Materials & Methods

CHEMICALS

Hydroxylamine, cumene H_2O_2, benzamide, gallotannin, trypan blue, pepstatin A, ALLN, NAD^+, agarose, caspase-3 assay kit, Annexin V-FITC/PI dual staining kit, APO-BRDU kit, Caspase-3 assay kit, 1,6 diphenyl 1,3,5-hexatriene (DPH), 4',6-diamidino-2-phenylindole (DAPI), 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$), 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA-AM), Protease Inhibitor Cocktail, anti-mouse IgG (whole molecule) FITC conjugate, anti rabbit IgG (whole molecule) TRITC conjugate, sheep anti-cyt c polyclonal antibodies raised against rabbit, anti sheep IgG (whole molecule) TRITC conjugate (Sigma, USA), anti-PAR mouse mAb (10H) (Calbiochem, Germany), N-succinyl-LLVY-AMC (Calbiochem, Germany), TRIZOL reagent (Invitrogen, USA), rabbit anti-AIF polyclonal antibodies raised against amino acids 151-180 of human AIF (Cayman Chemical, USA). Other reagents were of analytical grade from Merck and Himedia.

METHODS

D. discoideum culturing conditions

D. discoideum, Ax-2 strain which is an axenic derivative of Raper's wild type NC-4 (a mutant in at least two genes i.e. *axe* A and *axe* B, Newell *et al.*, 1982 and Deering *et al.*, 1988) was used. *D. discoideum* was grown under different culture conditions. The growing cells (unicellular) were maintained in a liquid suspension (HL5 medium). *D. discoideum* cells were grown in modified HL-5 medium, pH 6.5 with 150 rpm shaking at 22°C (Watts and Ashworth, 1970). Log phase cells at a density of ~2.5 X 10^6 cells/ml were used for experiments.

It is also maintained on a solid substratum containing Phosphate Buffered Agar (PBA). *D. discoideum* was also cultured on bacterial lawn of *Klebsiella* which is its natural food. For this a loop full of overnight grown culture of *Klebsiella* is taken and *D. discoideum* spores (4-5) are mixed with it. This 'mixture' of two cell types is then pour plated on PBA plates (90mm). *D. discoideum* cells feed on *Klebsiella* and when no more *Klebsiella* is left the *D. discoideum* cells undergo developmental changes and form fruiting bodies.

Preparation of Axenic Medium (Oxoid) – (modified HL5medium, Ashworth and Watts, 1970; Kosta *et al.*, 2001)

Proteose peptone	-	14.3 gm
Yeast extract	-	7.5 gm
Maltose	-	18 gm
$Na_2HPO_4\,2H_2O$	-	0.616 gm
$KH_2PO_4\,2H_2O$	-	0.486 gm

The above constituents were added to 1000ml of distilled water and pH was adjusted to 6.7. The medium (10 ml) was then dispensed into conical flasks and were autoclaved at 15 psi for 15 minutes. Care was taken so that the medium would not be charred by over heating.

Phosphate Buffer / Sorenson's buffer / KK_2 buffer (50X SB)

2mM Na_2HPO_4

15mM KH_2PO_4

pH 6.4

It was sterilized by autoclaving at 15 psi for 15 minutes. 1X SB buffer was obtained by adding 1 ml of 50X SB to 49 ml of autoclaved distilled water.

Phosphate Buffered Agar (PBA)

2 gm agar was added to 98 ml distilled water and autoclaved at 15psi for 15 minutes. After autoclaving 2 ml 50X SB was added.

Culture Maintenance

Growth of Ax-2 cells in Axenic medium (Levraud *et al.*, 2001)

i). 10 ml of the autoclaved HL-5 medium was taken and approximately 100 µl (inoculum size = 1% of medium volume, approx 0.05 X 10^5 cells/ml) of log phase culture with a cell density of $2X10^6$ cells/ml were used for inoculation in the medium under sterile conditions.

ii). The flasks were incubated at 22 ^{0}C under shaking conditions maintained at a speed of 150 rpm.

iii). Subculturing was done every week. Subculturing can be done twice (till one month), after which the culture must be revived from the spores. The first or second subculture from this spore revival was used for experimental purposes.

