Chapter 4:

Over expression of *ADPRT1A* and its effects on *D. discoideum* growth and multicellularity

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4.1 Introduction

Poly (ADP-ribose) polymerase 1 (PARP-1) is a well-known ADP-ribosylating enzyme, which was classically known to be activated by single-strand and double-strand breaks (Satoh *et al*., 1994; Langelier *et al*., 2008; 2010; Eustermann *et al*., 2011). However, reports now prove DNA independent activation of PARP-1 too (Jubin *et al*., 2016b). PARP-1 influences diverse biological processes namely DNA repair, cell growth, transcriptional regulation, differentiation and programmed cell death (Quenet *et al*., 2009; Hottiger *et al*., 2010; Messner *et al*., 2011; Mir *et al*., 2012). PARP-1 influences ~60-70% of genes controlling important processes like cell cycle, transcription etc. (Chaitanya *et al*., 2010). The role of PARP-1 is majorly identified as NAD^+ dependent enzyme that facilitates important steps in DNA damage response, transcription etc., however, its role during multicellularity and differentiation is yet to be fully understood. PARP homologs have been identified from lower to higher eukaryotes like protists, filamentous fungi, metazoans and plants with the notable exception of unicellular fungi, *S. cerevisiae* and *S. pombe* (Citarelli *et al*., 2010). There are scarce reports suggesting that PARP-1 regulates critical gene transcription and cellular events during development. Studies in *Drosophila* suggest the importance of PARP in chromatin loosening at ecdysone inducible regions thereby inducing purparium formation and metamorphosis (Fletcher *et al*., 1995; Tulin and Spradling., 2003). These results are also substantiated by mice studies wherein PARP-1 and PARP-2 double mutant mice are found to be not viable and die at the onset of gastrulation, establishing the importance of both the PARPs during early embryogenesis (de Murcia *et al*., 2003). Genetic studies on PARP-1 orthologs in fungus established defective development and decreased life span (Semighini *et al*., 2006; Kothe *et al*., 2010; Muller-Ohldach *et al*., 2011). However, more reports are essential to substantiate the role of PARP-1 under normal physiological conditions of growth and development.

D. discoideum provides us with the best model system with advantages of a fully sequenced genome, genetic and biochemical techniques to study various processes and also unicellular as well multicellular stages in its life cycle (Kawal *et al*., 2011; Schaap, 2011). Thus, the present study exploits these advantages for further studies. We have identified ADPRT1A as the PARP-1 ortholog in *D. discoideum.*

Our previous studies showed PARP's role in *D. discoideum* development by chemical inhibition of PARP activity with Benzamide (PARP inhibitor) and it resulted in delayed development. Also we have demonstrated that constitutive *PARP* down-regulation did not affect the growth of *D. discoideum,* nonetheless development was stalled at initial aggregation stage (Rajawat *et al*., 2007; 2011). Cell death and delayed development due to UV-C stress was also found to be rescued in *D. discoideum* via PARP inhibitor (Mir *et al*., 2015). Thus, we propose that PARP plays an essential role in *D. discoideum* development and cell death. We are thus, interested to further explore the role of ADPRT1A (PARP-1 ortholog) in *D. discoideum* growth and development.

In the present study, we analyzed the role of ADPRT1A in growth and development of *D. discoideum.* Our results substantiate that ADPRT1A and its regulated expression are essential for the growth and the proper cellular metabolism of *D. discoideum.* We also demonstrated ADPRT1A activity during *D. discoideum* development and confirmed its role in multicellularity.

4.2 Results

In order to unravel the novel role of ADPRT1A in growth and multicellularity, we cloned full length *ADPRT1A* with an aim to generate ADPRT1A overexpressing *D. discoideum* cells to study its effect on *D. discoideum* growth and development.

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4.2.1 Cloning of *PARP* (*ADPRT1A*) in EYFP vector

For generation of *ADPRT1A* overexpression construct, EYFP vector was used with *actin15* promoter- act15/Acg-Eyfp (Saran and Schaap, 2004). Full length *ADPRT1A* (3046 bp) was amplified from the genomic DNA and cloned in act15/Acg-Eyfp vector using *ADPRT1A*

specific primers as shown in Fig. 4.1. *Dictyostelium* genome being AT rich is difficult to amplify and hence modifications in PCR were made to amplify full length *ADPRT1A*. Extension temperature was standardized to 65^{o}C as opposed to 72^{o}C in standard PCR conditions. The purified PCR product digested with *Sac*I and *BamH*I was ligated in to act15/*Acg-Eyfp* from which the *Acg* was replaced by *ADPRT1A*. This ligation mixture was transformed into *E. coli DH5α* and the transformants were selected on Luria Bertani Agar containing ampicillin (100µg/ml). Randomly selected colonies were screened for the presence of recombinant plasmid EYFP-*ADPRT1A* and colonies giving a release of 3.04 kb on restriction digestion using *BamH*I and *Sac*I were selected (Fig. 4.2: Lanes 1-3). A second construct lacking EYFP (as EYFP interferes with FITC tagged antibodies used in our cell death studies) was constructed by restriction digestion of PCR product and act15/Acg-EYFP with enzymes *Sac*I and *Xba*I such that *Acg-EYFP* was replaced from the vector by *ADPRT1A* (Fig.4.2: Lanes 4 and 5).

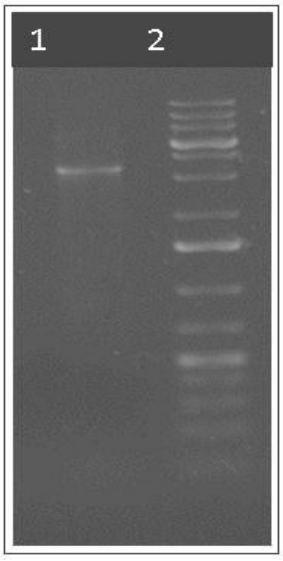


Fig. 4.1: PCR amplification of full length *ADPRT1*. Lane 1: *ADPRT1A* Amplicon*;* Lane 2: 1kb DNA ladder.

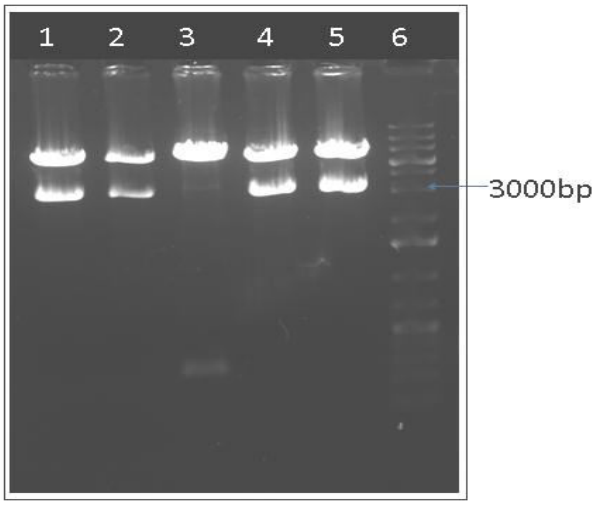


Fig. 4.2: Colony screening by Restriction digestion. Lanes 1-3: clones 1-3 digested with *BamH*I and *Sac*I; lanes 4 and 5: clones A , B digested with *Sac*I and *Xba*I; lane 6: 1 kb DNA ladder.

4.2.2 Confirmation of *PARP (ADPRT1A)* overexpression construct in EYFP using Restriction digestion

To confirm *PARP* (*ADPRT1A*) overexpression construct, resultant positive clone (EYFP-*PARP)* was re-digested with *BamH*I and *Sac*I giving a release of 3.04 kb. *Eco*RV is a restriction site present both in EYFP vector and insert (*ADPRT1A* gene) too. Presence of insert would give a release of 3.953 kb whereas absence of the insert would give a single linear band of 6.0 kb. *Eco*RV digested pattern with resultant clone gave a release of 3.953 kb confirming EYFP-*ADPRT1A* construct (Fig. 4.3) and untagged EYFP-*ADPRT1A* construct (Fig. 4.4). The confirmed clone containing full length *ADPRT1A* was used for transformation of *D. discoideum* cells to generate *ADPRT1A* overexpressing cell line.

