control. However, benzamide pretreatment had no effect on revival of spores formed after 10.4 J/m^2 UV-C exposure as shown in Figure 6.7. Also this second generation of UV-C exposed cells did not differ with respect to the damage in DNA (Fig. 6.8) ensuring that benzamide pretreatment did not interfere with the basal repair of the cells.

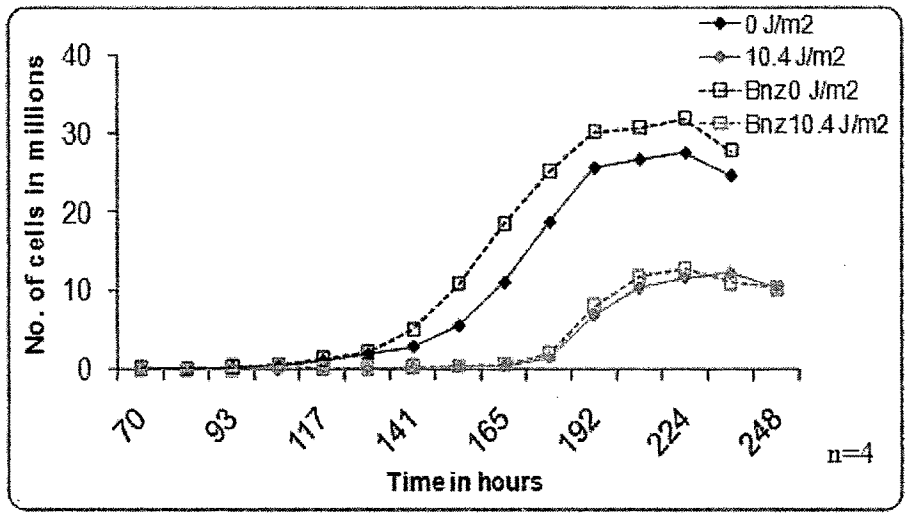

Figure 6.7: Effect of PARP inhibition on the fate of spores that were developed under UV-C stress. Spores of control cells revived within 81 hours whereas spores formed after 10.4 J/m^2 UV-C stress exhibited ~27 hours of delay in spore revival, which could not be rescued by benzamide pretreatment. Data is mean of 4 independent experiments.

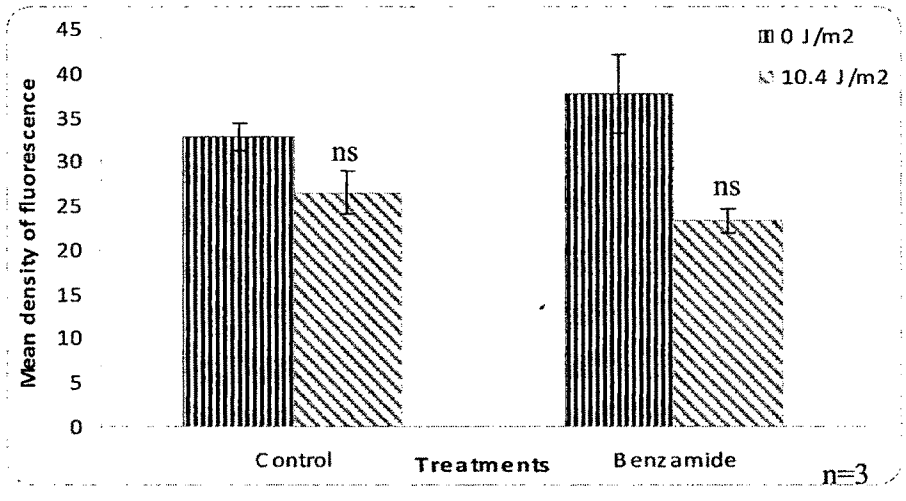

Figure 6.8: DNA damage monitored in second generation *D. discoideum* cells. No significant damage was observed in second generation cells.