Differentiation of *D. discoideum* cells on non nutrient agar (Levraud *et al.*, 2001)

i). Exponentially growing cells from HL-5 medium were pelleted down at 300g/5min/4^{0}C.

ii). The pellet was washed with 1X SB (Sorenson's buffer) thrice at 300g/5min/4^{0}C.

iii). The final cell density was adjusted to 2.5 X 10^6 cells / ml and 100µl of cell suspension was then spread on PBA (non-nutrient agar) containing 35 mm plate and allowed to differentiate at 22 ^{0}C to form fruiting bodies.

Spore Revival

- 5 ml HL-5 medium was dispensed in 25 ml conical flasks and autoclaved.
- The fruiting bodies were picked up from the surface PBA plates with a sterile Nichrome loop. The loop must not be allowed to touch the agar surface at any point, and all other precautions must also be taken to avoid bacterial contamination.
- Few fruiting bodies were inoculated in each flask, and then incubated under shaking conditions at 22 ^{0}C /150 rpm. Spores germinate with overnight incubation but log phase culture that can be used for further sub-culturing, could be obtained after about a week.

Cell viability test by trypan blue exclusion technique (Kosta *et al.*, 2001)

This is a vital staining procedure, where Trypan blue stains dead cells exclusively as live cells can pump out the dye. The stained dead cells then appear blue, while viable cells remain colourless. 20 µl of the culture to be tested is taken and 10 µl of 0.1% Trypan blue solution is added. A cell count is taken using the haemocytometer. The percentage viability is then calculated using the following formula

Number of dead cells X 100 = % Dead cells

Total number of cells

Note: Trypan blue is commercially available as 0.4% stock solution. Diluted it 4 times with 1X SB to make 0.1% working solution.

Induction of oxidative stress

Oxidative stress was induced in *D. discoideum* cells by *in situ* generation of H_2O_2 upon addition of HA or by exogenous addition of cumene H_2O_2 (Sigma), (Kono and Fridovich, 1983). Log phase cells at a density of ~2.5 X 10^6 cells/ml were exposed to different doses of HA (0, 1, 2.5, 4 mM) and cumene H_2O_2 (0, 0.03, 0.05 mM) in HL-5 medium at 22°C in a sterile flask. Cell viability was assayed using Trypan blue after 24 hours of HA or cumene H_2O_2 stress (Kosta *et al.*, 2001).

Assessment of cell death by AnnexinV-FITC/PI dual staining (Miller, 2004)

To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI was performed using Apoptosis detection kit (Molecular Probes, USA). Phosphatidyl serine (PS) exposure on the surface of the cells is one of the characteristic features of apoptotic cells. It is also a signal for macrophages to clear such cells by phagocytosis which is important to prevent inflammation and damage to the surrounding cells. PS has an affinity to bind to Annexin in a Ca^{+2} dependent manner. Annexin can bind to PS only when it is exposed outside as it cannot cross the membrane. Propidium Iodide (PI) is a DNA binding dye which can enter the cell only when membrane integrity is completely lost. Thus early apoptotic cells will be Annexin+ and PI- while late apoptotic and necrotic cells will be Annexin+ and PI+.

~2.0X10^6 cells were pelleted and washed twice with 1X SB. *D. discoideum* cells were then suspended in binding buffer provided in the kit and incubated with Annexin V and PI for 10 minutes in dark at 22°C. Fluorescence was monitored under 60X magnification using fluorescent microscope (Nikon Eclipse TE2000-S, Japan). Dose and time dependent study was done to standardize the apoptotic and necrotic doses for further experiments.