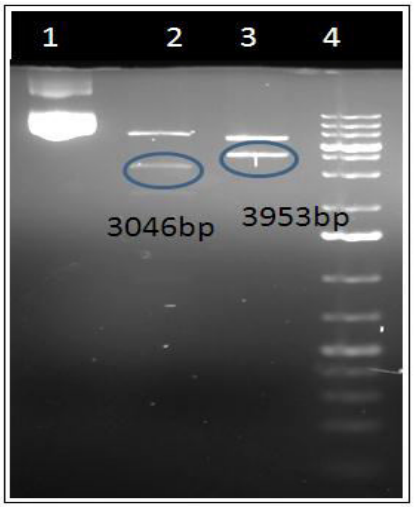


Fig. 4.3: Clone confirmation of ADPRT1A-EYFP by RE digestion. Lane 1: Uncut EYFP- *ADPRT1A* Lane 2: Digestion with *BamH*I & *Sac*I; Lane 3: Digestion with *EcoR*V; Lane 4: 1 kb DNA ladder.

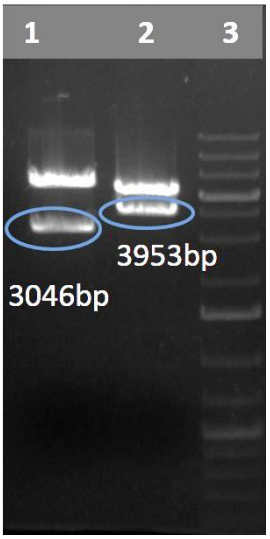


Fig. 4.4: Clone confirmation of untagged ADPRT1A construct by RE digestion. Lane 1: digestion with *Sac*I and *Xba*I; Lane 2: Digestion with *EcoR*V; Lane 3: 1 kb DNA ladder.

4.2.3 Transformation of EYFP-*ADPRT1A* overexpression construct in *D. discoideum*

Transformation of *D. discoideum* cells was done by electroporation method (Gaudet *et al*., 2007). Cells were transformed with EYFP-*ADPRT1A* using Gene Pulser Xcell™ electroporator (BioRad) at 650Volts, capacitance 25µF, 2 pulses with 15 sec gap between the 2 pulses. Geneticin 418 (G418) was used as a selection marker (100µg/ml). Cells were further stored as spore stock after developing into fruiting bodies on non- nutrient agar plate with 100µg/ml G418. Transformation of untagged *ADPRT1A* construct in *D. discoideum* was also carried out in similar manner.

4.2.4 Functional characterization and localization of *ADPRT1A* over expressing cells

Semi quantitative Reverse Transcriptase PCR (Fig. 4.5A) showed significantly higher *ADPRT1A* transcript levels in ADPRT1A-EYFP (AEOE) cells which were further confirmed by Real Time PCR (qPCR) studies wherein 61.10±7.354 fold higher expression of *ADPRT1A* transcripts were observed in AEOE cells as compared to control (Fig 4.5B). ADPRT1A OE (untagged- AOE) also showed 72.45±8.678 fold higher *ADPRT1A* transcript levels. A OE was used for all our fluorescence based studies in order to avoid interference of EYFP tag (Figs 4.5 A and B).

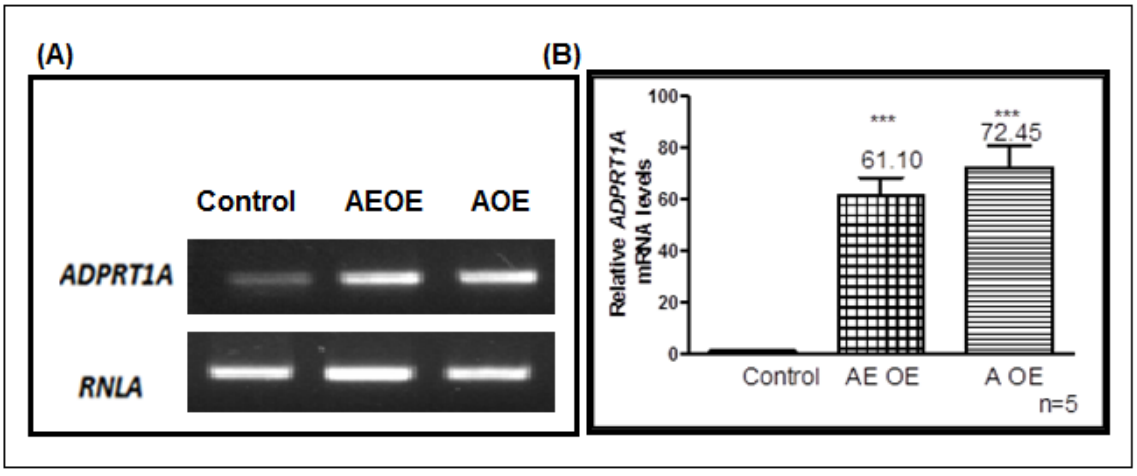


Fig. 4.5: Functional characterization of ADPRT1A overexpression

(A) Semi-quantitative RT-PCR of *ADPRT1A* in AE OE. AE OE exhibited increased ADPRT1A transcript levels as compared to control.
(B) Real time analysis in AE OE and A OE cells showed 61.10±7.354 and 72.45±8.678 fold overexpression of *ADPRT1A* transcripts as compared to control. ***p<0.001 as compared to control. Data is a representation of SEM values of five independent experiments.

In addition, the localization of cloned ADPRT1A was confirmed to be nuclear as YFP was co-localized with that of DAPI indicating that *ADPRT1A* is a nuclear localizing protein (Fig. 4.6A). As per our *in silico* analysis, ADPRT2 and ADPRT1B also showed homology to human PARP-1. Hence, to ensure that only *ADPRT1A* was overexpressed, the *ADPRT2* and *ADPRT1B* transcript levels were compared to their respective levels in the control cells. Fig. 4.6B shows that ADPRT1A transcript levels are 72.45 fold higher in A OE cells as compared to control cells. On the contrary, there was significant decrease in *ADPRT2* and *ADPRT1B* transcripts in A OE cells as compared to their respective expression in control cells reestablishing that *ADPRT1A* alone was overexpressed.