6.3. Discussion

6.3.1. *D. discoideum* growth is sensitive to UV-C in PARP dependent manner:

DNA damage is well reported to be followed by activation of the repair machinery as a part of cellular recovery mechanism. During repair the cells are subjected to cell cycle arrest so as to avoid the next generation of cells from receiving the damaged DNA.

UV-C is a well documented potent DNA damage inducer. It leads to cell cycle arrest (Gentile *et al.*, 2003) and cell death in a dose dependent manner (Fig. 6.1). This is also evident in the growth studies where dose dependent increase in the lag phase was observed with increasing UV-C dose (Fig. 6.3). Cells exposed to higher doses of UV-C (130 J/m^2) have longer lag phase (~100 hours). This lag phase could be due to seized cell cycle during which the repair mechanism becomes instrumental. Nevertheless, these cells do enter the log phase after coping with the damage induced by UV- induced by UV-C stress (Fig. 6.3).

PARP is activated post UV-C irradiation (Vodenicharov *et al.*, 2005). Our results further support these findings. Indirect immunofluorescence for PAR shows that UV-C treatment activates PARP in minutes (Fig. 6.2a). Such an early PARP activation implicates early DNA damage. Cells pretreated with benzamide show reduced PARP activity subsequent to UV-C stress (Fig. 6.2b). 10.4 J/m^2 UV-C exposed cells when pretreated with benzamide enter log phase earlier than the respective controls (Fig. 6.4), which shows that PARP inhibition reduces the lag phase induced by UV-C suggesting that UV-C activates PARP and that in turn plays a role in the repair and arrest of cell cycle. These cells also showed higher cell density compared to only 10.4 J/m^2 UV-C irradiated cells. Thus PARP inhibition during moderate cellular stress (10.4 J/m^2) is beneficial, but at higher UV-C doses this effect is not seen, as the cells take up cell death (Fig. 6.1).

6.3.2. UV-C alters *D. discoideum* development

D. discoideum during unicellular vegetative phase exhibited higher resistance to oxidative stress (Katoch and Begum, 2003) and other DNA damaging agents (Welker and Deering, 1978). Nevertheless, the effect of UV-C is more drastic on development. 13 J/m^2 and higher doses of UV-C blocked *D. discoideum* development completely whereas 10.4 J/m^2 UV-C irradiation led to delayed development (Fig. 6.5). Also these 10.4 J/m^2 UV-C

irradiated cells develop into smaller and fewer fruiting bodies as compared to control cells. This shows that *D. discoideum* cells could not cope up with the damage induced by 13 J/m^2 UV-C stress however, the cells do manage with the damage induced by 10.4 J/m^2 UV-C albeit only partly. Thus mild doses of UV-C i.e. doses below 39 J/m^2 UV-C which is paraptotic (Fig. 3.2) blocked development completely. Nevertheless during oxidative stress, a dose of 4 mM HA (hydroxylamine, a catalase inhibitor), higher than the paraptotic dose (1 mM HA), could only block *D. discoideum* development (Rajawat *et al.*, 2007).

Azzam *et al* (2003) demonstrated a cell type specific effect of UV-C irradiation on the expression of connexin 43 native isoform in human fibroblast cells and AG1522 cells. Experiments done with UV-C irradiation in human skin fibroblasts (Gentile *et al.*, 2003) showed that UV-C reduces transcription of certain proteins involved in adhesion and motility. Our results show that although adhesion proteins gp24 and gp 80 are not affected in UV-C treated cells however, expression of *countin* is markedly reduced in these cells without much alteration in countin2 mRNA levels. The *countin* null cells show increased group size (Gao *et al.*, 2004) similar to the larger aggregate size seen in case of UV-C treated cells (Fig. 6.5) which are however not able to progress further to fruiting bodies. Such cells have been reported to show decreased cAMP-induced cAMP pulse, and a decrease in cAMP-stimulated Akt/ PKB membrane translocation and kinase activity, which in turn decrease cell motility (Tang *et al.*, 2002; Brock *et al.*, 2003a; Gao *et al.*, 2004). In the present study also the expression levels of *car1* and *aca* are affected (Fig. 6.6) however this may not be mediated via the defect observed in countin as benzamide pretreatment that restores countin levels to normal is unable to affect car1 mRNA. Effect of UV-C on yakA, a protein crucial for growth to differentiation transition, may underlie the developmental delay. Benzamide pretreatment does not affect the reduced yakA expression. This partial rescuing effect of benzamide probably underlies its inability to restore the development of UV-C irradiated cells to complete normalcy. Taken together, UV-C irradiated cells undergo alteration in expression of genes associated with growth to differentiation transition, chemotaxis and aggregate size regulation and PARP inhibition rescues the expression of some of the size regulating genes (Fig. 6.6).