Measurement of PARP activity by indirect immunofluorescence (Cole and Perez-Polo, 2002)

PARP activity was assayed indirectly by using antibodies against the product of PARP i.e. PAR. For assaying PARP, indirect immunofluorescence was done using anti-PAR mouse mAb (10H) (Calbiochem, Germany) at a concentration of 0.5ug/ml and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:200. *D. discoideum* cells were pelleted and washed once with phosphate buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 minutes at -20 °C and then washed with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS) followed by incubation for 1 hour in primary antibody. After incubation the cells were washed 2-3 times with blocking solution and further incubated for 1 hour with FITC labeled secondary antibody. Finally these cells were washed 2-3 times with PBS and the fluorescence was observed at 490 nm under 60X magnification. PARP assay was performed at different time intervals as described in the experiments later.

ROS measurement by DCFDA dye (Esposti, 2002)

In order to observe the formation of reactive oxygen species a fluorescent dye is employed. The acetate ester form of 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA-AM) is a membrane permeable molecule that passes through the cell membrane. Once inside the cell, cellular esterases act on the molecule to form the non-fluorescent moiety H2DCFDA, which is ionic in nature and therefore trapped inside the cell. Oxidation of H2DCFDA by ROS converts the molecule to 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence signal over time.

$2X10^6$ cells were harvested and washed with 1X SB twice. DCFDA (50 nM) was added to cells and was incubated for 15 min at 22 ^{0}C with shaking, followed by two washes with 1X SB. Fluorescence unit was measured by fluorimeter (F7000, Hitachi, Japan) using 200 µl sample diluted 5 times using KK2 buffer. Excitation (λex) and emission (λem) wavelengths used for fluorimetric studies were 480 and 525 nm respectively.

Estimation of NAD^+ and ATP levels (Bernofsky and Swan, 1973; Leoncini *et al.*, 1987)

Intracellular levels of NAD^+ in *D. discoideum* were determined by enzymatic recycling method employing alcohol dehydrogenase to reduce NAD^+ to NADH. NAD^+ present in the cell is used to convert ethanol into acetaldehyde, the reaction being catalyzed by alcohol dehydrogenase. NADH produced in the first reaction is used to reduce MTT in to Formazan and NAD^+ is regenerated. In this way cellular NAD^+ keeps on getting recycled and thereby enhances the yield of Formazan, a chromophore which gives absorbance at 570 nm. In brief, cells were exposed to 0.05 and 0.1 mM cumene H_2O_2 for 1 hour and cultures were washed twice with ice cold PBS and NAD^+ was extracted with 1 ml of 0.5 M perchloric acid and then neutralized with 1 N KOH. NAD^+ levels were estimated by taking the absorbance at 570 nm following protein estimation with Lowry method (Lowry *et al.*, 1951).

ATP was determined by HPLC in aliquots extracted with alkali according to Leoncini *et al.*, (1987). Cells exposed to 0.05 and 0.1 mM cumene H_2O_2 were washed with cold saline solution (containing 10mM glucose and 2mM EDTA) after 1 hour of stress induction. Nucleotides were extracted by treating the cell pellet with 0.5 M KOH which were kept on ice for 2 minutes and immediately sonicated (four 10 sec bursts) to ensure maximal extraction. The extract was neutralized with 1 M KH_2PO_4, centrifuged at 8000g for 10 minutes and the supernatant was used for HPLC analysis using rp-C18 column (5μm) and UV detector. 1 M KH_2PO_4 was used as mobile phase and flow rate was maintained at 1 ml/min.

Evaluation of mitochondrial membrane potential (Koning *et al.*, 1993)

Potential sensitive dye $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide) (Sigma) was used to evaluate changes in mitochondrial membrane potential (MMP). $DiOC_6$ is a membrane permeable lipophilic cationic dye which binds specifically to mitochondria proportionally to the difference between the negativity of the cytoplasmic compartment and the mitochondrial matrix. Dissipation of the mitochondrial membrane potential reduces the affinity of binding of the dye.