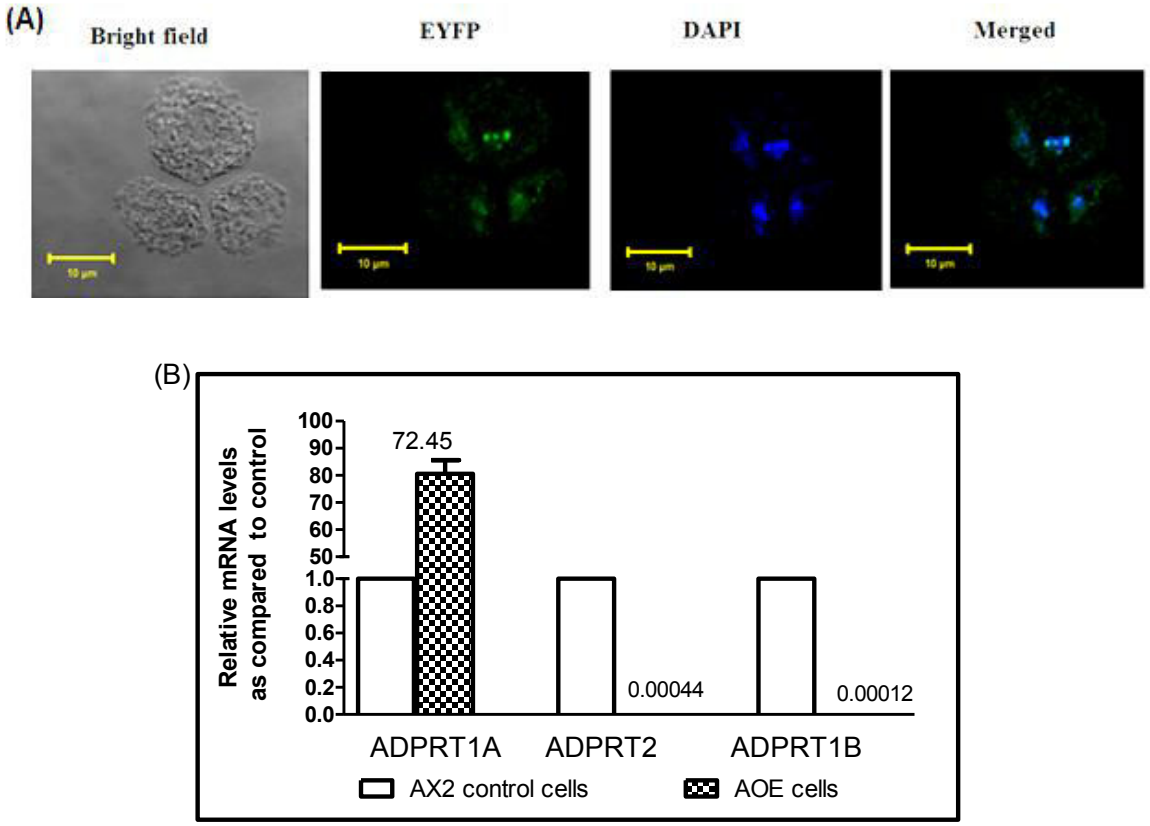


Fig. 4.6: Localization of ADPRT1A-EYFP in *D. discoideum* cells and transcript analysis of ADPRT isoforms in A OE. (A) Localization of ADPRT1A-EYFP in *D. discoideum* cells: EYFP merges with the DAPI stain demonstrating ADPRT1A to be a nuclear protein. Scale: 10 µm. Data is a representative of three independent experiments. Photographs were taken at 63X objective. (B) Relative mRNA levels of PARP isoforms – *ADPRT1A, ADPRT2* and *ADPRT1B* in AOE cells as compared to their respective levels in control cells. Data is a representation of SEM values of three independent experiments.

4.2.5 NAD^+ estimation in *ADPRT1A* over expression cell line

Further, it was essential to confirm the activity of the overexpressed ADPRT1A. Activation of PARP can lead to depletion of cellular NAD^+ pools which it uses as its substrate (Szabo and Dawson, 1998). Interestingly, the overexpressing cells show 60% reduction in NAD^+ levels in AE OE as well as A OE as compared to control cells,

confirming the ADPRT-1 activation in ADPRT1A overexpressing *D. discoideum* cells (Fig. 4.7).

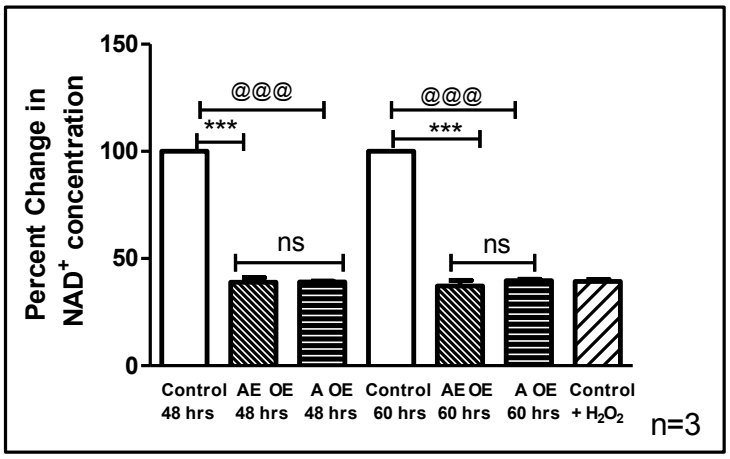


Fig. 4.7: Percent change of NAD^+ concentration in control and ADPRT1A-EYFP OE using Bernofsky's Enzymatic recycling method. Data is a representative of three independent experiments. *** *p* value for AE OE<0.001 as compared to control, $^{@@@}$ *p* value for AOE<0.001 as compared to control.

4.2.6 Effect of *ADPRT1A* overexpression on growth and cell cycle of *D. discoideum*

Followed by NAD^+ estimation, the effect of *ADPRT1A* overexpression on cellular proliferation of *D. discoideum* cells was studied. AEOE *D. discoideum* cells were significantly slow growing with doubling time of approximately 18.48 ± 1.65 hrs while AOE cells showed a doubling time of approximately 17.84 ± 1.45 hrs (Fig 4.8) while control cells and EYFP vector control cells divided at approximately every 12.41 ± 0.5166 hrs and 12.89 ± 1.126 hrs respectively. Thus, ADPRT1A overexpression significantly reduced cellular proliferation in *D. discoideum* cells.

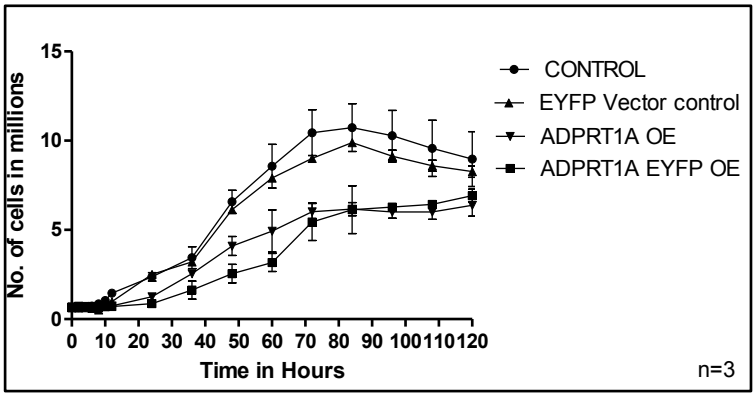


Fig. 4.8: Growth analysis of Control, ADPRT1A-EYFP OE (AE OE), ADPRT1A OE and EYFP vector control cells. Doubling Time in exponential phase for control cells was 12.41 ± 0.5166 hrs, EYFP vector control cells showed 12.89±1.126 hrs while that of AE OE cells was 18.48 ±1.65 hrs. Data is a representative of three independent experiments.

Since PARP-1 is known to be involved in cell-cycle regulation (Yang *et al*., 2013), we studied its effects on cell growth and also analyzed cell cycle phases using FACS by Propidium Iodide staining. We found that AE OE *D. discoideum* cells exhibited predominant population of cells in S and G2M phases as compared to population of cells in G2/M phase in control cells at 48 hrs and 60 hrs (log-phase cells) respectively (Fig. 4.9). Similar growth pattern and cell cycle profile was exhibited by A OE *D. discoideum* cells. These results suggest that PARP-1 homeostasis is indispensable for proper cell-cycle regulation and maintenance.

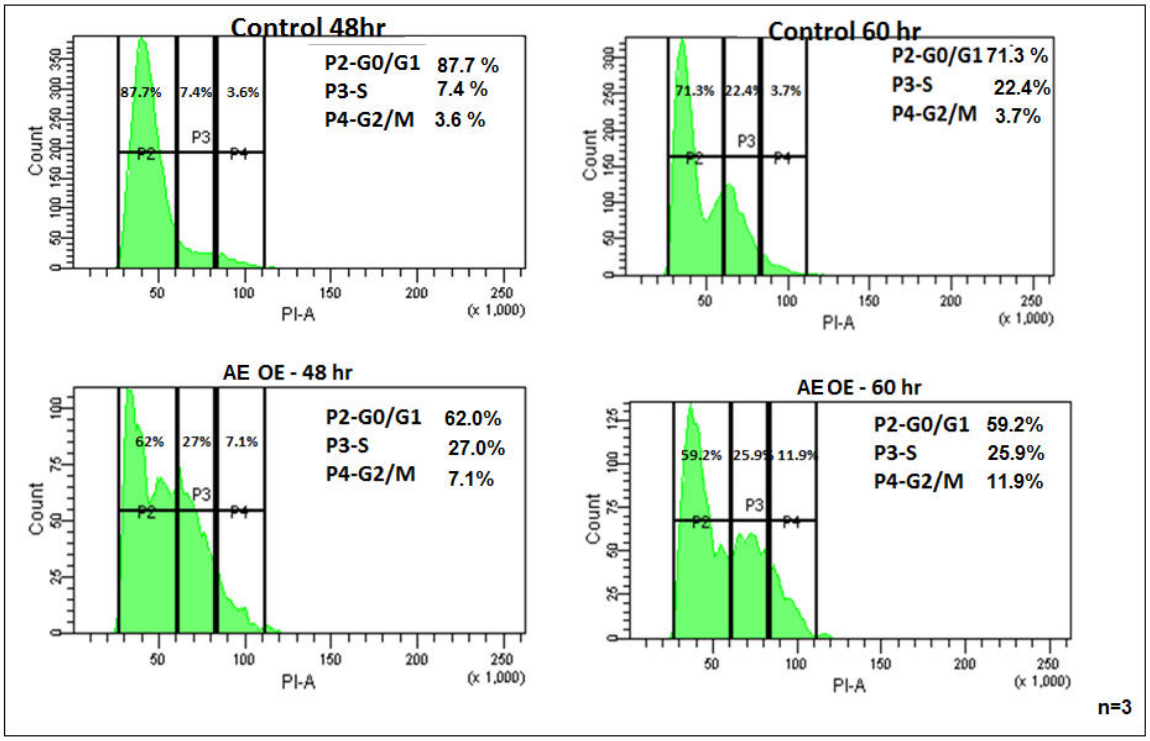


Fig. 4.9: Analysis of cells cycle by FACS using Propidium Iodide. AE OE cells show a marked population of cells in S and G2/M phase as compared to control, both at 48 and 60 hrs of growth. Data is a representative of three independent experiments.

