

Contents

SR NO.	TITLE	PAGE NO.
CH 1: INTRODUCTION		1-30
1.1	The Mitochondrion: the center for life and death	1
1.2	Apoptosis Inducing Factor (AIF)	2
1.2.1	AIF Structure	2
1.2.2	Transcript variants of AIF	3
1.3	Evolutionary origin and phylogeny of AIF	5
1.4	Apoptotic role of AIF	6
1.5	Non-apoptotic roles of AIF	8
1.5.1	Growth and development	9
1.5.2	Mitochondrial respiration	9
1.5.3	Regulation of ROS levels	11
1.5.4	Mitochondrial fission-fusion and mitochondrial DNA (mtDNA) content	11
1.6	<i>Dictyostelium discoideum</i>	15
1.6.1	Genes responsible for the transition from growth (unicellular) to development (multicellular)	17
1.6.2	<i>Dictyostelium discoideum</i> : a model organism	20
1.6.2.1	Multicellular morphogenesis and pattern formation	20
1.6.2.2	Chemotaxis and signal transduction	20
1.6.2.3	Phagocytosis	20
1.6.2.4	Host pathogen interaction	21
1.6.2.5	Cell death	21
1.6.2.6	Mitochondrial diseases	21
1.7	References	22
CH 2: MATERIALS AND METHODS		32-52
2.1	CHEMICALS	32
2.2	Materials and methods	32
2.2.1	<i>Dictyostelium discoideum</i> culture	32
2.2.2	Composition of modified HL-5 medium	33
2.2.3	Development of <i>D. discoideum</i> cells	33
2.2.4	Revival of <i>Dictyostelium</i> Spores	34
2.2.5	Genomic DNA Isolation from <i>D. discoideum</i>	34
2.2.6	Plasmid DNA isolation by alkaline lysis method	34
2.2.7	Transformation of plasmid DNA in <i>E.coli</i>	35
2.2.8	Generation of <i>AIF</i> antisense constructs (constitutive and prestalk specific)	35
2.2.8.1	PCR amplification	35
2.2.8.2	<i>AIF</i> constitutive and prestalk specific antisense constructs	36
2.2.9	Generation of <i>AIF</i> constitutive overexpression constructs	37
2.2.9.1	PCR amplification	37

2.2.9.2	<i>AIF</i> constitutive overexpression constructs	38
2.2.10	Transformation of plasmid DNA into <i>D. discoideum</i>	38
2.2.11	RNA Isolation and cDNA synthesis	39
2.2.12	Functional characterization of <i>AIF</i> downregulation	40
2.2.13	Cell growth profile	40
2.2.14	Cell cycle analysis	40
2.2.15	Induction of oxidative stress	41
2.2.16	Estimation of NAD ⁺ and ATP levels	41
2.2.17	Estimation of intracellular ROS generation	41
2.2.18	Electron Paramagnetic Resonance (EPR) spectroscopy	42
2.2.19	Estimation of Protein Carbonyl (PC) content	42
2.2.20	Fluorimetric estimation of intracellular calcium [Ca ²⁺] _i levels	42
2.2.21	DNA damage assay	43
2.2.22	Analysis of Mitochondrial Membrane Potential (MMP)	43
2.2.23	Assessment of cell death by AnnexinV-FITC/PI dual staining	44
2.2.24	Monitoring <i>AIF</i> release	44
2.2.25	Glucose dependency	54
2.2.26	Estimation of lactic acid	44
2.2.27	Transmission Electron Microscopy (TEM)	45
2.2.28	Estimation of Oxygen Consumption Rate (OCR)	45
2.2.29	Isolation of mitochondria from <i>D. discoideum</i> cells	46
2.2.30	Blue Native-PAGE (BN-PAGE)	46
2.2.31	Gene expression analysis by Real Time PCR	46
2.2.32	Estimation of mtDNA content (mtDNA/nDNA)	49
2.2.33	Data analysis and statistics	49
2.3	References	50