To observe the change in MMP, time dependent study was done using 0.05 mM cumene H_2O_2 dose (non-apoptotic dose) and 0.1 mM cumene H_2O_2 dose (necrotic dose) as standardized by PS-PI dual staining. ~$2.0X10^6$ cells were pelleted and

washed twice with 1X SB. Cells were stained with $DiOC_6$ (400 nM) for 15 minutes in dark and then washed once with 1X SB and monitored for the fluorescence as above.

Monitoring AIF release by indirect immunofluorescence (Bidere *et al.*, 2003)
Release of AIF from mitochondria to cytosol and its translocation to nucleus was monitored by detecting immunofluorescence at different time intervals. Same protocol as for PAR assay was followed using rabbit anti-AIF polyclonal antibodies raised against amino acids 151-180 of human AIF (Cayman Chemical) at 1:1000 dilution and anti rabbit IgG (whole molecule) TRITC conjugate (Sigma) at 1:400 dilution. Nuclear counterstaining with DAPI (1ug/ml) for 5 minutes was performed after the removal of excess secondary antibody and observed for the fluorescence.

Cytochrome c release (Granville *et al.*, 2001)
Release of cytochrome c from mitochondria into cytosol was monitored by detecting immunofluorescence at different time intervals. Same protocol as for AIF was followed using sheep anti-cyt c polyclonal antibodies (Sigma) raised against rabbit at 1:200 dilution and anti sheep IgG (whole molecule) TRITC conjugate (Sigma) at 1:400 dilution. Nuclear counterstaining with DAPI (1ug/ml) for 5 minutes was performed after the removal of excess secondary antibody and fluorescence was monitored.

Monitoring DNA fragmentation by agarose gel electrophoresis and TUNEL assay (Noegel *et al.*, 1985; Gavrieli *et al.*, 1992)
Genomic DNA and large scale fragments (>50kb) were run on 0.8% agarose gel. The DNA fragmentation creates a large number of 3'-hydroxyl sites at the DNA breaks. This property is used in the APO-BRDU kit (Sigma, USA) to identify apoptotic cells by labeling the 3'-hydroxyl sites with bromodeoxyuridine triphosphate (Br-dUTP). Br-dUTP is enzymatically attached to the 3-hydroxyl sites of double- or single-stranded DNA by terminal transferase (TdT). Non-apoptotic cells do not incorporate Br-dUTP due to the lack of available 3-hydroxyl sites.
Initial standardization of DNA fragmentation analysis was done at different time intervals. After exposure of cells to 0.05 and 0.1 mM cumene H_2O_2 for 6 and 3 hours respectively DNA isolation was carried out from the cells by the conventional method

as described by Noegel et al. (1985) and was subjected to electrophoresis using 0.8% agarose gel. TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) was performed according to the procedure supplied by the manufacturer (ApoBrdu DNA fragmentation kit; Sigma). Fluorescence was monitored by using Anti Brdu-FITC antibody.

PARP inhibition by benzamide (Szabo and Dawson, 1998)

Log phase culture of *D. discoideum* with a cell count of $2X10^6$ cells was incubated with different doses (1.0, 2.0, 3.0, 4.0 mM) of benzamide (Sigma, USA) for 24 hours. For further experiments, 1 mM benzamide was used as it showed 2% cell death only. Cells were preincubated with 1 mM benzamide and then treated with HA or cumene H_2O_2 and observed for different cell death parameters. To show the specificity of benzamide similar studies were also carried out with non inhibitory structural analog of benzamide, benzoic acid.

Detection of caspase activity (Olie *et al.*, 1998)

The substrate DEVD-AMC is an oligopeptide that is covalently linked with the fluorophore 7- amino 4- methyl coumarin (AMC). DEVD is the cleavage site for Caspases (3 and 7). The cleavage takes place at the C terminal of the last aspartate residue, thus liberating the fluorophore AMC which can be estimated and/or visualized under the fluorescence microscope (AMC λ_{ex} = 380 nm).