Since the redox status of the cell influences various cellular activities including growth and cell death, the reduced cellular proliferation rates in A OE cells may be a consequence of ROS generation (Suzuki *et al.*, 1997). Our studies revealed higher ROS levels in A OE cells as compared to that of control (Fig. 4.10A). Interestingly, we established that Benzamide, a PARP inhibitor, could alone rescue the delay in growth of A OE cells while N-Acetyl Cysteine (NAc), an antioxidant showed no significant effect on the growth of A OE cells, thus indicating that decrease in NAD^+ levels or accumulation of poly-ADP-ribose product could account for the phenotypes observed in *ADPRT1A* overexpressing cells (Fig. 4.10B).

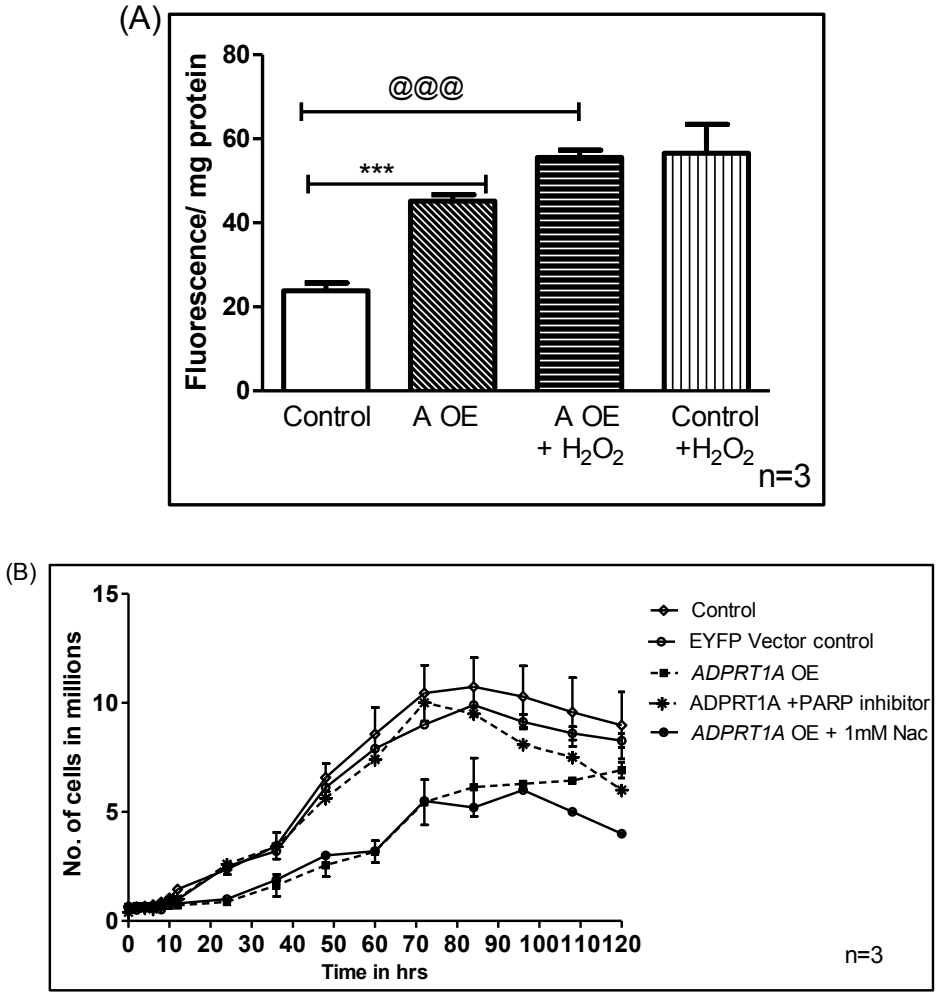


Fig. 4.10: ROS and PARP inhibition in ADPRT1A OE (A OE).

(A) Fluorometric analysis of ROS levels using DCFDA dye in control and A OE cells. A OE shows significantly higher ROS levels as compared to control. Data is a representative of three independent experiments. *** p value for A OE <0.001 as compared to control, $^{@@@}$ p value for A OE + H_2O_2 <0.001 as compared to control. (B) Rescue in slow growth of A OE on treatment with PARP inhibitor benzamide as compared to untreated A OE and control cells. However no change was seen in growth

when A OE cells were treated with antioxidant N-acetyl cysteine NAC as compared to untreated A OE and control cells. Data is representative of three independent experiments.

4.2.7 Effect of *ADPRT1A* overexpression on cell death of *D. discoideum*

Depending on the signal, PARP-1 either favors cell survival or activates cell death owing to extensive DNA damage, thus deciding cell fate. We thus, focused to study the effect of overexpression of ADPRT1A on cell survival during oxidative stress.

4.2.7.1 ROS estimation and DNA damage

ROS generation leads to DNA aberrations; the most abundant being base modification and gamma H2AX protein phosphorylation (Minami *et al*., 2005). It was seen that ROS levels in A OE cells peaked within 10 min after 0.03mM H_2O_2 treatment (standardized paraptotic dose in lab- Rajawat et al., 2014a) (Fig. 4.11) while in control cells, ROS levels peaked at 30 min. The effect of ROS on DNA damage in treated and untreated AOE cells was confirmed using pH2AX (Fig. 4.12). A OE cells treated with 0.03mM H_2O_2 showed significant DNA damage as compared to untreated control. However, it showed lesser fluorescence as compared to control cells treated with similar H_2O_2 dose.

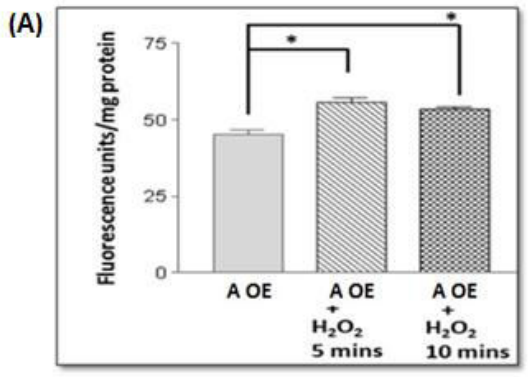


Fig. 4.11: Fluorometric analysis of ROS in control and ADPRT1A OE (A OE) cells levels using DCFDA dye. Data is representative of three independent experiments. **p* value< 0.05 as compared to control.

