STUDIES ON THE SIGNALING CASCADE, LEADING TO APOPTOSIS, UNDER OXIDATIVE STRESS IN *Giardia lamblia*

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CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled "**STUDIES ON THE SIGNALING CASCADE, LEADING TO APOPTOSIS, UNDER OXIDATIVE STRESS IN *Giardia lamblia***" submitted by Smt. **Rituparna Sarkar** who got her name registered on **4th February, 2018** for the award of Ph.D. (Science) Degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Sandipan Ganguly**, Senior Deputy Director (Scientist F) and Head, Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

22-Jan 2024

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PREFACE

This Ph.D. thesis was prepared at the **Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases, India** to fulfil the requirements for obtaining a Ph.D. degree. The thesis is titled **"STUDIES ON THE SIGNALING CASCADE, LEADING TO APOPTOSIS, UNDER OXIDATIVE STRESS IN *Giardia lamblia*"** and the research was conducted at **ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India**. The thesis was completed under the direct guidance of **Dr. Sandipan Ganguly**, Ph.D., Senior Deputy Director (Scientist F) and Head, Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India.

This thesis has been solely composed by the candidate and has not been submitted for any other degree, except where specifically acknowledged.

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Date: 24/01/24

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Rituparna Sarkar

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This thesis is dedicated to my family
and my teachers for their endless love,
support and encouragement
throughout my entire journey.

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A Ph.D. is not only an academic achievement, it's a transformative journey that moulds and defines who we become. It teaches us resilience, determination, and the importance of pursuing our dreams fearlessly. It is a dream, no matter how challenging, for in the end, it's not just about the destination but the person you become along the way. In this challenging yet inspiring journey many individuals have played important role and I would like to gladly acknowledge them.

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CONTENTS

ABBREVIATION

µM	Micromole
AA	Arachidonic acid
ACD	Accidental cell death
ADH	Alcohol dehydrogenase
ALT	Alanine-2-oxoglutarate transaminase
ARF	ADP-ribosylation factor
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
CO_2	Carbon dioxide
COX	Cyclooxygenase
CP	Cysteine protease
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMP	Embden-Meyerhof pathway
ER	Endoplasmic reticulum
ESV	Encystation- specific vesicles
GDH	Glutamate dehydrogenase
GFP	Green fluorescent protein
GPL	Glycerophospholipid
gPLB	*Giardia* PLB
H_2O_2	Hydrogen peroxide
HK	Hexokinase
K+	Potassium ion
kDa	Kilodalton
LOX	Lipoxygenase
lysoPA	Lysophosphatidic acid
lysoPI	Lysophosphatidylinositol
lysoPS	Lysophosphatidylserine
MDA	Malondialdehyde
NADH	Nicotinamide adenine dinucleotide hydrogen
NADP	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
O_2	Oxygen
OH	Hydroxyl radical
ONOO -	Peroxynitrite
PAF-Ahs	Platelet-activating factor acetylhydrolases
PCD	Programmed cell death
PDH	Pyruvate dehydrogenase
PEP	Phosphoenol pyruvate
PFGE	Pulse field gel electrophoresis
PFOR	Ferredoxin oxidoreductase
PGE2	Prostaglandine E2
PL	Phospholipid
PLA1	Phospholipase A1
PLA2	Phospholipase A2

PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PPDK	Pyrophosphate-dependent pyruvate phosphate Dikinase
PtdCho	Phosphatidylcholine
PtdGly	Phosphatidylglycerol
PtdEtn	Phosphatidylethanolamine
PUFAs	Polyunsaturated fatty acids
RCD	Regulated cell death
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
RNase	Ribonuclease
rRNA	Ribosomal RNA
SOD	Superoxide dismutase
SRP	Signal recognition particle
TCA	Tricarboxylic acid
TDP	Thiol-dependent peroxidise
TDR	Thiol-dependent reductase
Tim	Triosephosphate isomerise
UTR	Untranslated region

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Title: STUDIES ON THE SIGNALING CASCADE, LEADING TO APOPTOSIS, UNDER OXIDATIVE STRESS IN *Giardia lamblia*

Abstract

The causative agent of giardiasis in human and animals is the amitochondriate *Giardia lamblia* which posses a unique pathway for apoptosis under oxidative stress condition. We observed that exposing *Giardia* trophozoites to H_2O_2 led to an increase in lipid peroxidation compared to the control group, which was expressed in terms of menadione production as it is the marker for lipoperoxidation. Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms to produce arachidonic acid (AA). Despite of producing PLA2, our data suggested *Giardia* produces a unique 56kDa dimeric enzyme called Phospholipase B (PLB) in contrast to higher eukaryotes. The enzyme activity existed in a broad pH and temperature range but it is highly active at pH7.5 and 35°C.The enzyme is produced upon induction with oxidative (H2O2) stress, thus leading to prostaglandin E2 (PGE2) production. We also analyzed the expression of PLB protein in *G. lamblia,* which was significantly induced under increased oxidative stress condition. This specific enzyme was responsible for the production of intracellular free AA. Now, this free AA either reacylates to the cell membrane or deacylates to further produce prostaglandins. In normal un-induced controlled trophozoites the membrane reacylation process was dominant due the higher level of acyle CoA synthase (ACS) expression over the time. However, under the oxidative stressed condition the intracellular ACS expression was down regulated. This led to the increase in deacylation process. When AA deacylation becomes dominant over AA reacylation in cells, the free AA accumulates intracellularly. The free AA is an important bioactive molecule as it can be metabolized by three distinct enzyme systems – cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 (CYP) enzymes and generates a diverse spectrum of biologically active fatty acid mediators. Our data indicated that metabolism of the free AA in *Giardia* trophozoites occurred by the presence of prostaglandin specific synthase (COX). One of the lipid autacoids, derived from AA is prostaglandinE2 (PGE2). Oxidative stress in trophozoites increased the PGE2 production over the time with respect to the controlled one. After 9 hours of incubation, the PGE2 concentration in treated cells reached approximately 90 pg/ml,indicating that oxidative stress can stimulate PGE2 production. The control cells, on the other hand, maintained a relatively stable PGE2 production level throughout the incubation period. The oxidative stress and high intracellular PGE2 concentration decreased the intracellular K+ concentration over the time. *Giardia* trophozoites maintain a constant K+ concentration in un induced controlled condition (approx. 140 mM). But in case of high PGE2 concentration the intracellular K+ concentration decreased. This decreased K+ concentration enhanced cell shrinkage and externalization of phosphatidyl serine. Phosphatidyl serine exposure is the marker for apoptotic cells and it was measured by the fluorescence of the FITC.

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CHAPTER 1

INTRODUCTION

1.1. Introduction

Early-branched amitochondriate protozoa*Giardialamblia*, causes diarrheal diseases in humans and animals. Approximately 280 million cases of giardiasis are reported globally (Lane S *et al.,*2002) with a major impact on malnourished children in the developing world (*Savioli et al., 2006; Silva et al., 2009*). *Giardia* infection can be asymptomatic or symptomatic presenting clinical manifestations like diarrhoea, abdominal discomfort, vomiting, malabsorption, and weight loss (*Kamdaet al.,2009*). Previous epidemiological studies showed that humans, cattle, and other mammals can be the hosts for this parasite and chronic infection has a negative impact on the global economy (*Giangaspero et al., 2005*). Available data supports the zoonotic transmission of this parasite among different host species through contaminated water (*Bajer et al.,2008, Monis et al.,2003, Smith et al., 2007*).The life cycle of this intestinal parasite consists of two morphological forms: trophozoites and cysts. The trophozoite form colonizes in the small intestine, whereas the resistant cysts are responsible for the oral transmission of the disease through contaminated food and water.Inside the host gut environment, the trophozoites are exposed to complex and ever-changing hydrogen ion concentrations and comparatively high oxygen species levels (60 µM)(*Davenport 1977*, *Atkinson et al.1980*) to fight off the parasite. It is quite perplexing how *Giardia*, lacking conventional eukaryotic antioxidant machinery (e.g. catalase, superoxide dismutase and glutathione peroxidase), can cope with the oxidative stress in this environment (*Showgyet al.,2015*). Initially, *Giardia* can consume O_2, however within 90 min at O_2 concentrations above 50 µM this activity ceases (*Lloyd et al., 2000*). The ability of *Giardia* to respire in O_2 (*Weinbach et al., 1980*) signifies that O_2 can serve as a terminal electron acceptor (*Brown et al., 1998*). Free oxygen radicals (O_2^-) and hydrogen peroxide (H2O2) are produced upon O_2 reduction, which can react with endogenous iron (Fe^{2+}) to produce toxic hydroxyl radicals (OH) (*Brown et al., 1998*). Reactive oxygen species (ROS)

production is an inevitable consequence of normal oxidative metabolism. This parasite is highly vulnerable to both O_2 and ROS, due to: (i) the lack of conventional ROS-scavenging enzymes, such as catalase, super-oxide dismutase, and glutathione (GSH) peroxidase; (ii) the expression of the ROS-generating NAD(P)H:menadioneoxido-reductase [so-called DT-diaphorase (2)]; and (iii) the O_2-labilityof key metabolic enzymes, such as pyruvate-ferredoxinoxido-reductase (*Mastronicola D et al. 2011*). The above aspects of *Giardia* metabolism imply that O_2 and its metabolic products will have detrimental effects on *Giardia* viability. Indeed, the evidence shows that oxidative stress in *Giardia* affects plasma membrane permeability, cell volume, homeostasis of the thiol pool and induces cell cycle blockage leading to trophozoite death (*Lloyd et al., 2000*,; *Paget et al., 2004*). When in high levels ROS exerts many direct and indirect effects on cell signalling pathways, which finally result in the induction of apoptosis or necrosis (*K. England et al.,*2005). One of the effects of ROS on cells is lipoperoxidation, i.e., the oxidative modification of polyunsaturated fatty acids (PUFAs).Cellular membranes are especially susceptible to ROS damage, which is called lipid peroxidation, due to their high PUFAs (*Su et al., 2019*).

In the process of lipid peroxidation electrons from lipids are removed by free radical species such as oxyl radicals, peroxyl radicals, and hydroxyl radicals and subsequently produce reactive intermediates that can undergo further reactions (*Su et al., 2019*). The process damages phospholipids directly and can also act as cell death signal which induces programmed cell death. The lipid peroxidation is a chain reactions and the product of which display high biological activity including destruction of DNA, proteins, and enzyme activity and acts as molecular to activate signaling pathways initiating cell death (*Zarkovic et al., 2017; Łuczaj et al., 2017*). Biomembranes are prone to undergo lipid peroxidation, via nonenzymatic or enzymatic pathway. Non-enzymatic phospholipid (PL) autoxidation is iron-dependent lipid peroxidation and divided into three stages. On the other hand, enzymatic

peroxidation occurs in one kind of phospholipid and catalyzed by lipoxygenase (LOX).Arachidonic (C20: 4) is the most abundant polyenoic fatty acid that serves as substrates for LOX using molecular oxygen to form hydroperoxyl groups at different carbon position of acyl chains(*Su et al., 2019*) . This type of lipid peroxidation predominantly occurs at the sn-2 position of phospholipids, as most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. This position is also targeted by phospholipase A2 (PLA2) for cleavage (*Cummings et al.,2000)*.Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms (*Sapirsteinet al.,2000*). Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites (*Maria et al. 2006*,*Mark et al 1989)*. In*Trypanosoma,* PLA2 activity is stimulated by Ca2+, and the release of AA by the PLA2 enzyme provides positive feedback for eicosanoid metabolism (*Eintracht, J. et al. 1998)*. Despite the fact that PLA1 and PLA2 are essential for AA production in *Giardia lamblia*, either of these enzymes or their corresponding genes are absent in this parasite *(Vargas-Villarreal et al., 2007).*After the genome database (Giardiadb) survey, we could identify a unique enzyme called phospholipase B (PLB) which has the capability to catalyze the hydrolytic cleavage of both sn-1(like PLA1) & sn2 (like PLA2) acyl ester bonds of glycerophospholipids, forming free fatty acids and lysophospholipids*(Aloulou et al., 2018)*. Studies on fungal and bacterial phospholipase B revealed that it has hydrolase and acyl transferase activities. A detailed study on cryptococcal Phospholipase B showed that the phospholipids of the host cell membrane are a potent site for its lipolytic activity *(Shiell et al., 2007).*Our study focuses on exploring the impact of *Giardia* PLB (gPLB) in generating AA in response to oxidative stress. Our rationale for investigating this is based on literature indicating that PLB can hydrolyze the sn-2 position of membrane phospholipids, such as PLA2, resulting in the

release of AA.To comprehensively investigate this enzyme, we cloned the PLB gene from Giardia lamblia, obtained the recombinant protein through purification, and performed biochemical and bioinformatic characterization on it.Upon cellular stimulation activated PLA2 shifts the cycle toward the accumulation of free AA, which then leads to eicosanoid synthesis (*Pérez et al., 2006*). AA mobilization mediated by Ca^{2+}-dependent PLA_2 enzymes generally appears to involve the activation of kinase cascades and intracellular calcium movements, AA mobilization by Ca^{2+}-independent PLA_2 enzymes appears to require neither of these (*María et al., 2006*). In our data it can be established that the present gPLB is Ca^{2+} dependant.