~$2.0X10^6$ cells were harvested and washed with PBS. Cells were resuspended in 1ml PBS and an aliquot of 100 µl was taken from it and 10µl of DEVD-AMC substrate (1mg/ml) was added. After an incubation of 1 hour cells were observed under the fluorescence microscope. AMC liberated from the fluorogenic substrate was measured at 380 nm using UV filter. Caspase activity as a function of AMC fluorescence was monitored at different time intervals. Effect of caspases on cell death was also studied by using caspase-3 specific inhibitor. The entire procedure was carried out using Caspase-3 assay kit (Sigma) as per manufacturer's instructions.

Characterization of apoptotic vesicles (Gautam and Sharma, 2002)

Isolation of apoptotic vesicles was performed from *D. discoideum* culture after 16 hours of 1mM HA stress as mentioned by Gautam and Sharma (2002). The culture

was centrifuged at 1000g for 4minutes at 4°C; the supernatant was collected and centrifuged at 21,000g for 45minutes at 4°C. The obtained pellet was washed once with 1X SB and used for further analysis. Formation of apoptotic vesicles was monitored at different time intervals. Isolated vesicles were stained separately with a fluorescent membrane probe, 1,6 diphenyl 1,3,5-hexatriene (DPH) at a concentration of 1μM, 4',6-diamidino-2-phenylindole (DAPI), PS-PI and then observed under fluorescence microscope.

Investigation of proteases involved in cell death

Cells were pre-incubated with protease inhibitor cocktail (Sigma, USA) followed by HA stress and MMP changes and PS exposure were checked. Similarly MMP changes, PS exposure and vesicle formation were followed in HA treated cells pre-incubated for 12 hours with 0.5 μg/ml pepstatin A, a cathepsin D inhibitor and 10 μM ALLN, a Calpain inhibitor.

Calpain activity (Moubarak *et al.*, 2007)

Calpain activity present in total cell lysates of $2X10^6$ cells of control and oxidative stressed *D. discoideum* was determined by cleavage of the fluorescent substrate N-succinyl-LLVY-AMC (Calbiochem, Germany). Calcium dependent fluorescence was measured after 30 min incubation at 37 °C in buffer containing 63 mM imidazole-HCl, pH 7.3, 10 mM β-mercaptoethanol, and 5 mM $CaCl_2$. Fluorescence was recorded in a fluorimeter.

Genomic DNA Isolation from *D. discoideum* (Pilcher *et al.*, 2007)

10^7cells/ml were harvested and washed once with 1X SB, followed by one wash with ice cold 0.2 % NaCl (0.034M). One volume 2% SDS was added and incubated at 65 °C for 15'. One volume TE buffer (pH 9.5) was added and then extracted with one volume of Phenol: Chloroform (1:1). The upper aqueous layer was collected after centrifugation at 9000g for 5'and extracted it with one volume of chloroform. Centrifuge at 9000g for 5' and collect the upper aqueous layer. $1/10^{th}$ volume of sodium acetate and one volume of isopropanol was added. Complete precipitation was done by incubating at -20 °C for more than 30'. The pellet was obtained by

centrifuging at 9000g for 5'. Washed the pellet once with 75% ethanol. Dried the pellet in oven and resuspended it in autoclaved DDW (~20 µl). The DNA preparation was stored at -20 °C till further use.

PCR Standardization for Amplification of PARP

PCR reaction was setup as follows:

DNA	3.0µl (100 ng)
Fordward Primer	0.5µl (10 pmol)
Reverse Primer	0.5µl (10 pmol)
Master Mix (2X)	12.5µl
DDW	8.5µl
Total	25µl

Reaction parameters were as follows:

Reaction parameters	**Temperature and Time**
Initial Denaturation	94°C for 6 min
Denaturation	94°C for 45 sec
Annealing	60°C for 30 sec
Polymerization	72°C for 45 sec
Repeat cycle 29 times	
Final Extension	72°C for 10 min
Hold	22°C