CH 3: EFFECT OF CONSTITUTIVE AND PRESTALK SPECIFIC DOWNREGULATION OF APOPTOSIS INDUCING FACTOR (*AIF*) ON GROWTH AND DEVELOPMENT OF *D. DISCOIDEUM* **53-69**

3.1	Introduction	53
3.2	Results	54
3.2.2	Confirmation of <i>AIF</i> antisense constructs using restriction digestion	56
3.2.3	Transformation of pTX- <i>AIF</i> and pEcmB- <i>AIF</i> antisense construct in <i>D. discoideum</i>	56
3.2.4	Functional characterization of <i>AIF</i> antisense	57
3.2.5	Relative expression of AIFB, AIFC and AIFD	58
3.2.6	Cell growth profile	59
3.2.7	Analysis of cell cycle	60
3.2.8	Cell Morphology	61
3.2.9	Effect of <i>AIF</i> downregulation on development	61
3.2.10	Effect of <i>AIF</i> downregulation on cell differentiation	64
3.3	Discussion	66

3.4	References	66
CH 4: EFFECT OF CONSTITUTIVE AIF DOWNREGULATION ON OXIDATIVE STRESS MEDIATED CELL DEATH OF <i>D. DISCOIDEUM</i>		70-86
4.1	Introduction	70
4.2	Results	71
4.2.1	Assessment of relation between AIF and ROS	71
4.2.2	Analysis of DNA damage and Protein Carbonyl (PC) content	75
4.2.3	Free cytosolic calcium [Ca ²⁺] levels	76
4.2.4	Mitochondrial membrane potential (MMP)	77
4.2.5	AIF translocation under oxidative stress	79
4.2.6	Annexin V-FITC and PI staining under oxidative stress	80
4.3	Discussion	81
4.4	References	83
CH 5: OXIDATIVE PHOSPHORYLATION STUDIES IN CONSTITUTIVE AIF DOWNREGULATED <i>D. DISCOIDEUM</i> CELLS		87-107
5.1	Introduction	87
5.2	Results	88
5.2.1	Estimation of Oxygen Consumption Rate (OCR) in constitutive AIF dR cells	88
5.2.1.1	OCR studies during vegetative stage	89
5.2.1.2	OCR studies during developmental stages	90
5.2.2	Investigating the effect of glutathione (GSH) on OCR	91
5.2.3	Total ATP and NAD ⁺ levels	92
5.2.4	Glucose dependency	92
5.2.5	Analysis of the assembly of electron transport chain complexes	95
5.2.6	Estimation of mtDNA content	99
5.3	Discussion	100
5.4	References	103
CH 6: EFFECT OF CONSTITUTIVE AIF DOWNREGULATION ON MITOCHONDRIAL FUSION-FISSION MECHANISM OF <i>D. DISCOIDEUM</i>		108-118
6.1	Introduction	108
6.2	Results	109
6.2.1	Analysis of the transcript levels of mitochondrial fusion-fission genes in vegetative cells	109
6.2.2	Analysis of transcript levels of mitochondrial fusion-fission genes in starving cells	111
6.2.3	Mitochondrial morphology	113
6.3	Discussion	115
6.4	References	117
CH 7: EFFECT OF AIF OVEREXPRESSION ON MITOCHONDRIAL PARAMETERS OF <i>D. DISCOIDEUM</i>		119-130
7.1	Introduction	119
7.2	Results	120

7.2.1	Cloning of <i>AIF</i> (<i>AIFA</i>) in EYFP vector	120
7.2.2	Confirmation of <i>AIF</i> overexpression construct in EYFP using Restriction digestion and PCR	121
7.2.3	Functional characterization of <i>AIF</i> overexpression	123
7.2.4	Cellular ROS levels in <i>AIF</i> OE cells	123
7.2.5	Analysis of transcript levels of mitochondrial fusion-fission genes in <i>AIF</i> OE cells	124
7.3	Discussion	126
7.4	References	128
CONCLUSION		131-134
APPENDIX		