Arachidonic acid (AA), a cis-polyunsaturated fatty acid, is liberated from plasma membrane through activation of phospholipase A_2 due to lipid peroxidation. AA is the intermediate in the Lands cyclewhich is adeacylation/reacylation cycle of membrane phospholipids. In this process the hydrolyzed fatty acid by phospholipase A2,is reincorporated into the phospholipids by the concerted action of fatty acyl-CoA synthetase and lysophospholipid acyltransferase (*Lands WE., 2000*; *Chilton et al., 1996*).The reacylation pathway is dominant over the phospholipolytic step in un-induced cell keeping AA levels very low. However, whenAA deacylationis dominant over AA reacylation in cells the accumulation of free AA occur (*Chilton et al., 1996*).Then this Free AA is metabolized to prostaglandins by cyclooxygenase (COX) (*Chen K C., et al. 2009)*. AA induced apoptotic cell deaths of tumour cells are hallmark of this ROS generation (*Chen K C et al., 2010; Dymkowska D et al.,2009; Liu W H et al.,2009)*. Absence of active conventional antioxidant machinery leads to accumulation of intracellular ROS and cell damage; finally cell death by programmed cell death (PCD) process.PCD is well regulated cellular process that has been extensively characterized in multicellular organisms but inmany unicellulareukaryotes PCD is also observed in which *Giardia* is the newly added amitochondrial eukaryote. With increasing

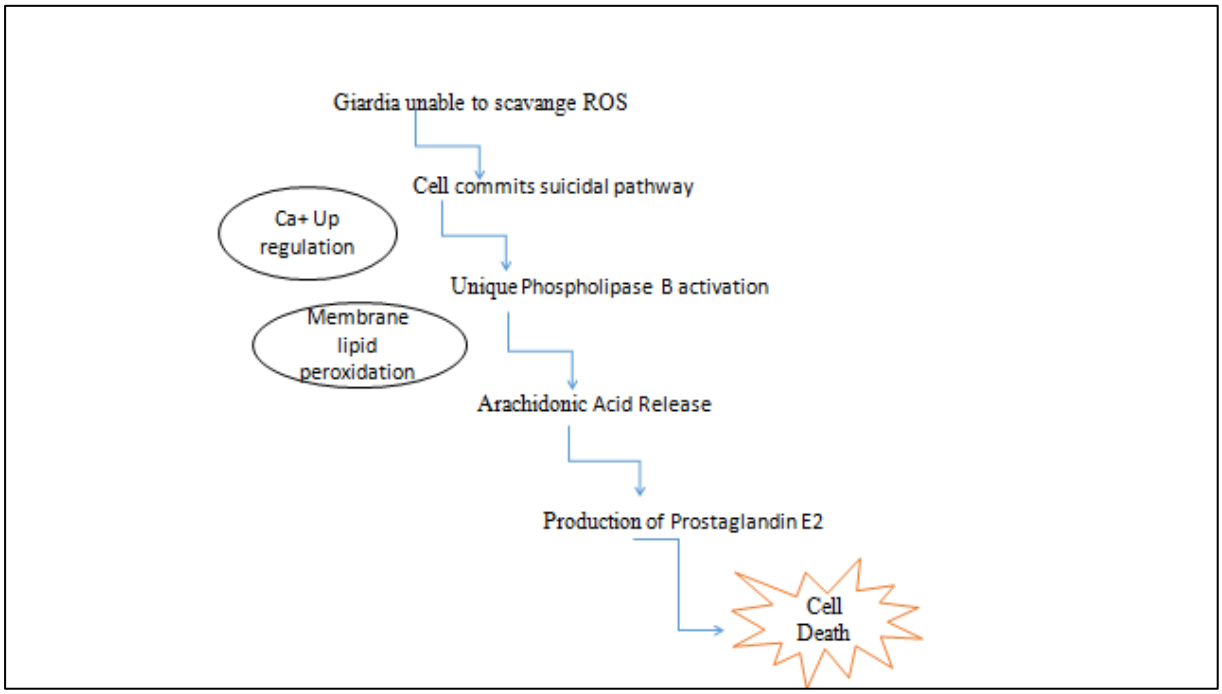
evidence of PCD in single celled organism including Bacteria *(Kooninet al., 2002)* it is important to understand how these processes evolved with respect to the pathways and also the rational for such systems in unicellular organisms. Although controversial, the majority of researchers consider *Giardia* as early branched eukaryote which diverged at or just after mitochondrial acquisition. This has made this parasite an important organism for research into the understanding of evolution in eukaryotic cells (*Thompson, 2004*). *Giardia* possesses two forms of PCD – apoptosis and autophagy (*Correa et al (2009)*. Most common type of PCD known is apoptosis which involves series of events including activation of caspase, cell shrinkage, DNA fragmentation, phosphatidylserine exposure, formation of apoptotic bodies, loss of mitochondrial membrane potential with cytochrome c release (*Menna-Burreto et al. 2009*). In the process of apoptosis lipid peroxidation plays an important role. The products of peroxidized lipid interact with membrane receptors and transcription factors/repressors to induce signalling for both intrinsic and extrinsic apoptotic pathway *(Su et al., 2019).*Both of the suicidal pathways require different type of caspase machinies.In case of *Giardia*, under oxidative stress condition though phosphatidylserine exposure occurs but no caspase activity is detected (*Bagchi et al.,2012*).

Recent studies established the fact that many unicellular eukaryotes possess caspase independent apoptotic pathway via mitochondrial programmed cell death. Evidential reports also highlighted the involvement of metacaspase in an apoptotic-like PCD in yeast (*Mazzoni et al.,2008*). Due to lack of typical mitochondria, caspase independent apoptosis is unlikely to happen in *Giadia.* The parasite, however, have a relic structure termed as mitosome but this organelle has no clear metabolic or signaling role related to apoptosis (*Rosa et al.,2008*). One of the many characteristic of ROS generation is AA production and ultimately apoptosis induced by AA and its metabolites *(Chen K C., et al. 2009)*.

Literature survey revealed that Prostaglandine E2(PGE2), one of the metabolites of AA, activates the erythrocyte cation channels and thus lead to erythrocyte shrinkage and phosphatidylserin exposure.Injuries to erythrocytes such as osmotic shock, oxidative stress, or energy depletion can induce the formation of prostaglandin E2, which then activates the Ca^{2+} permeable cation channel. This increase in Ca^{2+} activates the K+ channel, leading to hyperpolarization, cell shrinkage, and the exposure of phosphatidylserine, which is a marker for apoptosis (*lang etal.,2005*).

1.2. Hypothesis of work

In our studies we have discovered similar phenomenon as phosphatitdylserine exposure under ROS induction confirming it as apoptosis. Though the process of cell death is apoptotic type, the *Giardia*trophozoiets does not follow the conventional apoptotic pathways due to absence of caspase machineries. So we proposed that the apoptosis of *Giardia* was AA and its metabolite induced unique one. The pathway is dependent upon the activation and the synthesis of PGE_2.



In the present study, we used three conditions in *Giardia* trophozoites to mimic the intracellular oxidative stress generation. First, hydrogen peroxide (H2O2) was to generate free oxygen radicles easily *(*Brown *et al., 1998).Giardia*trophozoiteswere killed by 2h-incubation with H2O2≥50μM(Hill et al.,1987) andamaged by 1h-incubation with sub-lethal H2O2concentrations(*Crouch et al.,1991*), but the mechanisms underlying H2O2toxicity are not fullyunderstood yet (*Lloyd et al., 2000).* Secondly, the most common and effective drug against giardiasis, metronidazole was used to induce stress in trophozoites. Finally, a modified medium which was devoid of cysteine and ascorbate, was used. Cysteine plays a protective role by protecting crucial thiol groups and ascorbic acid protects trophozoites under high PO2 (*Tekwani et al., 1999*).

1.3. Objective

The aim of this study is to unravel the mechanism of apoptotic death of this unicellular protist under high oxidative stress condition (lacks the conventional apoptotic machinery like caspases). The major objective of this proposed study is to identify the signaling cascade leading to the apoptosis like death in Giardia and to determine the expression pattern of enzymes which involves Phospholipid metabolism under oxidative stress condition at both transcriptomic and proteomic level. The objective is also to characterize the role of phospholipase B (PLB) enzyme [contains both PLA1 &PLA2 activity] in initiating the downstream pathway to activate lipid signaling cascade involving apoptosis in Giardia. For this purpose, following objectives will be considered:

i. **In vitro production of stress in the parasite will be standardized using different drugs and chemicals.**

ii. **Correlate between Prostaglandine E2 formation and Phosphatidylserine exposure**

iii. **Cloning, expression, and Characterization study of PLB.**

iv. **Determination of the expression level of PLB under different oxidative stress condition at both transcriptomic and proteomic level** and **study the activation status of PLB and downstream molecule.**

v. **Finally determining the fate of Prostaglandin E2 (PGE2) and the mechanism of apoptosis via PGE2 signaling.**

CHAPTER 2

REVIEW OF LITERATURE

2.1.Introduction

Giardia lamblia, a binucleate flagellated intestinal protozoan parasite is a common cause of diarrhoea in humans and other mammals throughout the world. It has four pairs of flagella arise from the superficial organelles on the ventral side of the body. Apart from its medical importance the parasite is fascinating in its own right because trophozoites contain two nuclei and well developed cytoskeleton but lack mitochondria, peroxisome and the components of oxidative phosphorylation. The primitive nature of the organelles and metabolism ,as well as the small subunit rRNA phylogeny ,has led to the proposal that *Giardia spp.* are among the most primitive eukaryotes(*Adam et al. 2001*).*G.lamblia*probablyhas a ploidy of 4 and a genomic size of approximately 10 to 12 Mb divided among five chromosomes.