Plasmid isolation from *E. coli*

Alkaline lysis method for isolation of plasmid from *E. coli* has been followed (Sambrook and Russell, 2001):

2 ml over night grown culture was pelleted down. Washed once with 0.25 volume of STE and 100 µl of AL-1 was added and vortex vigorously. 200 µl AL-II was added and mixed well. 150 µl AL-III was added and mixed well, stored on ice for 10 min. Centrifuged at 9K/5' and collected supernatant. It was extracted with one volume of Phenol: Chloroform (1:1). Centrifuged at 9K/5' and the upper aqueous layer was transferred in fresh eppendorf. It was then extracted with one volume of Chloroform. Centrifuge at 9K/5' and again collect the upper aqueous layer. $1/10^{th}$ volume of Sodium acetate and one volume of Isopropanol was added to it and incubated at -20

°C for 30'. Centrifuge at 9K/5' and supernatant was discarded. Washed once with 75% ethanol and evaporated the ethanol completely. Finally pellet was resuspended in autoclaved DDW (~20 µl).

Transformation of plasmid DNA in *E.coli* (Sambrook and Russell, 2001)

Inoculated 3 ml LB with *E.coli DH5α* and incubated at 37°C till it reaches OD ~ 0.4 - 0.6 (log phase). 3 ml log phase culture of *E.coli* was pelleted down at 5000g/5min. Added 1.8 ml 80 mM $MgCl_2$ + 20 mM $CaCl_2$ and kept it on ice for 20'. Pelleted the cells and washed once with 1 ml 100 mM $CaCl_2$. Added 100 µl 100 mM $CaCl_2$ and incubated on ice for ~ 1 hour to prepare competent cells. Added DNA and incubated on ice for 30 minutes. Heat shock was given at 42 °C for 90 seconds and immediately transferred on ice for 2-3 minutes and then added 800 µl of sterile luria broth (LB) and incubated at 37 °C for 45 minutes under shaking conditions. Pelleted down the cells and resuspended in 40 µl LB. Spread it on luria agar plates containing ampicillin.

Transformation of plasmid DNA in *D. discoideum* (Gaudet *et al.*, 2007)

Pelleted down $5x10^6$ cells. Washed twice with ice cold H-50 buffer. Added 2 µl (1-10 µg DNA) of cloned plasmid DNA. Incubated on ice for 5 minutes. The cell suspension was transferred to a cold 0.2 cm electroporation cuvette. Electroporated in cold at 0.8kV/25 µF pulse, twice for about 5 second gap between 2 pulses. Incubated the cuvette on ice for 5 minutes. Set up 10cm Petri dish for selection with sterile 10ml HL5 and cells were transferred out of cuvette with a few ml of HL5 to the plate. Incubated in BOD at 22 °C for overnight.

Added antibiotics from the next day with medium change as follows:

	1st day	3rd day	5th day	Alternate day
Ampicillin	1µg/ml	1 µg/ml	1 µg/ml	1 µg/ml
G418	5 µg/ml	10 µg/ml	15 µg/ml	15 µg/ml

RNA Isolation Protocol (Pilcher *et al.*, 2007)

Harvested 2 x 10^7 cells and washed with 1 ml KK2. The pellet can be stored at -80 °C. Re-suspended the pellet in 500 µl Trizol reagent (Invitrogen, USA) by pipetting

up and down. Incubated at room temperature (RT) for 5 min. Added 200 µl chloroform and stored at RT for 5 min. Centrifuged for 10 min at 10,000 rpm. The upper phase was transferred in a new tube and added 1 ml 100% ethanol. Incubated for 20 min at -20 °C. Centrifuged for 5 min at 13,000 rpm and then removed the supernatant completely. Air dried the pellet for 5 min without allowing the pellet to completely dry out. The pellet was dissolved in 10 µl nuclease free water. Measured the RNA content in a spectrophotometer (used a 1:100 dilution) and also checked RNA quality with an agarose gel.