Although benzamide restores countin levels it was observed that expression of gp24 and gp80, cell adhesion proteins increased in the presence of benzamide (Fig. 6.6).

This is in accordance with various studies that show association of PARP activity with the expression of adhesion proteins (Lukowicz *et al.*, 2008). As increased adhesion proteins are known to hinder chemotaxis (Jang and Gomer, 2008) and hence effect of benzamide on the gp(s) may mask its effect on development via restoration of countin levels. These results also implicate that interplay between PARP and UV-C holds significance with respect to adhesion protein expression and hence is associated with certain developmental defects induced by UV-C.

6.3.3. Delayed spore germination after UV-C irradiation is independent of PARP

Cells irradiated with 10.4 J/m^2 UV-C undergo delayed development followed by delayed revival compared to control spores (Fig. 6.7). This could be due to lesser number of cells combining to form a smaller fruit as the number of fruiting bodies is regained on increasing the cell density (Fig. 7.1).

As can be seen from Figure 6.7, 10.4 J/m^2 UV-C irradiated cells with benzamide pretreatment did not show any significant change in the revival of spores compared to 10.4 J/m^2 UV-C treated cells. This result is intriguing as PARP inhibition corrects the growth defects induced by UV-C however, when the same cells' spores are put for revival, PARP inhibition did not show any change. This implicates that PARP may not be involved in increasing the dormancy of spores induced by UV-C. Interestingly, the cells germinated from spores did not show any significant damage (Fig. 6.8) signifying that the basal activity of PARP (Fig. 6.2) may be sufficient to repair the DNA damage induced by 10.4 J/m^2 UV-C. UV-C induces prominent, dose dependent down regulation of transcription of selective genes (Gentile *et al.*, 2003). Delayed revival of spores formed from UV-C irradiated cells (Fig. 6.7) could be attributed to such specific down regulation of certain genes involved in the revival of *D. discoideum* spores.

Interestingly, these effects of PARP inhibition on UV-C induced changes in *D. discoideum* growth and development differs from our oxidative stress response results (Rajawat *et al.*, 2007). The spores formed under oxidative stress exhibited delayed revival compared to benzamide pretreated cells suggesting that PARP inhibition during oxidative stress not only corrects the affected development but also retains the normal spore revival time (Rajawat *et al.*, 2007). However, the present study suggests that PARP inhibition and UV-C treatment do not have any effect on the spore development of second generation cells. This in addition to the observation that lower doses of UV-C cause the

developmental defects in the *D. discoideum* cells, a few of which are unaffected by PARP inhibition raises a question at the therapeutic significance of PARP inhibition in various oxidative stress and DNA damage related diseases, where PARP is known to be overactivated (Virag and Szabo, 2002). Hence the concept of PARP inhibition being beneficial in various DNA associated diseases should be considered cautiously.

D. discoideum shows differential effects of oxidative and UV-C stress on development and spore germination. Hence this organism in spite of being a lower eukaryote has the complex signaling machinery that deals with different stresses i.e. oxidative stress and UV-C in diverse ways. This fact emphasizes the importance of studies of signaling pathways in *D. discoideum* which is simple and easy to handle compared to mammalian cell lines.

6.4. References

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