2.2.History and nomenclature of *Giardia*

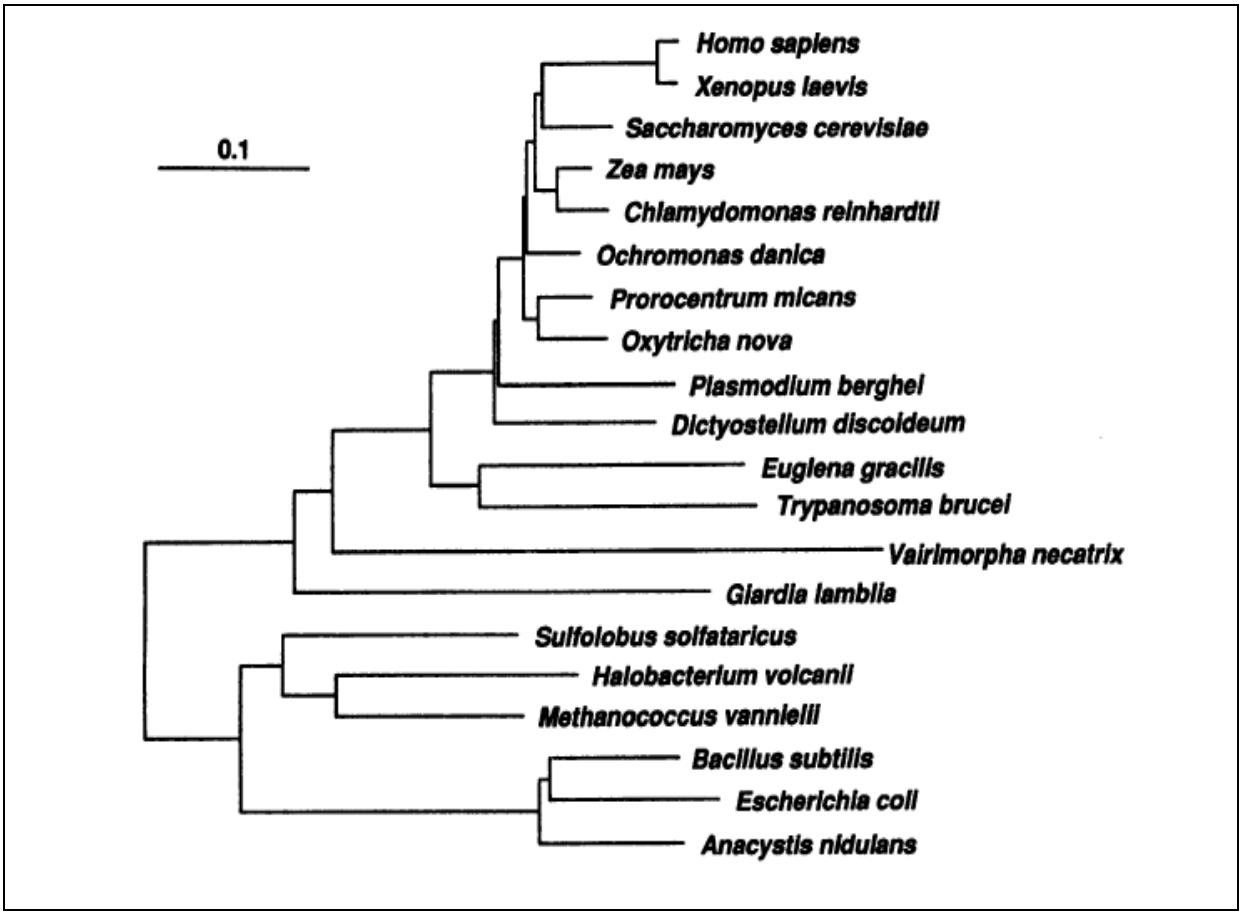
Giardia gain attention as a human pathogen after the outbreaks of 1960 although the parasite captured the attention of Dutch microscopist Auto Van Leeuwenhoek in 1681. The first morphological report of *Giardia* was published by Lambl (1859) and Cunningham (1881). Despite huge contradiction regarding the genus and species name of the parasite, the genus name was established as Giardia by Kunstler (1882). Fianly stiles named this parasite as Giardia lamblia at 1915.

Traditionally, all living organisms have been classified as prokaryotes or eukaryotes, but some argue still persists for retaining the two major divisions (*Mayer et al., 1998*). Recent widely accepted classification system considers Archaea (archaebacteria), Bacteria (eubacteria), and Eukarya (eukaryotes) as three major dividion, which can then be divided into kingdoms (*Woese et al.,1990*). Either of the classification system, places *G. lamblia*as a eukaryotic organism belonging phylum protozoa, the more “animal-like” of the unicellular eukaryotes.*Giardia* species is categorized with other flagellated protozoa that are pathogenic

to humans and further subdivided into flagellates, ciliates, amoeboid and sporozoa, depending on their morphology. Thus, *G. lamblia*was classified with the flagellated protozoans, including the kinetoplastids (e.g., *Leishmania* sp. and *Trypanosoma* sp.), parabasalids (e.g., *Trichomonasvaginalis*), and *Dientamoeba*(e.g., *Dientamoeba fragilis*) (*Levine et al.,1980*). *Giardia*belongs to theorder Diplomonadida (two karyomastigonts, each with four flagella, two nuclei, no mitochondria, and no Golgi complex; cysts are present, and it can be free-living or parasitic) and the family Hexamitidae (six or eight flagella, two nuclei, bilaterally symmetrical, and sometimes axostyles and median or parabasal bodies), along with the mole parasite *Sppironucleusmuris*and the free-living organism *Hexamitainflate (Januschka et al., 1988)*. One recent classification system with one prokaryotic and five eukaryotic kingdoms has retained Protozoa as one of the six kingdoms (*Cavalier-Smith et al., 1998*), but most recent proposals have suggested abandoning the term "protozoa" to be more general but at the same time more precise term "protista" (*Corliss et al., 1981; Corliss, et al., 1984; Whittaker et al., 1978*).

Recently, the eukaryotic microbial organisms are classified on the basis of molecular comparisons. An ideal molecular classification system requires a gene(s) that is present in all life form as well as highly conserved across all forms of life. The accurate alignments of these specific genes allow comparison and classification of all organisms. As rRNA sequences are highly conserved across life, it has been proved to be the most useful gene for molecular comparisons. Therefore, the most widely accepted classification scheme has been based on SS (18S) rRNA sequences. *G. lamblia*was proposed as one of the most primitive eukaryotic organisms based on the rRNA sequence comparison. The use of SS rRNA sequences to place *Giardia* as an earlybranching eukaryote has been criticized because of the high GC content of the SS rRNA of *Giardia* (75%) and because *Giardia* spp. Are parasitic organisms; artifacts may be introduced by high rates of mutation accompanying host

adaptation. An analysis of the early-branching eukaryotes suggests that the basal position of *Giardia* is an artifactual result of long-branch attraction due to a greater evolutionary rate of *Giardia* (*Stiller et al., 2000*). Analysis of the large subunit of RNA polymerase II and reanalysis of the eF1 and eF2 sequences also support the idea of the long-branch 13nflate13 (*Holberton et al., 1988*). However, no such effect is shown for the eRF3 tree. The absence of the highly conserved N-terminal domain of eRF3 in *G. lamblia,* found in other eukaryotes including *T. Vaginalis* suggests*G. lambli*aisdiveregedbefore the acquisition of the N-terminal domain (*Inagaki et al., 2000*). It should also be noted that the phylogenetic placement of *Hexamita*, a free-living organism in the same family (as determined by morphologic criteria), avoids artifacts due to high GC content or parasitism. The GC content of the *Hexamita13nflate*SS rRNA is 51%, and *H.13nflate*is found to be monophyletic with *Giardia* (*Leipe et al., 1993, van Keulen et al., 1993*). The classification of *H. 13nflate*with *G. lambli*a is also supported by a comparison of the 13nflate13dehydes-3-phosphate dehydrogenase sequences of *G. lamblia, Trepomonasagilis, H. 13nflate*, and *Spironucleus*sp. (*Rozario et al., 1996*). A comparison of the SS rRNA sequences of all diplomonads like *Giardia, Hexamita, Trepomonas*, and *Spironucleus*, showed all are phylogenetically related but that the last three are grouped in one clade while *Giardia* occupied another clade (*Cavalier-Smith et al., 1996*).



Source: Sogin et al., 1989

Fig.1.Multikingdom tree based on the sequence analysis of 16s-like rRNAs. Using a computer-assisted method, 16s like rRNAs sequences from represtatives of the eubacteria, archaebacteria and eukaryote were aligned which considered the evolutionary conservation of both primary and secondary structure features (*Elwwod et al., 1985*). To infer the unrooted multikingdom tree the distance matrix method.

A phylogenetic tree of one of the genes (*cpn60*), probably mitochondrial origin, suggests that *Giardia* is an early-branching eukaryote (*Germot et al.,1996; Roger et al.,1998*). Cytoskeletal genes give mixed results, with actin giving an early divergence but tubulin yielding a divergence that is later than that of *Entamoeba histolytica*. Among other classes of genes, HSP70 and cathepsin B phylogenies suggest an early divergence for *Giardia*. Thus, most of the data suggest that *G. lamblia*and the other diplomonads are among the most basalof the extant eukaryotes.

Systematic Position (Source: -*López et al., 2022*)

PHYLUM: Metamonada

CLASS: Fornicata

ORDER: Diplomonadida

FAMILY: Hexamitidae

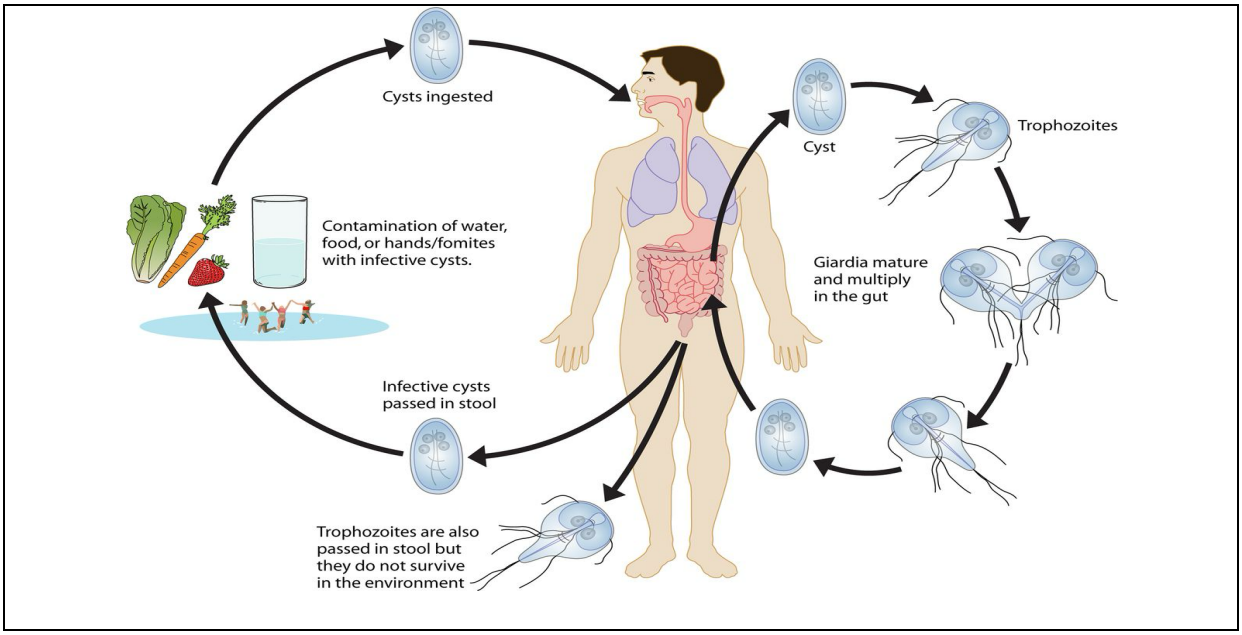
GENUS: Giardia

SPECIES: lamblia

2.3. Life Cycle:

The life cycle of this intestinal parasite consists of two morphological forms: the vegetative trophozoites stage and the infective cyst stage. The trophozoite form colonizes in the small intestine, whereas the resistant cysts are responsible for the transmission of the disease through contaminated food and water. Infection normally follows ingestion of cysts in focally contaminated water or via foods (*Adam et al., 1991*). All *G.lambliа*infections are symptomatic, and many people can unknowingly serve as carriers of the parasite. The life cycle begins with the excretion of a non-infective cyst with thefaeces of an infected individual. The hardy cyst, which is protected from heat and cold, desiccation, and infection from other organisms possess distinguishable characteristic of four nuclei and a retracted cytoplasm. Once ingested by a healthy host, the excystation of cysts is activated by low ph in stomach (*Bingham et al., 1979*). Then the process is stimulated by the slightly alkaline pH and proteolytic activity of duodenum fluid *(Rice et al., 1981)*.