Expression analysis of *aca, cAR1, hspD* and *countin2* by RT-PCR

D. discoideum cells were exposed to oxidative stress as mentioned earlier. After one hour pretreatment cells were pelleted and washed with 1X SB and finally resuspended in 1X SB. Total RNA was isolated from the cells at two time points (0 and 9 hour) using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The expression kinetics of *aca*A, *cAR*1, *hsp*D was examined by RT-PCR and *rnl* A was used as an internal control. The reactions were performed according to the manufacturer's instructions (Fermentas, Ontario, Canada). DNA fragments were amplified 24 cycles after reverse transcription and signal intensities were analyzed on 2% agarose gel.

cDNA synthesis protocol:

Mixed the following:

1-5 µg total RNA
1 µl oligo dT primer (supplied at 100uM)
1 µl 10 mM dNTPs
Added water to 10 µl total volume
Incubated 10 min at 65°

In the meantime prepared:
4 µl 5x Reaction Buffer
1 µl Ribonuclease Inhibitor
4 µl water
Added 9 µl of the mixture to 10 µl samples and incubate 5 min at 37°C

Added 1 µl M-MuLV Reverse Transcriptase.
Incubated 60 min at 37°C. Stopped the reaction at 70°C for 10 min.
1 µl of the cDNA should be around 50-100 ng and can be used in a usual PCR with gene specific primers

Developmental studies

Effect of GSH/ benzamide/ gallotannin on the growth of HA treated *D. discoideum* cells

0.5 x 10^6 cells were treated with the respective chemicals for 1 hour. The cells were then exposed to different concentrations of HA (1.0, 2.5, 4.0 mM) for 1 hour. Following this, the cells were harvested, washed with 1X SB and finally resuspended in 4 ml of sterile HL5 containing flask and growth was monitored for 6 days at an interval of 12 hours under shaking conditions at 22°C.

Effect of continuous exposure of glutathione on *D. discoideum* development

2.5 x 10^6 cells were harvested and exposed to antioxidants (1 mM GSH) for 24 hours. After the incubation period the cells were pelleted and plated on phosphate buffered agar (PBA) plate and monitored for development.

Effect of GSH/ benzamide on HA induced *D. discoideum* development

D. discoideum cells were subjected to 1 mM GSH/1mM benzamide treatment for 1 hour, then subjected to 1 hour HA treatment (1.0, 2.5, 3.0, 4.0 mM.) without removing the glutathione. Cells were then washed and the pellet was resuspended in 100µl of Sorenson's buffer (1X SB) and spread on PBA plate (Sroka *et al.*, 2002).

Measurement of cAMP levels in *D. discoideum* cells subjected to oxidative stress

5 x10^6 cells were harvested by centrifugation at 700g/5 minutes/ 4^0C and subjected to oxidative stress. Cells were resuspended in 1X SB for 6 hours. Cells were then collected and extracellular cAMP was estimated in the buffer by ELISA kit method as per the manufacturer's instructions (Calbiochem, USA).

Effect of exogenous addition of cAMP (1 μM and 10 μM) on oxidative stress induced changes in *D. discoideum* development

2.5 x 10^6 cells were harvested and processed for HA treatment (1.0, 2.5, 3.0, 4.0 mM). Washed the cells thrice with 1X SB, transferred to 4 ml of 1X SB containing 1 μM/ 10 μM cAMP and the flasks were kept at 120 rpm/2.5 hours /22°C and then subjected to development.

Nitric oxide estimation in HA treated *D. discoideum* cells (Green *et al.*, 1982)

Nitric oxide generation was estimated according to Green et al (1982). HA treated (1, 2.5, 4 mM) 5 x 10^6 cells were suspended in 1 ml 1X SB and incubated at 21°C for 20 minutes to allow the accumulation of NO generation. 1 ml Griess reagent was added and mixed well. Again incubated at 21°C for 15-30 minutes and took absorbance at 546 nm.

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