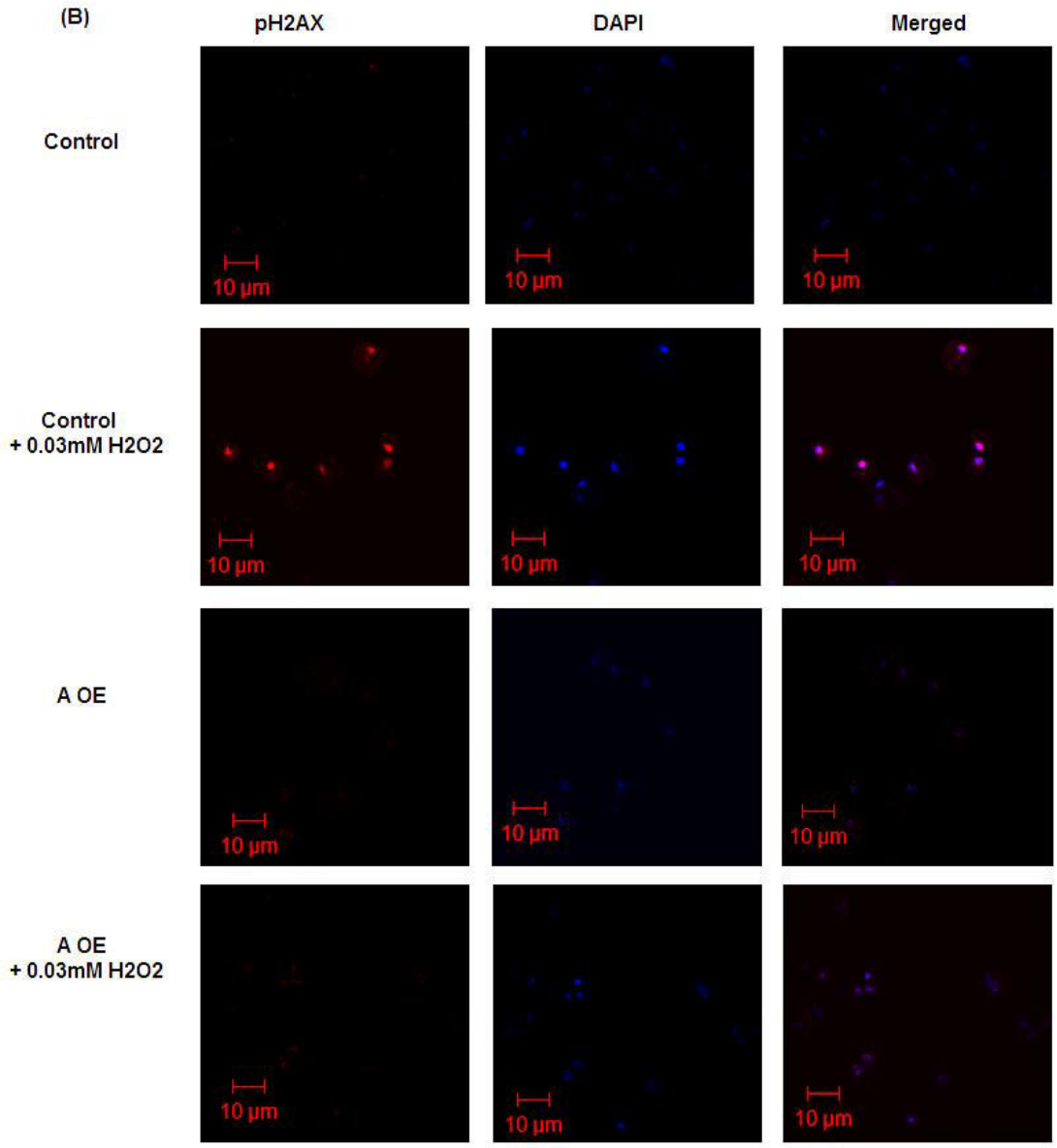


Fig. 4.12: DNA damage was observed in control and A OE 5 min post 0.03mM cumene H_2O_2 stress by immunofluorescence using antibody against pH2AX. Data is a representative of three independent experiments. Scale: 10 µm.

4.2.7.2 PARP activation under oxidative stress

Subsequent to ROS estimation, PARP activation was checked after 0.03mM H_2O_2 treatment at 2 min, 5 min and 10 min in A OE cells as well as in the control cells. A OE cells exhibited higher basal poly ADP-ribosylation activity (Fig. 4.13) as compared to control without any oxidant treatment. Moreover, in presence of oxidative stress, there was significant increase in fluorescence indicating PARP activation within 5 min (Fig. 4.13). However, in control cells highest PARP activation signals were observed at 10 min (Fig. 4.13). It was thus observed that endogenous PAR levels were higher in A OE cells. Moreover, under oxidative stress, AOE cells showed earlier PARP activation as compared to control cells.

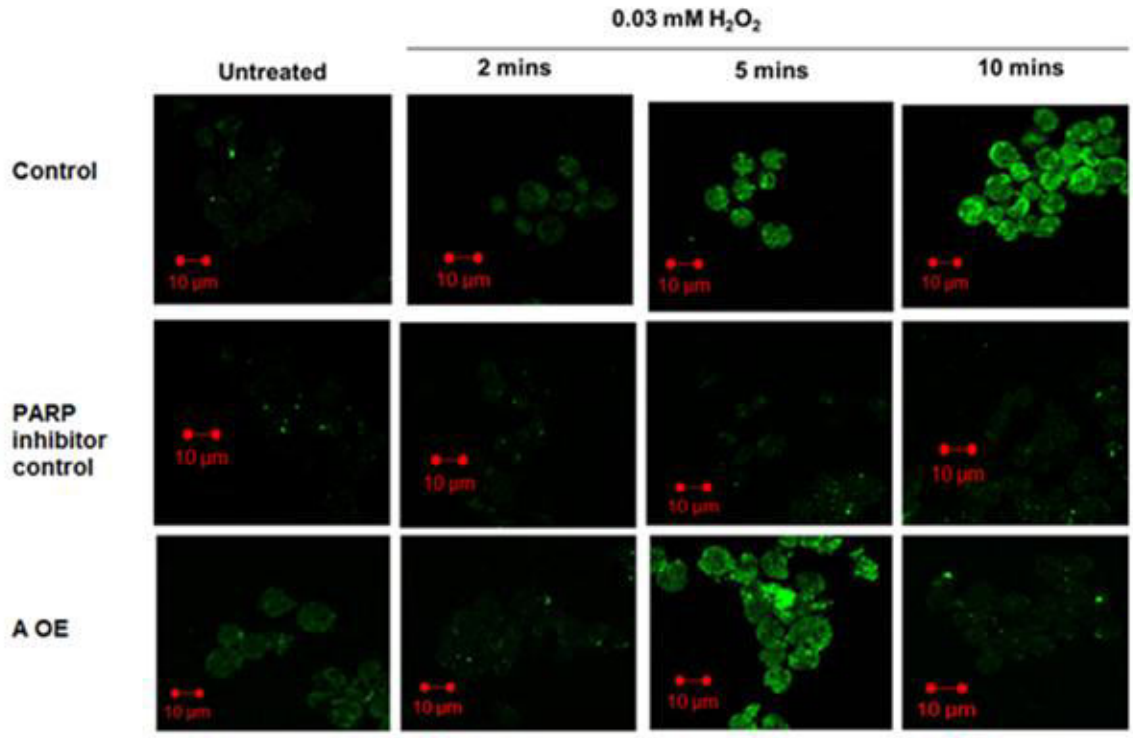


Fig. 4.13: PARP activation post 0.03mM cumene H_2O_2 treatment using anti-PAR antibodies in control, PARP inhibitor control and A OE cells by confocal microscopy at 63X magnification. PARP activity peaked at 10 min post 0.03mM cumene H_2O_2 stress in control cells as compared to 5 min in A OE cells. Data is representative of three independent experiments. Scale: 10 µm.

4.2.7.3 Mitochondrial membrane potential

The loss of mitochondrial membrane potential (MMP) is a characteristic feature of cell death and with earlier reports on PARP-1's role in cell death, overexpression of *ADPRT1A* was hypothesized to change the MMP in *D. discoideum* cells. This parameter was monitored using $DiOC_6$ dye after subjecting A OE cells to oxidative stress of 0.03mM H_2O_2 dose after 3 hrs and 5 hrs. A OE cells showed marked decrease in fluorescence within 3 hrs (Fig. 4.14A) signifying a significant loss of MMP while in control cells loss of MMP was observed at 5 hrs of 0.03mM H_2O_2 dose as opposed to PARP inhibitor treated cells wherein there was no significant loss of mitochondrial membrane potential even at 5 hrs.

MMP changes were quantified using $DiOC_6$ dye by FACS too. Population of cells showing MMP loss post 3 hrs of 0.03mM H_2O_2 dose in A OE cells were observed to be ~81% (P1 + P2), out of which 40.1% (P1: lowest fluorescence) cells (Fig. 4.14B) had very less fluorescence intensity suggesting significant mitochondrial membrane potential loss. On the other hand, control cells after 3 hrs of 0.03mM H_2O_2 dose did not show complete MMP loss while cells treated with PARP inhibitor showed no significant loss in MMP.