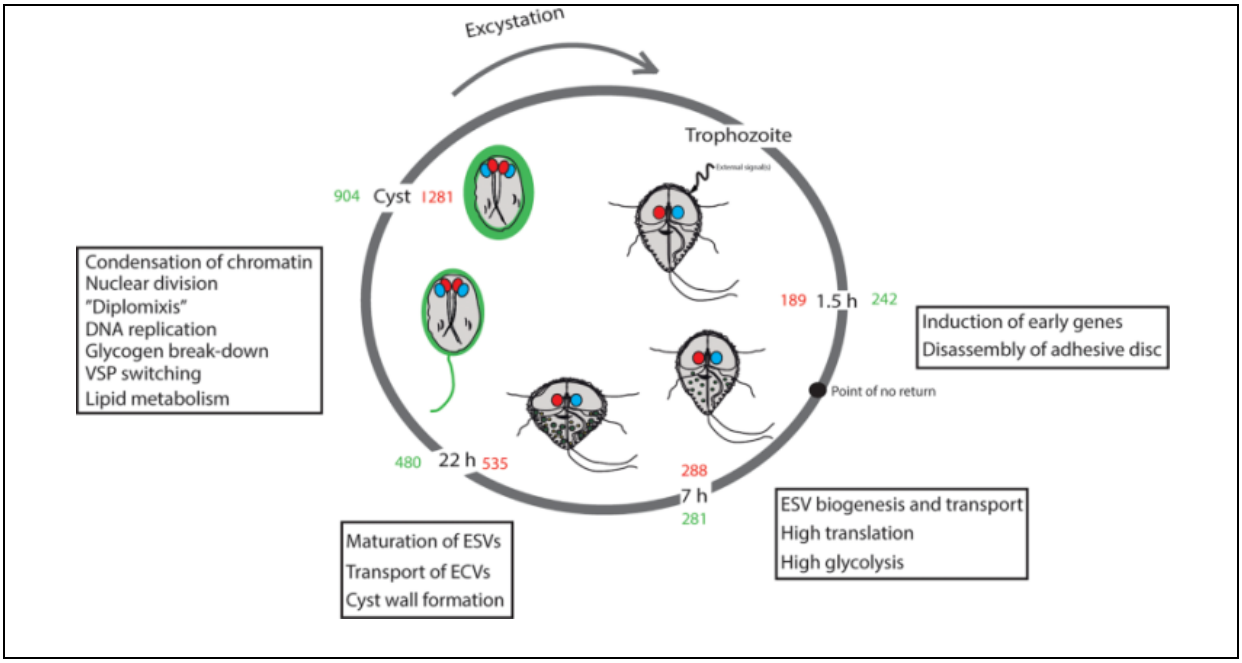
The release of cysteine protease, stored in the peripheral vesicles of the trophozoites, in to the space between trophozoites and cell wall during excysation is crucial for infection. After the emergence parasite quickly divides into equivalent binucleate trophozoites by binary fission and colonise the human small intestine. They swim in the lumen fluid with four pairs of flagella and also adhere to mucus strand in vivo and in vitro (*Adam et al., 1991*).



Source: *Esch et al., 2013*

Fig.2. *Giardia* sp. life cycle. *Giardia* cysts shed in the feces are infectious. Infection occurs after the ingestion of cysts either through the fecal-oral route or through the ingestion of contaminated water or food. Cysts are environmentally resistant and can persist for months in soil or water. Excystation occurs within the small intestine. Trophozoites remain either free in the intestinal lumen or attached to villous enterocytes, causing clinical signs. Trophozoites encyst upon movement toward the colon, becoming infectious oocysts, and are shed in the feces.

The parasite can attach to intestinal epithelial cells after the penetration of the mucus layer via their unique ventral adhesive disc (*Nemanic, 1979*).Here they feed on the content of the hostgut by micropinocytotic vesicles andmultiply by binary fission.Some trophozoites are



Source*: Einarsson et al., 2016*

Fig.3. Summarizing image showing the transformation from the motile trophozoite via encyzoite to the final cyst stage. The trophozoite monitors the external environment and encystation is induced via intracellular pathways that remain largely unknown. The cell passes a "point of no return" during early encystation after which it is no longer possible to relapse to the proliferating stage. Transcription factors (e.g. Myb2) activates encystation-specific genes among them are cyst wall proteins (CWP1-3). An overall increase of translation could be observed early in encystation as the production of CWPs is dramatically increased and the transportation in encystation vesicles (ESVs) begins. The vesicles undergo maturation steps after leaving the ER. The other component of the cyst wall, the UDP-GalNAc sugar (giardin), is also synthesized and secreted via encystation positive carbohydrate vesicles (ECVs). The enzymes involved in giardin synthesis are induced during encystation. During late encystation, the cell changes shape as it enters dormancy and the ventral disc together with the flagella are disassembled as the construction of the cyst wall proceeds. But the mechanism behind the assembly is still unknown. Often pre-cyst stages with a "tail" can be observed in encystation. Two rounds of DNA replication occur without cytokinesis rendering a cyst with four nuclei each with the genome ploidy of 4N. Interconnections between the nuclei in the cysts are formed and genetic material can be exchanged through the process "diplomixis". During excystation, each cell receives one pair of non-sister nuclei (indicated as red and blue).

Carried downstream by the intestinal fluid and they encyst as a result of elevated pH and high bile concentration of the intestinal lumen (*Gillin et al.,1988*). During encystations trophozoites gradually round up and detaches, lose mobility and become retractile.The *Giardia* cyst wall is found to increase by 3-5folds during encystations.

2.4.Genotypes of *Giardia*

To accurately understand the pathogenesis and host range of *Giardia* isolates obtained from humans and a variety of other mammals, the molecular classification tool is essential.The restriction fragment length polymorphism (RFLP) analysis of 15 Giardia isolates, using random probes results in description of three Giardia groups (*Nash et al. 1985*).For more conclusive result, sequence of the small-subunit rRNA, glutamate dehydrogenase (GDH) and triosephosphate isomerase (tim), genes of Giardia isolates, were compared in different studies (*Baruch et al.,1996; Ey et al.,1997; Lu et al.,1998;Monis et al.,1996;Monis et al.,1999*).

Species name	Hosts	Morphology by:		Molecular data
		Light microscopy	Electron microscopy	
G. agilis	Amphibians	Long and slender; teardrop-shaped median body		NA[a]
G. muris	Rodents	Short and rounded; small rounded median body		Distant from *G. lamblia*
G. lamblia	Numerous mammals, including humans	Pear shaped; one or two transverse, claw-shaped median bodies		Clade with multiple genotypes
G. ardeae	Herons	Same as *G. lamblia*	Ventral disk and caudal flagellum similar to *G. muris*	Closer to *G. lamblia* than to *G. muris*
G. psittaci	Psittacine birds	Same as *G. lamblia*	Incomplete ventrolateral flange, no marginal groove	NA
G. microti	Voles and muskrats	Same as *G. lamblia*	Cysts contain two trophozoites with mature ventral disks	Similar to *G. lamblia* genotypes

[a] NA, not available.

Source: *Adam et al., 2001*

Table.1. Occurance of different species of *Giardia* along with their host organisms and the morphology.

All the molecular studies have confirmed the division of *G. lamblia*human isolates into two major genotypes. The first consists of Nash groups 1 and 2, Mayrhofer assemblage A, groups 1 and 2, and the Polish isolates, while Nash group 3, Mayrhofer assemblage B, groups 3 and 4, and the Belgian isolates form the other major genotype. The analysis of *tim*nucleotide sequences analysis shows that the group 1 and 2 isolates diverged by1% and 2% in the protein coding region and flanking regions respectively,while groups 1 and 3 diverged by

19% and the flankingregions (*Lu et al., 1998*).The small-subunit or 18S rRNA (SS rRNA) sequence reveals 1% divergence between groups 1 and 3, as it is highly conserved sequence (*van Keulen et al., 1995*). In addition to their marked genetic differences, the two genotypes may have a number of important biologic differences. More recently, a number of additional assemblages (genotypes) have been proposed for *Giardia* isolates from a variety of mammals. These isolates are morphologically identical to human *G. lamblia*, but sequences of their protein-coding regions differ. The assemblages are summarized in the table underneath.

Proposed designation	Nash group (251)	Mayrhofer assemblage (209)	Origin (137)	Hosts
Genotype A-1	1	A (group 1)	Poland	Human, beaver, cat, lemur, sheep, calf, dog, chinchilla, alpaca, horse, pig, cow
Genotype A-2	2	A (group 2)		Human, beaver
Genotype B	3	B (groups 3 and 4)	Belgium	Human, beaver, guinea pig, dog, monkey
		C		Dog
		D		Dog
		E (or A-livestock)		Cow, sheep, alpaca, goat, pig
		F		Cat
		G		Rat

Source: *Adam et al., 2001*

Table.2. Table consists of proposed designation of different existing *Giardia* genotypes along with their hosts and origin.

2.5. Epidemiology and transmission

2.5.1. Faecal-oral transmission

Predominantly *Giardia* is transmitted via faecal-oral contamination. So, poor sanitation and hygiene in tropical and subtropical environments aggravate the process of transmission with only 10 cysts or single trophozoites (*Rendtorff, 1954*) (*De Carneri et al., 1977*). Giardiasis is one of the major concerning issues regarding public health among the disadvantaged group of developing countries (*Brucker, 1989; Thompson, 1992b*).In such circumstances, long term

control requires education and improved levels of hygiene. Food-borne transmission usually results from the contamination during food preparation, which is also a well recognized source of *Giardia* infection (*Barnard and Jackson, 1984; Petersen et al., 1988*). Direct transmission between homosexual men is being increasingly recognized as significant in the epidemiology of giardiasis (*Phillips et al., 1981*).

2.5.2. Water-borne transmission

In the USA, numerous *Giardia* infections are reported which are associated with contaminated water and most frequently diagnosed as water-borne disease (*Levine et al., 1990*). Water-Borne *Giardia* outbreaks have been reported from different parts of the world like Canada, Europe, Australia and New Zealand (Wallis et al., 1986; Jephcott et al., 1986; Ampofo et al., 1991; Fraser and Cooke, 1991). Incase of traveller's giardiasis, water-borne transmission is well documented *(Jokipii and Jokipii, 1974).* Endemic water-borne giardiasis in the USA is caused from contaminated water supply due to contamination with human sewage, ineffective filtration or inadequate water treatment *(Craun, 1986; Schantz, 1991*).

As chlorination alone is insufficient without high concentrations of chlorine to remove Giardia, filtration is necessary (*Schantz, 1991*). Although, Human giardiasis have been reported from the consumption of stream water which are away from urban environments. This indicates some wild animals, particularly beavers and muskrats, are the reservoirs of infection *(Davies and Hibler, 1979; Craun, 1990)*. However it is not determined if animals serve as the original source of contamination or amplify the numbers of the originally contaminating isolate *(Bemrick et al., 1988).*

2.5.3.Zoonotic transmission

There are some *Giardia* isolates which are not host-specific and there is evidence that can prove that humans and other animals share the parasite. Therefore event of *Giardia* infection

can be regarded as a zoonosis (*Acha et al., 1987; Schantz, 1991*). Studies on the *Giardia* isolates from variety of mammals like dogs and cats and birds reveal that these isolates can be a potential reservoir of human infection (*Swan et al., 1986; Baker et al., 1987; Collins et al., 1987; Winsland et al., 1989; Castor et al., 1990; Tonks et al., 1991; Thompson, 1992; Meloni et al., 1993).*It can be a strong circumstantial evidence of zoonotic transmission if all family members and the pets are found to be infected with *Giardia (Cribb et al., 1986).* A recent study in Canada shows *Giardia* is prevalent in numerous ruminants like 17.7% in sheep, 10.4% in cattle, 35.6% in lambs and 27.7% in calves *(Buret et al., 1990)*. Some authors suggested that birds can be a source of zoonotic contamination of *Giardia*in open water (Bemrick, 1984).Although it is now well accepted that *Giardia*posses potential for zoonotic transmission but evidence for this incident outside the laboratory is very difficult to obtain as *Giardia* isolates are morphologically indistinguishable (*Thompson et al., 1990)*.Using molecular characterization procedures (enzyme electrophoresis and DNA hybridization using specific probes), it has been found that some mammalian isolates are similar to human ones *(Meloni et al., 1989).* However, *Giardia* from Western Australia cat and isolates from Europe cat are quite different *(Meloni et al., 1992)*. Hence, the genetic identity between isolates of *Giardia* from humans and other animals of in one endemic area can vary from another area *(Thompson et al., 1990)*. Comparative studies using molecular characterization data for zoonotic transmission must compare isolates at a local level. If genetic identity of *Giardia* isolates of same location from humans and other animals is found to be similar, it can be a conclusive data of cross- transmission *(Archibald et al., 1991).* However, it does not necessarily follow that nonidentity means that zoonotic transmission is not occurring, because particular species of host are known to be susceptible to more than one genetically distinct variant of Giardia *(Andrews et al., 1989; Meloni et al., 1989; Thompson et al., 1990).*

2.6. Pathogenesis

For many years the mechanism of *Giardia* infection was unknown but recently the report about the pathogenic factors has been emerged involving increased epithelial cell turnover, epithelial damage, disaccharidase deficiency and villous shortening *(Yardley et al., 1964; Ament et al., 1972; Gillon et al., 1984; Buret et al., 1990a,b)*.Detailed clinical manifestations of the *Giardia* infection have been studied using rodent model for both *Giardia muris* and *Giardia duodenalis*.Experimental data suggests brush border enzyme deficiencies along with epithelial microvilli damage can be reversed if the infection is removed.Brush border injury,in terms of lowered disaccharidase activity and decreased microvillous surface area result in impaired digestion, malabsorption and reduced growth. The brush border injurey level is the limiting factors for disaccharidase activity which in turn is dependent on parasite load *(Buret et al., 1991)*.This mechanism of pathogenesis could account for both of the major clinical features diarrhoea and failure to thrive. The physical damage occurredduring the adherence of *Giardia* to the gut plays some part in the disease process but the role of other factors such as toxins is uncertain. Two major proteolytic enzyme activitieshave been demonstrated in Giardia: 40 and 50 kDacystein proteases*(Parenti, 1989, Hare et al., 1989)*. The cleavage property of 40kDa protease has been proposed as a virulence determinant but no evidence has been made in the pathogenesis in the host. The outcome of the *Giardia* infection is determined by both the characteristics of parasite and host. The patients who are very young, immunocompromised, or malnourished are more prone to symptomatic giardiasis which indicates that the host factors are important.However, there is also evidence that a particular strain of *Giardia* may have a greater potential to cause disease in a particular host (*Thompson et al., 1990a*). Difference in virulence factors among *Giardia* isolates is the cause of different surface antigen expression *(Nash et al., 1991).*

2.7. Diagnosis and treatment:

2.7.1. Diagnosis methods

2.7.1.1. Traditional methods

Traditionally infected stool samples are microscopically examined to detect *Giardia* cyst or trophozoites and so far it is a reliable indicator of giardiasis *(Wolfe, 1990)*. Single day stool examination is able to detect 50-75% of positive patients whereas three faecalsamples collected on non-consecutive days gives more accurate result (*Wolfe, 1990; Adam, 1991)*. More invasive and costly methods like the Entero-Testsor duodenal fluid/biopsy aspirate are also available for the detection of infection (*Beal et al., 1970; Hall et al., 1988*). Many authorities are employing filtration and concentration before microscopic examination to be done.The light microscopy examination of faecal sample is facilitated by concentrating the sample using zinc sulphate or formol-etherstaining the slide by trichrome or iron haematoxylin stains. Hence, it is tedious process and relies on the manual identification process (*Baker et al., 1987)*. Recently the old traditional microscopic method is upgraded by using the fluorogenic dye fluorescein diacetate to mark viable cyst and propidium iodide from non-viable cycts. Though this method is not 100% accurate but can be used for a large through-put of samples using fluorescence-activated cell sorters (*Schupp etal., 1987; Smith et al., 1989)*. These two dyes have also been shown to be useful quantitative markers of the killing of trophozoites, using two-colour flow cytometry (*Heyworth et al., 1989)*.

2.7.1.2. Immunological techniques

Recent development of immunological tests based on *Giardia* antigens open a new avenue for the detection of giardiasis as the antigen based detection method is well equipped to perform in clinic or in the field with water sample or other environmental samples (*Ungar et*

al., 1984; Green et al., 1985; Sauch, 1985; Vinayak et al., 1985; Nash et al., 1987b; Janoff et al., 1989; Engelkirket al., 1990; Hopkins et al., 1993).Recently Giardia antigens of molecular mass 30 and 66 kDa, which is of similar molecular antigen present in the *Giardia* infected human sera, is used in an ELISA test (CELISAs Cellabs, Sydney, Australia) *(Taylor et al., 1987; Janoff et al., 1988, 1989)*. The specificity of the test was 95% for canine samples and 91 % for human samples and sensitivity 100% for human samples whereas only 55-64% for canine samples (*Hopkins et al., 1993*). It has been reported that a 21-39 kDa and 66-103 kDa protein located in encystation specific vesicles (ESV) is one of the most prominent cyst antigens are from a hetero-disperse group (*Reiner et al.,1989*). Major surface antigens of different molecular masses are also identified as immune components (*Einfeldet al., 1984*). A commercially available ELISA uses a glycosylated glycoprotein, GSA 65 which is 96% sensitive and 100% specific for giardiasis detection*(Rosoff and Stibbs, 1986a; Rosoff et al., 1989).*

2.7.1.3. Alternative approaches

In 1990 a polymerase chain reaction (PCR) technique using DNA probe was developed to detect live *Giardia* cyst (*Mahbubani et al., 1991*). However, sensitivity has been low and, until recently, only 1 ng or more of *Giardia* DNA (equivalent to 5 x lo3 trophozoites) has been detectable *(Butcher and Farthing, 1989).* This is a very sensitive and specific assay for *Giardia* detection based on the amplification of beta giardin gene by PCR. Further improvement in the protocol has made it a powerful tool for environmental detection which can detect a single Giardia cyst (*Abbaszadegan et al., 1991*). The PCR technique is also able to distinguish between pathogenic and non-pathogenic Giardia strains (*Mahbubani et al., 1992*).

2.7.2. Treatment

The several drugs commonly used for therapy of giardiasis worldwide have in general been effective. However, in those regions of the world where *Giardia* is endemic there are some drawbacks associated with long dose regimes, poor palatability and potential toxicity, especially in children who may require treatment often because of reinfection. Most often used drugs are -

Drug	**Treatment duration**	**Possible side effects**
Metronidazole	5–7 days	Metallic taste; nausea; vomiting; dizziness; headache; disulfiram-like effect; neutropenia
Tinidazole	Single dose	Metallic taste; nausea; vomiting; belching; dizziness; headache; disulfiram-like effect
Nitazoxanide	3 days	Abdominal pain; diarrhea; vomiting; headache; yellow-green discolouration of urine
Albendazole	5 days	Dizziness; headache; fever; nausea; vomiting; temporary hair loss

2.8. Cultivation of parasites

Karapetyan (1960, 1962) first demonstrated successful and reproducible method of culturing Giardia trophozoites but it was monoxenicculture and presence of yeast was required for the survival of trophozoites. The method of axenic cultivation was first reported by Meyer (1970) from the rabbit, chinchilla and cat and later human (1976) using HSP-1 medium, subsequently modified in 1976, contained phytone peptone, glucose, L-cysteine HCL, Hank's solution and human serum (*Meyer, 1976*). Successful establishment of axenic culture is made majorly with the isolates of *Giardia lamblia,* though single reports of axenisations of

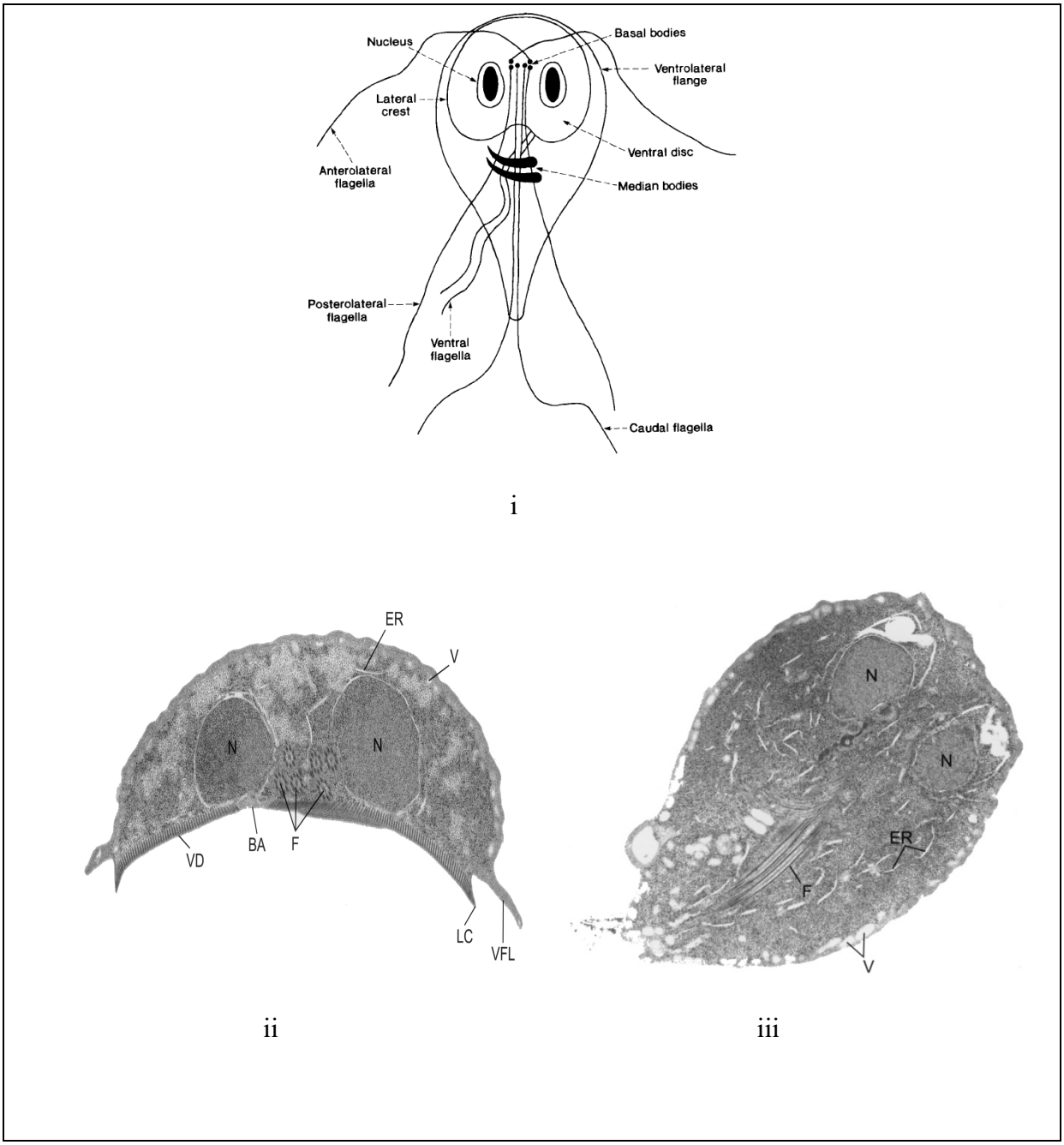
Giardiamuris and *Giardia ardeae* are also present *(Gonzalez-Castro et al., 1986; Erlandsen et al., 1990b)*. Upgradation to bile supplemented medium for *Giardia* enables invitro excystation techniques for cultured trophozoites which is essential for maintenance, isolation and amplification of a large number of different isolates in the laboratory *(Keister, 1983; Radulescuet al., 1990; Thompson et al., 1990a)*. Next development in inducing successful encystation of *Giardia* trophozoites in vitro enables to study the complete life cycle of it *(Boucher et al., 1990)*.Portland-1 and WB, an isolate obtained from a symptomatic human who probably acquired his infection in Afghanistan (*Smith et al.,1982a*), belong to the same genotype and have been used for many of the studies of *G.lamblia.* The growth medium has subsequently been modified from the first used medium, and currently the most commonly used medium is modified TYI-S-33 (*Keister,1983*). The essential requirements for axenic growth are a high cysteine concentration, exogenous lipids which are acquired from decomplemented serum, with a low O_2 concentration. At 37^0C trophozoites are able to adhere to the glass walls and grow. This adherence is dependent on glycolysis and on contraction of proteins of the ventral disc (*van Keulen et al.,1998*).