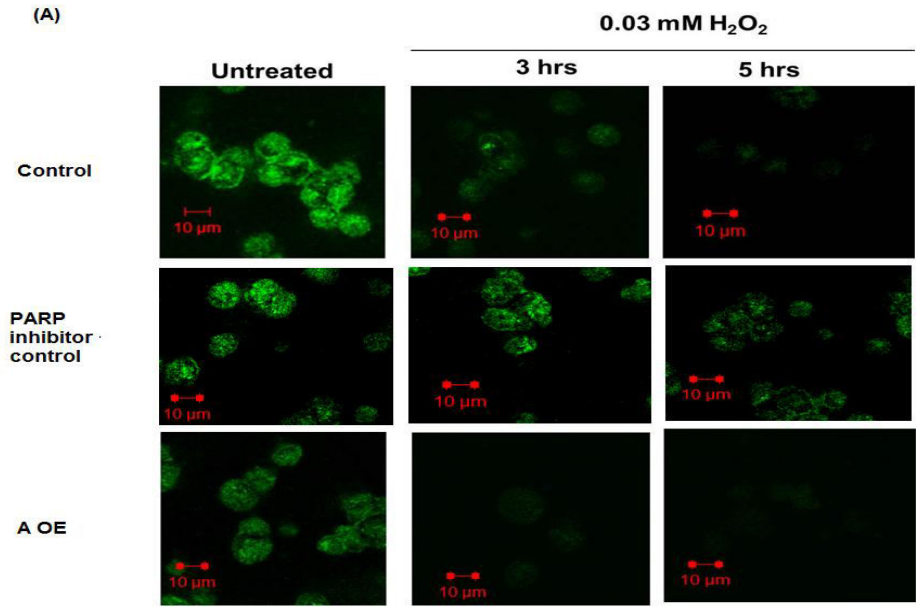
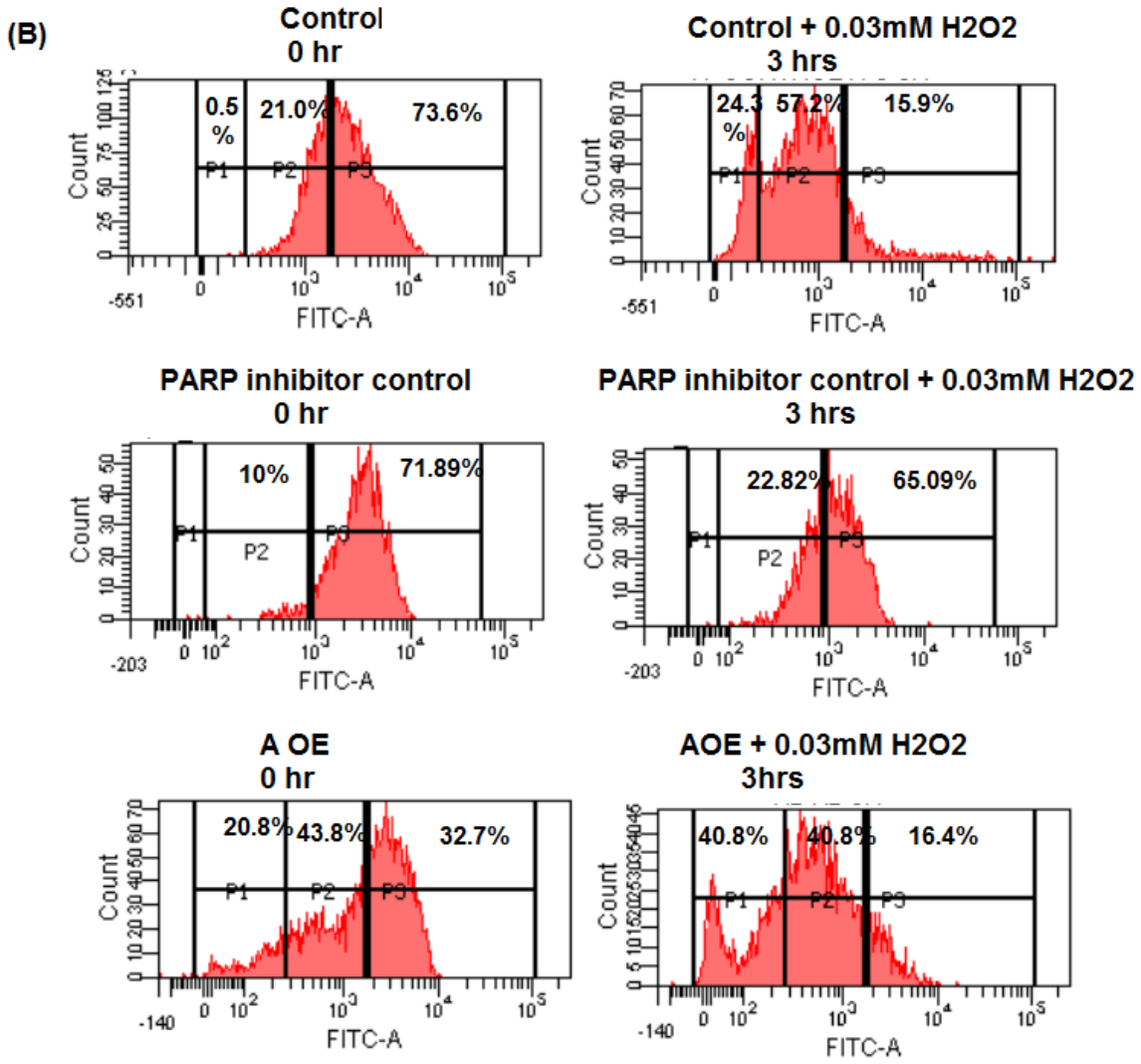


Fig. 4.14: Mitochondrial membrane potential changes in control, PARP inhibitor treated and ADPRT1A OE (A OE) cells at 3hrs and 5hrs post 0.03mM cumene H_2O_2 treatment.

(A) Mitochondrial membrane potential changes by $DiOC_6$ staining in control, PARP inhibitor treated cells and ADPRT1A OE (A OE) cells at 63X magnification by confocal microscopy. Data are representative of five independent experiments. Scale: 10 µm.



(B) FACS analysis of MMP change in A OE and control cells post 3 hrs of 0.03mM cumene H_2O_2 treatment using $DiOC_6$ dye. Data are representative of three independent experiments.

4.2.7.4 Annexin V FITC-PI

PARP-1 on over activation causes severe NAD^+ and ATP depletion resulting in energy crisis and thus results in necrosis (Edinger and Thompson, 2004). However moderate PARP-1 activation favors programmed cell death or apoptosis. All above cell death parameters exemplified *ADPRT1A* OE cells to demonstrate an oxidative stress sensitive phenotype. However, the mode of cell death in an ADPRT1A overexpressed background remains to be studied. AOE and control cells after 0.03mM H_2O_2 treatment exhibited both Annexin V-FITC and Propidium Iodide (PI) staining, however, A OE cells showed early Annexin V-FITC staining (due to earlier exposure of phosphatidyl serine) as well as early PI staining (1 hr and 3 hrs respectively) (Fig 4.15). Control cells, on the other hand, showed Annexin V- FITC and PI staining at 3 hrs and 12 hrs respectively (Fig 4.15). Collectively, these results indicate that A OE cells are susceptible to oxidative stress as 0.03mM H_2O_2 dose led to paraptotic cell death in control cells. Nevertheless, A OE cells exhibited necrotic cell death at the same dose.