2.9. Morphology of *Giardia lamblia*

2.9.1.Trophozoite Structure

G. duodenalis trophozoites are 12 to 15 μm long and 8 μm wide with a pear-shaped body. They possess a median body, four pairs of flagella (anterior, posterior, caudal, and ventral). The ventral side of the parasite is concave and contains a ventral disk which is surrounded by the lateral crest and a flange (Fig.5.). The ventral disc is important for the organisms as it allows the parasite to attach to the intestinal epithelium. It has a convex dorsal surface with two symmetrically placed nuclei in the anterior half.Trophozoites have anteriorly located, symmetric two nuclei without nucleoli. Lysosomal vacuoles, as well as ribosomal and

glycogen granules, are found in the cytoplasm. Golgi complexes become visible in encysting trophozoites but have not been confirmed to be present in vegetative trophozoites (*Gillin et al.,1996*). However, stacked membranes suggestive of Golgi complexes have been demonstrated (*Lanfredi et al., 1999; Soltys et al., 1996*).



Source: *Adam et al., 2001*

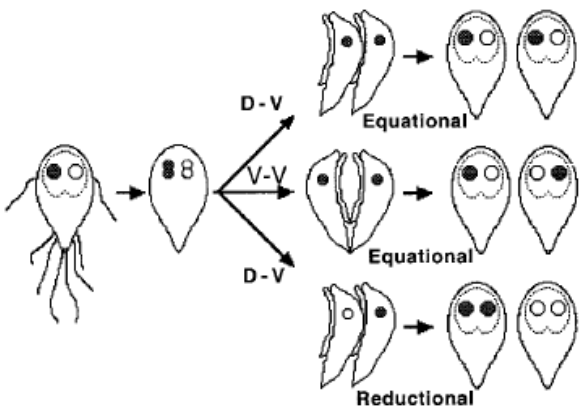
Fig.5.i. Diagram showing the general morphology of the trophozoite of *Giurdia.***ii.**Trophozoite coronal section. A coronal view of a trophozoite demonstrates the

nuclei (N), endoplasmic reticulum (ER), flagella (F), and vacuoles (V). A mechanical suction is formed when the ventral disk (VD) attaches to an intestinal or glass surface. Components of the ventraldisk include the bare area (BA), lateral crest (LC), and ventrolateral flange (VLF). **iii.** Trophozoite cross section. A cross-sectional view of a trophozoite demonstrates the nuclei (N), flagella (F), vacuoles (V), and endoplasmic reticulum (ER).

2.9.2.Nuclear Structure and Replication

*G. lamblia*trophozoites have two nuclei that are nearly identical in appearance. They replicate at approximately the same time (*Wiesehahn et al.,1984*) and are both transcriptionally active as determined by uridine incorporation into nuclear RNA. Both nuclei have approximately equal numbers of rDNA genes as determined by in situ hybridization using the rDNA probe (*Kabnick et al.,1990*). Both have approximately equal amounts of DNA as determined by the intensity of nuclear staining with 49,6-diamidino-2-phenylindole (DAPI) or propidium iodide (*Kabnick et al.,1990; Bernander et al., 2001*), although the possession of equal amounts of DNA by the two nuclei has been questioned. It is generally assumed that the two nuclei have the same complement of genes and chromosomes, and results from our laboratory using fluorescence in situ hybridization with single-copy genes support this assumption. These features all differ from those of the ciliated protista that are binucleate, such as *Tetrahymena* and *Paramecium* (*Lanzendo¨rfer et al., 1992*). These organisms have a smaller micronucleus that contains the genomic DNA but is not transcriptionally active. DNA from the micronucleus is amplified into multiple copies, forming the macronucleus, from which transcription occurs. *Giardia* spp. and the other diplomonads appear to be unique in their possession of two nuclei that are identical by the above parameters. The nuclei of higher eukaryotes have readily visible nucleoli which are the sites of rRNA transcription. Fibrillarin is a major component of nucleoli and is required for pre-rRNA processing as well as for viability in *Saccharomyces cerevisiae* (*Jansenet al., 1991; Schimmang et al., 1989*,*Tollervey et al., 1993*). Nucleoli have not been identified in *G. lamblia*nuclei, and antibody to *G. lamblia*fibrillarin diffusely stains the nuclei of *G. lamblia*, suggesting that rRNA transcription

and processing are not localized to certain regions of the nuclei (*Narcisi et al., 1998*). Axenic cultures have not been synchronized, and the division time is very brief, so studies of cellular and nuclear division have been limited to observations of trophozoite replication based on static microscopy. On the basis of these observations, it has been proposed that during cellular division, the nuclei move laterally followed by nuclear replication, resulting in trophozoites with four nuclei. The trophozoites then divide in the longitudinal plane in such a way as to maintain the left-right asymmetry (*Cerva et al., 1992, Filice et al 1952; Kabnick et al., 1990*). Electron micrographs of excysting organisms undergoing cytokinesis have shown cells that appear to be joined laterally or with the ventral disks opposing each other (*Hetsko et al., 1998*). Laterally joined cells would be consistent with the above mechanism of cytokinesis, but replication that results in opposing ventral disks would invert the left-right asymmetry with each division. Thus, it is not yet possible to be certain of the morphology of cytokinesis. A number of drugs that arrest the cell cycle of mammalian cells have been tested for their effect on trophozoites. Colchicine (mammalian G21M) and gamma irradiation (mammalianG21M) had no effect, while hydroxyurea (mammalian G21M) and razoxane (mammalian G1/S, sometimes G21M), arrested the cell cycle in the G21M phase (*Hoyne et al.,1989*).



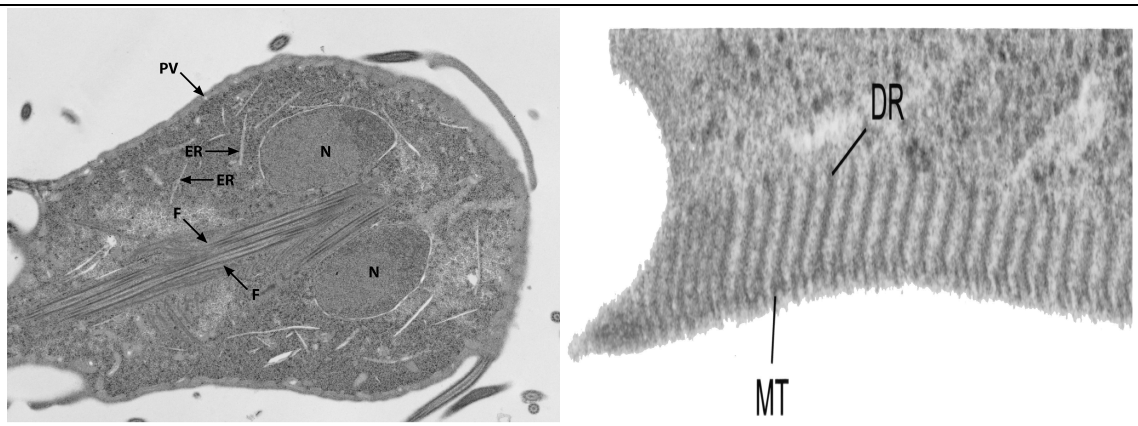
Source: *YU et al.2002*

2.9.3.Cytoskeleton and Motility

Trophozoites colonize the small intestine of their host, predominating in the mid-jejunum. They attach by their concave ventral surfaces (ventral adhesive disk) to the intestinal wall, where they obtain the necessary nutrients and avoid transport beyond the jejunum. The ventral disk mediates a mechanical attachment not only to the intestinal wall but also to the surface of the container used for axenic growth. Neither cellular invasion nor receptor-mediated attachment has been documented for *Giardia* spp. Therefore, the cytoskeleton and especially the ventral disk play a key role in the survival of the organism in the intestine of the host. The ventral disk is a unique and important component of the *G. lamblia*cytoskeleton. Grossly, it appears as a concave structure with a maximum depth of 0.4 mm covering the entire ventral surface. The edge narrows into a lateral crest, and a more flexible ventrolateral flange surrounds the disk (*Erlandsen et al., 1974*). The disk contains the contractile proteins actinin, a-actinin, myosin, and tropomyosin (*Feely et al.,1982*) as the biochemical basis for the contraction of the disk involved in adherence. Attachment depends on active metabolism and is inhibited by temperatures below 37°C, increased oxygen levels, or reduced cysteine concentrations (*Gillin et al., 1981,Gillin et al., 1982*). Ultrastructurally, the ventral disk includes a set of microtubules containing 13 protofilaments and linked to the ventral membrane. These microtubules form the base of the microribbons (dorsal ribbons) that extend nearly perpendicular from the membrane. The protein constituents of the dorsal ribbons (microribbons) include a set of giardins, which are alpha-coiled-helix proteins approximately 29 to 38 kDa in size. The giardins line the edges of the microribbons but are not found in the microtubules (*Peattie et al., 1989*). Although 23 forms of these proteins have been separated by two-dimensional electrophoresis, the N-terminal sequences of some variants are identical, suggesting that posttranslational modification accounts for some of the forms. Several giardins, a1-giardin , a2-giardin, b-giardin, and g-giardin, have been cloned,

and their sequences confirm the alpha-helical structure of these proteins. The a1-giardin and a2-giardin sequences demonstrate approximately 80% identity at the nucleotide and amino acid levels , while the other giardins have no significant sequence similarity *(Peattie et al., 1989; Alonso et al.,1992; Aggarwal et al., 1989; Holberton et al., 1973)*. A protein with immunologic cross-reactivity with b-giardin, but significantly larger, has also been identified as a cytoskeletal protein (head stalk protein; HPSR2) (*Marshall et al.,1995*). This 183-kDa protein has a long coiled-coil stalk and an N-terminal hydrolytic domain. Tubulins are not found in the microribbons but are found in the microtubules (*Soltys et al.,1994*). The microtubules of the ventral disk, as well as those of the flagella, are presumably composed of ab-tubulin. Posttranslational modifications of the *G. lamblia* tubulins include acetylation and polyglycylation (*Soltys et al.,1994;Weber et al.,1996;Weber et al.,1997*). Benzimidazoles are compounds used primarily as antihelminthic agents, but they also have significant in vitro activity against *G. lamblia*as well as in vivo efficacy for giardiasis (*Chavez et al.,1992;Dutta et al.,1994;Edlind et al.,1990;Hall et al.,1993;Morgan et al.,1993*). Their effect is thought to be mediated by their interaction with b-tubulin. *G. lamblia*trophozoites exposed to albendazole (or other benzimidazoles) lose their ability to adhere despite normal flagellar movement (*Edlind et al.,1990*). This suggests a difference between the tubulin found in the ventral disk and that found in the flagella. This, as well as the observation that adherence can occur in the absence of flagellar function (*Feely et al.,1982*), suggest that the ventral disk is important for adherence but the flagella are not. With more prolonged exposure (e.g. 24 h) to albendazole, the ventral disk becomes fragmented, with dislocation of the microribbons and microtubules of the ventral disk. Substantial electron-dense precipitates can be found in the microtubules and microribbons and to a lesser extent in the other tubulin-containing structures, such as the median body and flagella (*Chavez et al.,1992*). The pronounced effect of albendazole on the microribbons, despite the apparent lack of tubulin in the microribbons,

raises the possibility that the benzimidazoles are reacting with proteins other than tubulins, such as the giardins. Vinculin is a protein that binds a-actinin and mediates the attachment of actin filaments to membrane sites. Its location in attachment regions of the ventral disk has led to the suggestion that it may be involved in the attachment process (*Narcisi et al.,1994*). Further functional studies are required to confirm or refute this proposal. The trophozoite has four pairs of flagella that begin at two sets of basal bodies that are near the midline and anteroventral to the nucleus. They emerge from the anterior, posterior, caudal, and ventral regions of the trophozoite. Paraflagellar rods extend along one side of the two ventral flagella (*Feely et al.,1990, Holberton et al.,1973*). Nine pairs of microtubules encircle two microtubules to form the flagella. The flagella appear to be important for motility but not for attachment. In addition, their early emergence through the cyst wall during the process of excystation suggests their importance in excystation (*Buchel et al.,1987*) (see "Excystation" below). The median body is a component of the cytoskeleton that is located in the midline and dorsal to the caudal flagella and consists of a group of microtubules in a tight bundle. It is unique to *Giardia* spp., and its morphology helps define the morphologic characteristics of the different *Giardia* species. *G. lamblia*trophozoites typically have two median bodies that are shaped like claw hammers. The median body has been proposed as the assembly site for microtubules to be incorporated into the ventral disk (*Meng et al.,1996*). Caltractin/centrin, a calcium binding protein that is responsible for basal-body orientation, has been identified in the basal bodies as well as the paraflagellar rods and median bodies (*Belhadri et al.,1995*; *Meng et al.,1996*). A 101-kDa coiled-coil protein has also been localized to the median body by immunofluorescence microscopy (*Marshall et al.,1993*).



I **II**

Source :*Belhadri et al.,1995*

Fig.6.: **I.**A coronal view through the nuclei (N) also demonstrate the multiple flagellar axonemes (F) traversing through posteriorly. The endoplasmic reticulum (ER) and peripheral vesicles (vacuoles) can also be seen. **II**. Close-up of the ventral disk. A magnified view of the ventral disk shows the microtubules (MT) and microribbons or dorsal ribbons (DR)

2.9.4. Ventral disk

The Importance of ventral disk lies on the process of parasite attachment to the host intestine. It is composed of tubulins as well as another one, termed giardins, which are unique to Giardia (*Dawson, 2010*). From the electron microscopic view it is revealed that the ventral disk is formed with regularly spaced rows of microtubules and trilaminar microribbons composed of a set of giardins, and uniformly spaced cross bridge structures that connect the adjacent microribbons*(Dawson, 2010)*. At the process of attachment, the peripheral lateral crest of ventral disk first makes the contact with the biological or inert surface. Initial contact with the surface changes the shape of more flatten ventral disk to a dome shaped architecture *(House et al., 2011).*The ventral disk microtubules are resistant to microtubule-targeted drugs that can either stabilize or destabilize microtubules *(Dawson et al., 2007),* which implies the fact that these microtubulesdo not undergo rapid polymerization or depolymerisation. While in the process of encystations, there is a gradual change in its shapefrom ribbon like structure

to fragmented one and finally in matured cyst it is completely absent *(Midlej&Benchimol, 2009)*.

2.9.5.Median body

The median body, located at the dorsal side, slightly posterior to the ventral disk, serves as an assembly site for microtubules that are incorporated into the ventral disk (*Woessneret al., 2012*). Its morphology differs from one species of Giardia to another. It is claw-hammer shaped in G. lamblia, teardrop shaped in Giardia agilis, and rounded in Giardia muris (*Adam et al., 2001*). Median body microtubules are highly dynamic and their dynamicity is regulated by the microtubule depolymerizing kinesin motor protein, kinesin 13 *(Dawson et al., 2007)*. Depending on the stage of the cell cycle, the median body may or may not be visible; it disappears completely after mitosis and before disk assembly, and re-appears after cytokinesis. Its structure and function remains poorly defined and only two protein, the median body protein, MBP, and a regulator of microtubules, EB1, have been localized to it (*Dawson et al., 2010*).

2.9.6.Endosome-lysosome vacuoles

The eukaryotes have a system of endosomes and lysosomes to degrade and recycle endogenous proteins or internalized protein from the extracellular space. Early endosomes internalize endocytosed proteins to return to the cell membrane or transport to late endosomes (or, alternatively, maturation of early into late endosomes) followed by degradation by the lysosomes. Both are acidic, with a pH of 6 and pH 5 respectively. *Giardia*posses numerous vacuoles along the periphery of the cell,thathas the criteria of endosomes and lysosomes. These vacuoles are acidic, as shown by their uptake of acridine orange (*Feely et al.,1991, Kattenbach et al.,1991*). They have potential role in endocytosis (*Bockman et al.,1968;Lanfredi-Rangel et al.,1998*). *G. lamblia* virus particles appear to be concentrated

into the vacuoles by an endocytotic mechanism (*Tai et al.,1993*). Pulse-chase labeling with horseradish peroxidase showed early and persistent labeling of vacuoles, suggesting that there is no distinction between early and late endocytic vesicles as is found in higher eukaryotes (*Kattenbach et al.,1991*). Labeling of a smaller portion of the vacuoles with chemicals similar to the labelling agent of ER (*Kattenbach et al.,1991*), as well as EM using anti-BiP antibody (*Soltys et al.,1996*), have suggested a continuity of these vacuoles with the ER. The vacuoles also contain acid phosphatase, proteinase, and RNase, indicating their lysosomal characteristics (*Feelyet al.,1987; Lindmark et al.,1988*). Hence, the vacuoles may function as early and late endosomes and lysosomes and can be functionally associated with the ER as well (*Kattenbach et al.,1991*). The major cysteine proteinase activity of *G. lambli*atrophozoites has been found in proteins with molecular masses of 40 and 105 kDa (*Hare et al.,1989*), 35 and 95 kDa (*Werries et al.,1991*), and 38-kDa thiol (*Parenti 1989*) in the endosome-lysosome vacuoles (*Lindmark et al.,1988*). A family of three cysteine protease genes (CP1, CP2, and CP3) have been found to be members of the cathepsin B subgroup of the peptidase family C1 (*Ward et al.,1997*). These cysteine proteinases are approximately 30 kDa in size, perhaps corresponding to the smaller of the above-noted proteinase activities. Only CP2 and CP3 are expressed; CP2 is found in the vacuole and is involved in excystation.

2.9.7.Proteasomes

The proteasome is a large complex which is able to degrade improperly folded endogenous proteins. The molecular mass of 20S proteasome complex is about 700 kDawith protease activity. With the addition of regulatory activity proteins the complex can be transformed to a 26S complex with the size of 2,000 kDa. Unlike archaea the eukaryotic 20S proteasome contains seven different alpha and seven different beta subunits but their organization pattern is similar to the Archaea. Though *Giardia. lamblia* has certain similarities to the Archaea but

20S proteasome of *Giardia* demonstrates a distinctly eukaryotic pattern with 14 subunits (*Emmerlich et al.,1999*). Proteins are targeted to the proteasomes by ubiquitin, which appears to be present in *Giardia* as a single-copy gene, in contrast to the multiple gene copies found in other eukaryotes (*Krebber et al.,1994*).

2.9.8.Nucleic acids

The characteristics of giardial DNA, RNA species and the associated genetics have been recently reviewed (Adam, 1991). Attempts to resolve the genome size and ploidy in Giardia have yielded rather conflicting data.

2.9.8.1. DNA and chromosomes

The estimated guanine (G) and cytosine (C) content of *Giardia* genome is 42 and 48% respectively *(Nash et al., 1985; Boothroyd et al., 1987).* So far sequenced protein coding genes have G-C content ranging from 49 to 60% and the non coding sequence is rich in adenine (A) + thymine (T). Five sets of chromosomes have been identified by using pulse field gel electrophoresis (PFGE) which are of varying sizes between 1 x $l0^6$ to 4 x $l0^6$ base pairs (bp) *(Adam et al., 1988a)*. According to the results of PFGE separation it is suggested that two nuclei of trophozoites are consist of 30-50 chromosomal DNA molecules *(Adam et al.,1988b)*. The differences in the number of chromosomes between the ranges of 650-800 kb have been discovered by Field inversion gel electrophoresis (FIGE) *(Upcroftet al1989)*.

Light microscopy findings of four chromosomes, of 1-4 Mb in size per nucleus, would not explain the Ci analysis of 3 x lo7 and 8 x lo7 or the apparent presence of 3&50 chromosomal DNA molecules per trophozoite *(Adam, 1991)*.

2.9.8.2. Ribosomal RNA

The giardial ribosomal RNA (rRNA) transcripts are smaller than those of other eukaryotes as well as eubacteria which make it unique *(Boothroyd et al., 1987; Edlindet al., 1987).* The sequence of small (16s) subunit of *Giardia*rRNA is more similar to archaebacteria than to the corresponding sequence of other eukaryotes *(Edlindet al., 1990a)*. The 5566 bp rDNA gene of *Giardia lamblia* is tandemly repeated in the genome *(Healey et al., 1990)*. Due to lengthy intergenic spacer region *Giardia muris* and *Giardia ardeae*rDNA genes are 7600 bp long *(van Keulen et al., 1991a, b)*. The coding region of the 5.8s rRNA and 3' portion of the large 28s subunit rRNA are also different in these *Giardias(van Keulen et al., 1991a, b)*. Copies of rDNA genes were identified on multiple chromosomes with extensive variation between otherwise closely related isolates. It has been shown that rDNA repeats are present in telomeric region of Giardia isolates and can rearrange frequently within cloned lines of WB strain *(Le Blancqet al., 1991*). Investigation of three *G . duodenalis*telomeric clones have revealed abrupt transition from rDNA to telomeric repeats, TAGGG, two from the same location at the beginning of the 28s subunit and another at the beginning of the intergenic spacer with the sequence CCCCGGA being present at each of the breakpoints *(Adam etal., 1991)*.

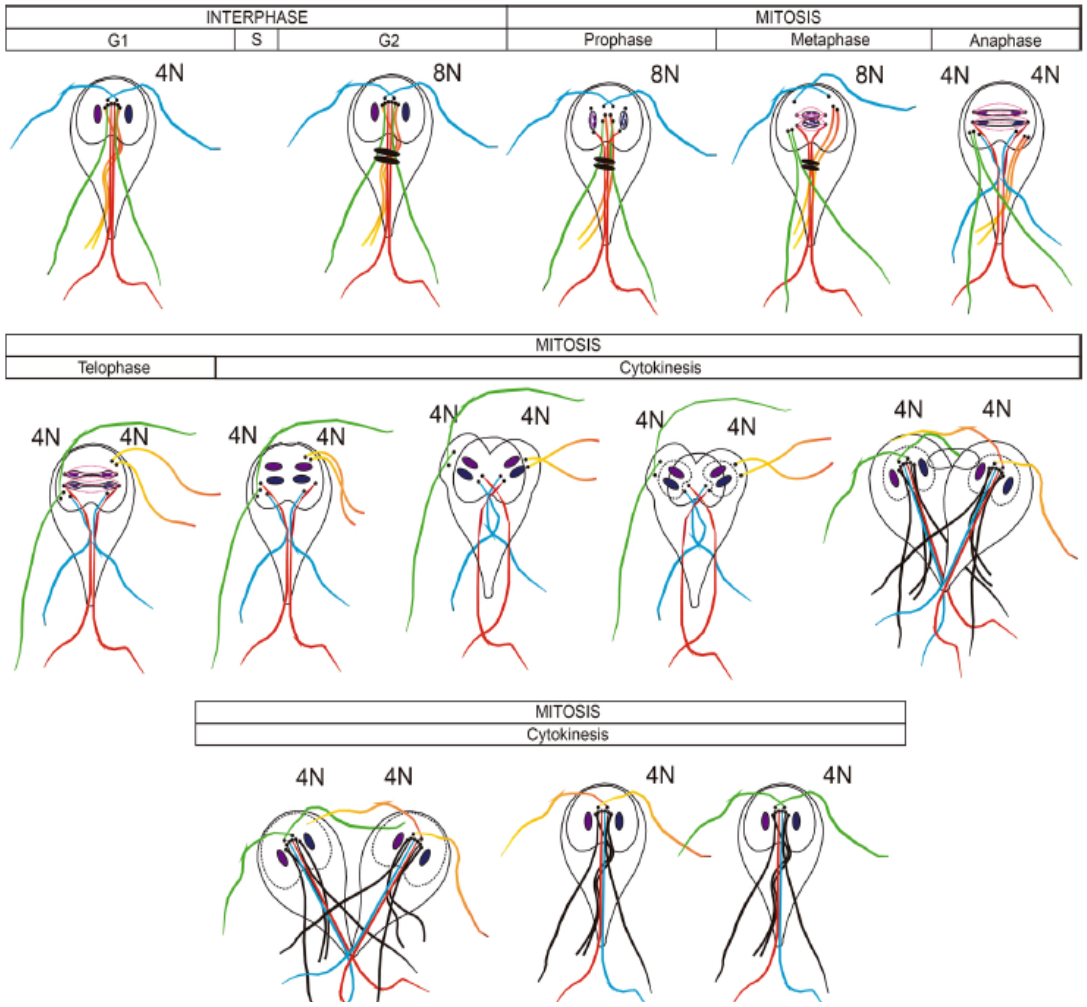
2.9.9. *Giardia* Cyst Structure

Encystation occurs after organisms have undergone nuclear replication but before cytokinesis; therefore, cysts contain four nuclei. They are approximately 5 by 7 to 10 mm in diameter and are covered by a wall that is 0.3 to 0.5 mm thick and composed of an outer filamentous layer and an inner membranous layer with two membranes. The outer portion of the cyst wall is covered by a web of 7- to 20-nm filaments (*Erlandsen et al.,1990*). Four major proteins have been identified in the outer cyst wall, 29, 75, 88, and 102 kDa in size