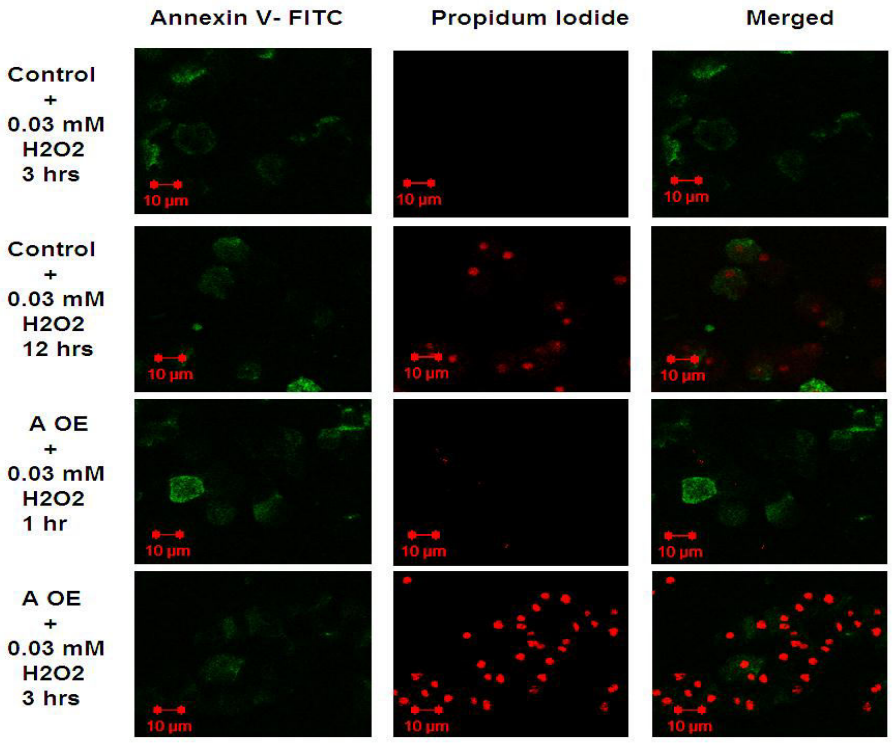


Fig. 4.15: Phosphatidylserine (PS) exposure (Annexin V-FITC Staining) and PI staining in control and *ADPRT1A* OE (A OE). Cells were observed at 63X magnification under confocal microscope. A OE cells showed both PS (denoted by green fluorescence) and PI (red) positive cells as early as 3 hrs post 0.03mM cumene H_2O_2 treatment indicating necrotic mode of cell death while control cells showed only PS (green fluorescence) positive cells at 3hrs indicating paraptotic mode of cell death under the same oxidative stress. Data are representative of three independent experiments. Scale: 10 µm.

4.2.8 Effect of *ADPRT1A* overexpression on *D. discoideum* development

Developmental morphogenesis involves large scale changes in gene expression. PARP-1 is stated to regulate gene expression via transcriptional control thereby controlling growth and differentiation (Ji and Tulin, 2010). To identify the role of *ADPRT1A* during *D. discoideum* development, control and A OE cells were starved followed by study of developmental morphogenesis. Interestingly, A OE cells showed delay at aggregation

stage. Streaming started in these cells at around 8 hrs thereby forming loose aggregates at 10 hrs and tight aggregates at 14 hrs, tipped aggregates at 18 hrs followed by slug formation at 22 hrs. However, A OE cells remained in the slug stage until 28 hrs and also showed much delay in fruiting body formation at 38 hrs as opposed to 24 hrs in control cells (Fig. 4.16). Thus the aggregation stage seems to be affected in *ADPRT1A* OE cells. Hence these cells required prolonged time to form the culminants.

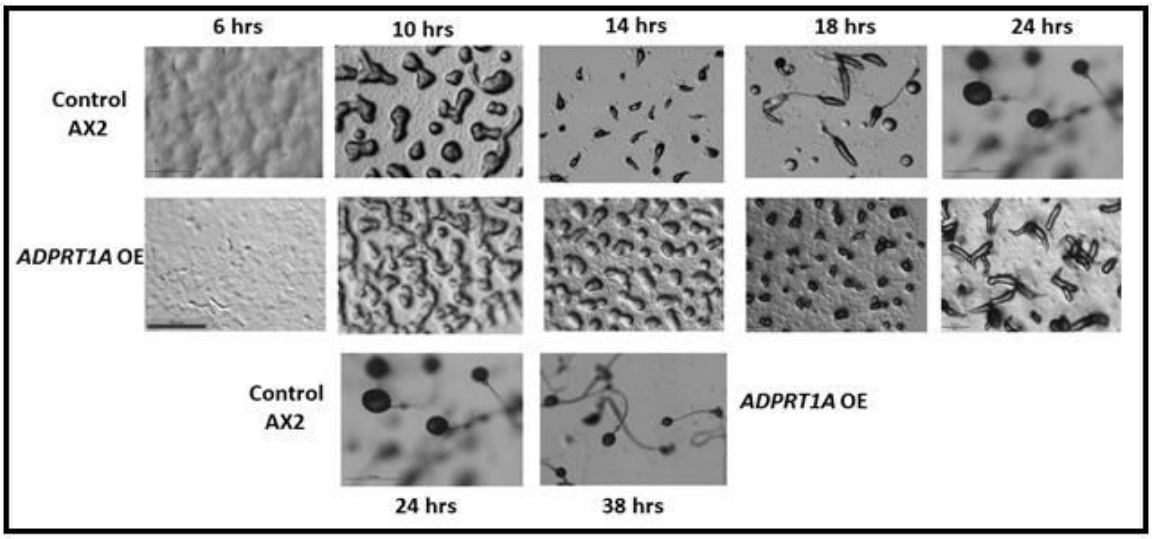


Fig. 4.16: Role of ADPRT1A in *D. discoideum* development: Development of control and *ADPRT1A* OE cells were developed on non-nutrient agar and allowed to develop at 22^{o}C followed by synchronization. Photographs were taken at various time points. Scale: 100µm. Data are a representative of three independent experiments.

4.2.9 *ADPRT1A* transcript levels and PARP activation during *D. discoideum* development

Additionally, to understand whether ADPRT1A expression is developmentally regulated in *D. discoideum,* the expression pattern of *ADPRT1A* during the growth and developmental stages were examined by Real Time PCR. Interestingly, *ADPRT1A* transcript levels were highest at the loose aggregate stage followed by significant decrease at the tight aggregate stage after which an increase was seen at the migrating slug stage. The final culminant stage *i.e* the fruiting body of *D. discoideum* exhibited the

lowest transcript levels of *ADPRT1A* (Fig. 4.17). However, during development, there is a significant reduction in *ADPRT1A* mRNA levels as compared to vegetative stage. Although the expression was seen to decrease at the onset of development, PARP activity was found to be significantly higher in cells subjected to nutrient starvation from 2 hrs till 6 hrs than the vegetative stage implicating a strong role of PARP during developmental morphogenesis of *Dictyostelium* and precisely during aggregation (Fig. 4.18). Also, these results are consistent with the above results wherein aggregation stage seems to be affected more due to ADPRT1A overexpression. These results and the existing literature suggest ADPRT1A homeostasis may be essential for the aggregation process in *Dictyostelium.*

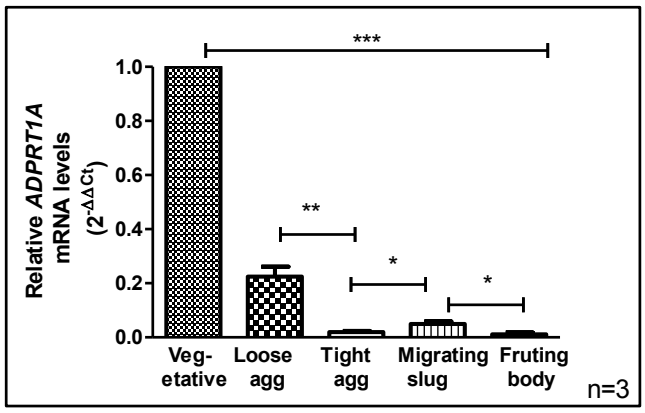


Fig. 4.17: Developmental gene expression pattern of *ADPRT1A*. Change in gene expression was analyzed by Real time PCR using total RNA samples from *D. discoideum* control cells in the growth and developmental phases as templates. Relative mRNA levels ($2^{-\Delta\Delta Ct}$) as compared to *ADPRT1A* transcript levels in the vegetative phase were plotted graphically. Data are representative of three independent experiments.

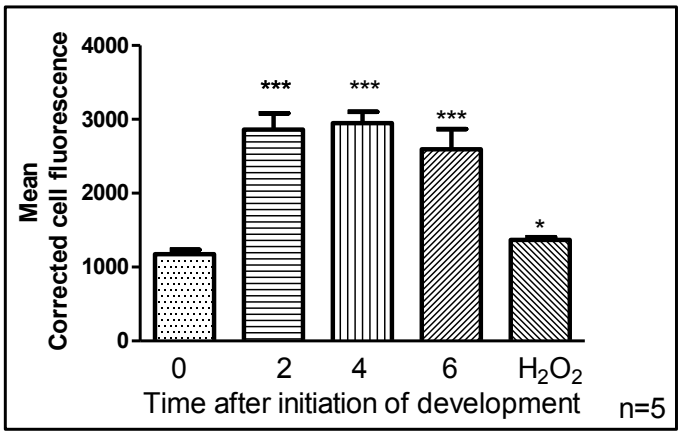


Fig. 4.18 Densitometric analysis of PARP activation up to 6 hrs of nutrient starvation. Mean corrected cell fluorescence for cells showing PARP activity was checked using FITC labelled Anti-PAR antibodies by confocal microscopy. *** *p* value<0.001, * *p* value<0.05 as compared to control. Data are a representative of five independent experiments.

In conclusion, the above results illustrate a definite role of *ADPRT1A* in *D. discoideum* growth and multicellularity.

4.3 Discussion

PARP-1 is an abundant and ubiquitous nuclear enzyme involved in many diverse functions like DNA repair, transcription regulation, cell death etc. In addition, recent reports suggest a novel PARP-1 function in multicellularity and also differentiation. PARP deletion mutants in Drosophila develop only up to larval stages due to chromatin remodelling defects and regulation of gene expression (Tulin and Spradling, 2003) suggesting that PARP activity is crucial for normal development. In addition, overexpression of *PrpA* (homolog of PARP) leads to increased spore production and colonies largely consisting of aerial hyphae and a fluffy phenotype, reflecting PARP's role in regulating the expression and/or proteins activity which are essential in its development (Semighini *et al*., 2006). Interestingly, our earlier reports are also indicative of the role of PARP-1 in multicellularity in *D. discoideum* since downregulation of PARP

using antisense against the conserved catalytic domain ensued in stalled development at the aggregate stage of *D. discoideum* (Rajawat *et al*., 2011). Thus, an understanding of how the PARP-1 ortholog (*ADPRT1A)*, functions during growth and multicellularity of *D. discoideum* would help us to pinpoint its less studied functions that still remain obscure.

The *Dictyostelium* genome codes for eight PARP proteins (Kawal *et al*., 2011). The i*n silico* analysis by phylogenetic tree construction and domain organization prediction studies identified ADPRT1A as the human PARP-1 ortholog in *D. discoideum*. It is already known that PARP activation results in the drop of NAD^+ levels upon excessive DNA damage (Berger, 1985) and hence triggering NAD^+ turnover (Houtkooper *et al*., 2010). Similar results were seen in ADPRT1A overexpressed cells wherein there was significant NAD^+ depletion (Fig. 4.7). This suggests that PARP is not only overexpressed but also activated in ADPRT1A overexpressed cells. This result is consistent with our PARP activation results where basal PARP activity was higher as compared to control cells (Fig. 4.13). Higher basal levels of PAR were also reported during PARP overexpression in transfected hamster cell lines thus indicating PARP activation (Gool *et al*., 1997). In addition, it was seen that *ADPRT1A* overexpression led to the downregulation of the other PARP isoforms *ADPRT1B* and *ADPRT2* (Fig. 4.6). This decrease in *ADPRT2* and *ADPRT1B* levels could be the cells' mechanism to circumvent further reduction of the common substrate for all the isoforms *i.e.* NAD^+, thereby keeping the cells viable. This is reinforced by the growth curve of the AOE cells wherein in spite of the high NAD^+ consumption; the cells are viable but exhibited delayed growth. The decrease in ADPRT isoforms is supported by a report wherein overexpression of the Na^+/K^+ ATPase α2 elicited downregulation of the other isoform α1 such that the total levels of $Na^{+/}K^+$ ATPases were preserved (Correll *et al.,* 2014).

NAD^+ and ATP depletion can be seen as a result of PARP activation in response to DNA damage due to ROS (Canto *et al*., 2013). Our results are consistent with the fact that despite having high ROS levels (Fig. 4.11), AOE cells showed negligible cell death. This could be due to the presence of more PARP molecules in ADPRT1A overexpressed cells

as compared to control, making the cells more equipped to combat the increased DNA damage caused by ROS as seen in Fig. 4.12 wherein untreated AOE cells do not show significant DNA damage. Reports suggest that ADPRT1A is involved in double stranded break repair while ADPRT1B and ADPRT2 are required for tolerance to single stranded breaks (Tulin and Spradling, 2003). Couto group also explained that in absence of ADPRT2, ADPRT1A signals single strand DNA lesions to promote resistance of the cells to DNA damage (Couto *et al.*, 2013). Likewise, our results clearly indicate lower *ADPRT1B* and *ADPRT2* levels in AOE cells as compared control cells (Fig. 4.6). Hence, overexpressed *ADPRT1A* may compensate for the other isoforms. Together, these results put forward that ADPRT1A homeostasis is very essential, and any disruption/change in ADPRT1A levels and over-activated PARP may hamper the normal functioning of the cell.

Higher NAD^+ and ATP reduction along with high basal ROS thus justify increased doubling time (~18 hrs) in *ADPRT1A* overexpressed cells (Fig. 4.8). Therefore, decreased availability of energy would result in slow growth of *ADPRT1A* overexpressed cells. Apart from NAD^+ and ATP reduction, cell cycle analysis also showed that population of cells in S and G2/M phase was higher in *ADPRT1A* OE cells as compared to control (Fig. 4.9). Thus ADPRT1A also seems to affect cell-cycle regulation in *ADPRT1A* OE cells. This goes in accordance with literature which suggested that PARP-1 over-expression results in an increase in the number of cells in the diploid population (Bhatia *et al.*, 1996). Additionally, PARP-1 is vital for the induction of G1 arrest and is also involved in the regulation of G2 arrest (Masutani *et al.*, 1995). Conversely, PARP-2 is reported to regulate cell cycle-related genes independent of poly (ADP-ribosyl)ation (Liang *et al.*, 2013). This study thus provides the first report in *Dictyostelium* wherein ADPRT1A plays a role in cell cycle control via poly (ADP-ribosyl)ation.

PARP-1 is a well-established mediator of necrotic mode of cell death (Edinger and Thompson, 2004; Rajawat *et al.*, 2014b). It is known that in *D. discoideum*, high oxidative stress leads to DNA damage and subsequent PARP activation (Rajawat *et al.*, 2007). PARP-1 depletes cellular NAD^+ and ATP levels that cause necrotic cell death

instead of apoptosis (Ha and Snyder, 1999). Likewise, *ADPRT1A* overexpressed *D. discoideum* cells showed susceptibility to oxidative stress during 0.03mM H_2O_2 insult resulting in necrosis (Fig. 4.15).

In addition to PARP's role in growth, few evidences also propose that it might play a key role in development and differentiation. *PARP-1* and *PARP-2* double knockouts mice were found to be embryonically lethal (Henrie *et al*., 2003), suggesting the role of PARP in multicellularity. Constitutive *PARP* downregulation obstructs development at the aggregate stage (Rajawat *et al*., 2011). Interestingly, *ADPRT1A* OE cells showed delayed streaming, loose aggregation and early culminant stages (Fig. 4.16). Aggregation defective phenotypes of *D. discoideum* show disruption in cAMP signaling (Bader *et al*., 2006; Sawai *et al*., 2007). PARP-1 is identified to interact with histones and chromatin modifying enzymes to control their activity at target gene promoters, ultimately regulating gene expression (Frizzell *et al*., 2009).

Thus, our results show that analysis of endogenous *ADPRT1A* transcript level during *D. discoideum* development display highest *ADPRT1A* transcript levels at aggregation stage (Fig. 4.17). Furthermore, the developmental stimulus of nutrient starvation not only led to increased transcripts but also caused a significant increase in PARP activity in the aggregation stages (Fig. 4.18). Masutani *et al*., also report significant change in PARP mRNA expression throughout the developmental stages of *Sarcophaga peregrina* (Masutani *et al*., 2004). In conclusion, it can be established that *ADPRT1A* has a definite role in development and may affect the cAMP signaling which is an important signaling molecule for aggregation. Our results for the first time reveal that there is increased PARP activity and *ADPRT1A* expression is developmentally regulated during *D. discoideum* development. Thus, it would be interesting to further explore how ADPRT1A affects *D. discoideum* developmental morphogenesis which would be addressed in the next chapter.

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4.4 References

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