(*Erlandsen et al.,1990*). The sugar component of the outer portion is predominantly galactosamine in the form of N-acetyl galactosamine (GalNAc) (*Adam 1991*). Earlier claims that the cyst wall is composed of chitin (*N*-acetylglucosamine) have been refuted (*Paget et al., 1989*). As the environmentally stable form of the life cycle, cysts have a metabolic rate only 10 to 20% of that found in trophozoites (*Gillin et al.,1988*). The respiration of both cysts and trophozoites is stimulated by ethanol, while trophozoites are stimulated only by glucose.

2.10. Cell Cycle

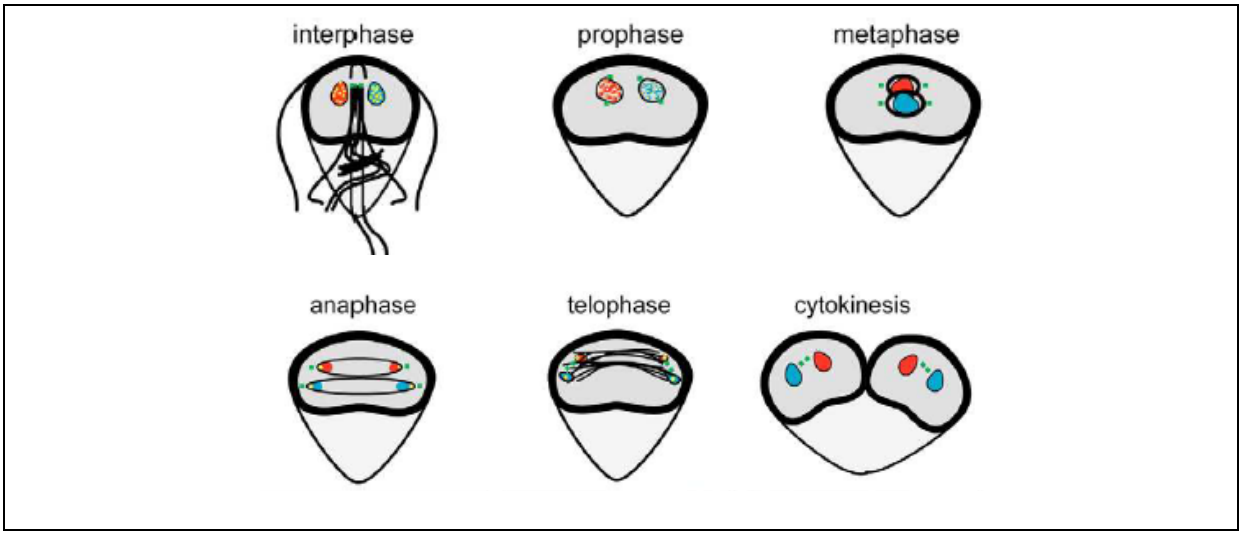
In the process of Giardia trophozoites cell division it is necessary to duplicate and partition of both diploid nuclei as well as the multiple cytoskeletal structures. On the basis of the conventional flow cytometric analysis of the DNA content of encysting cells, early and late cysts, and a cells after excystment, a model illustrating the relationship between cell cycle and life cycle has been proposed *(Bernander et al., 2001)*. During the cell cycle, the ploidy of the Giardia trophozoites alternates between tetraploid (2 × 2 N ploidy) in the G1 stage and octoploid (2 × 4 N ploidy) in the G2 stage. Upon induction of encystation, a G2 Giardia trophozoite (with 2 × 4 N ploidy) forms a cyst wall and divides its nuclei, resulting in a cell with four nuclei with 2 N per nucleus (4 × 2 N ploidy). Each nucleus then undergoes another round of DNA replication without cell division (cytokinesis) so that the resultant 16 N cyst will contain four 4 N nuclei (4 × 4 N ploidy). When the cyst "hatches" during excystation, a form of Giardia called the excyzoite emerges and divides to generate two G2 trophozoites (each with 2 × 4 N ploidy) that re-enter the cell cycle and then immediately undergo cytokinesis leading to four G1 cells (each with 2 × 2 N ploidy) *(Bernander et al., 2001).*



Source:*Lagunas-Rangel et al., 2021*

Fig.6.:Dynamics of cytoskeletal structures during the *Giardia* cell cycle. The cell division of *Giardia* is a complex process that requires coordination of several events during prophase, metaphase, anaphase, telophase and cytokinesis. In this figure we focus on the changes that occur in the nuclei, the flagella and the adhesive disc during the cell cycle. All images show the dorsal view of the cell. The ventral disc located in the upper half of the trophozoite is outlined by a solid line in the parental cell, whereas it is outlined by a dotted line in each daughter cell. Basal bodies are indicated as black dots above the nuclei. The parental anterior flagella are represented in blue, caudal flagella in red, posterior flagella in green and ventral flagella in yellow. The pair of parental anterior flagella (blue) are transformed into the right caudal flagellum in each of the daughter cells. The parental posteriorlateral flagella (green) are transformed into the anterior flagella in one daughter cell whereas the parental ventral flagella (yellow) are transformed into the anterior flagella in the other daughter cell. In contrast, both caudal flagella (red) maintain their type and are always positioned on the left side in each of the resultant daughter cells when viewed on the dorsal side.

G2 phase of interphage of *Giardia* trophozoites cell cycle is the longest one*(Bernander et al., 2001)*. During this phase, *Giardia* chromatin is decondensed and basal bodies are located in the region immediately above both nuclei. In *Giardia* trophozoites mitosis is semi-open. The two extranuclear central spindles and microtubules enter the nuclei through polar openings in the nuclear envelopes (*Sagolla et al., 2006)*. In prophase, condensation of chromatin into chromosomes starts*(Markova et al., 2016)*in absence of the H1 linker histone *(Wu et al., 2000; Yee et al., 2007)*.Next, the flagellar basal bodies duplicateand the nucleation of the four spindle poles begins *(Hardin et al., 2017; Sagolla et al., 2006)*. A repositioning of both nuclei above the other along the dorsal-ventral axis the trophozoites occurs near the end of the prophase.Chromosomes are segregated along the left-right (L-R) axis before cytokinesis along the longitudinal axis – perpendicular to the spindle and the daughter cells inherit one copy of each parent nucleus.Meanwhile, spindle microtubules and the flagellar basal bodies are gradually placed around each nucleus, and the microtubules are organized in the polar spindles, elongating until they surround each nucleus (*Sagolla et al., 2006*).



Source:*Sagolla et al., 2006*

Fig.7.: Chromosome segregation during cell division. During mitosis two autonomous spindles are responsible for chromosome segregation after nuclei migrate to the cell center. The centromere-specific histone cenH3 marks centromeres (cenH3:GFP in green, A-C,G-I), and centrin antibodies mark spindle poles (green, D-F,J-L). TAT1 (anti-tubulin) labels the

microtubule cytoskeleton including the mitotic spindles (all cells, in red) and DAPI labels chromatin (all cells, in blue). Behavior of left (red) and right (blue) nuclei is diagramed above each stage.

2.11. Trascription and Translation

Transcription in *G.lamblia* is distinctly eukaryotic in nature; nonetheless it has a number of features that are more characteristic of prokaryotics such as amanitin-resistant transcription using RNA polymeraseII. As in all eukaryotes, the transcript is produced in the nucleus and transport/rted to the cytoplasm for translation. The polyadenylationof transcripts are typical for eukaryotes, but the short 5' UTRs and general lack of introns are more characteristics of prokaryotes (although introns may be relatively infrequent in the unicellular eukaryotes). Also, in the contrast to other eukaryotes, most *G.lamblia*transcripts do not appear to be capped at their 5' ends. Transcripts have been analysed to determine whether they have 7-methylguanosine or other caps at the 5' ends. Total polyadenylated RNA was analyzed to determine if the 5'ends were susceptible to T4 RNA ligase (*Yu et al., 1995*). They were resistant to 5' phosphorylation unless pretreated with calf intestinal alkaline phosphatase to remove the 5' phosphates. These results suggested that the RNA was phosphorylated but not capped.Treatment with the decapping enzyme tobacco acid pyrophosphatase did not increase phosphorylation, also indicating a lack of 5' capping. This result suggests that most polyadenylated RNA does not have a 7' methylguanosine cap. However, since the studies were done using total rather than transcript-specific mRNA, it is still possible that individual transcript could be capped.

Small nuclear RNA molecules are involved in the splicing of nuclear pre-mRNA (snRNA U1,U2, U4, U5, U6) and pre-rRNA (sno-RNA U3,U8, U14, snR10 and snR30) to produce the mature RNA (145). Many of these molecules from other eukaryotes have a tri methylguanosine cap at the 5'end. A number of candidate snRNAs from *G.lamblia*were

immuno-precipitated by anti trimethylguanosine antiserum. Caps were confirmed by the susceptibility of the antibody reactivity to the decapping effect of tobacco acid pyrophosphatase. The exact roles and identities of the candidates of snRNAs have not yet been determined. It will be of special interest to determine whether splicing of mRNA occurs since introns have not been identified in the *G.lamblia* genes, although very recently *Nixon et.al.* has proposed a spliceosomal introns in *G.lamblia* (*McArthur et al.,2000*).

2.12.Protein Transport and Degradation

The endoplasmic reticulum (ER) and Golgi complex are part of a eukaryotic endomembrane system involved in protein folding and translocation. In higher eukaryotes, proteins destined for secretion have a signal sequence that directs them to the ER as they are translated in the ribosomes. The signal sequence binds to the signal recognition particle (SRP). This complex then binds to the SRP receptor (SR) on the cytoplasmic portion of the ER. The SR is a dimeric protein consisting of the membrane anchored SRb and the GTPase, SRa. After their translocation to the ER, chaperonins such as BiP (the HSP70 homologue found in the ER) aid in folding and further transport. Although structures consistent with ER had previously been identified by electron microscopy (EM), there was some doubt until recently about the existence of ER in *G. lamblia*. However, the cloning and characterization of SRa, as well the identification of an extensive membrane system labeled with antibody to BiP, has clearly demonstrated the existence of the ER (*Soltys et al.,1996*). One of the important aspects of protein folding includes the correct formation of disulfide bonds, which is accomplished in the lumen of the ER by protein disulfide isomerase. Three protein disulfide isomerase genes from *G. lamblia*have been cloned and characterized, and their products have been localized to the ER (*Marshall et al.,1993*). Golgi complexes have not been detected in trophozoites by standard microscopic techniques but have been demonstrated in encysting organisms (*Gillinet al.,1996*;*Reiner et al.,1990*). More recently, transmission and freeze fracture EM of

nitrobenzoxadiazole (NBD) ceramide-labeled log phase trophozoites demonstrated heavy staining in the perinuclear region in a pattern similar to that of the Golgi complex from other organisms (*Lanfredi-Rangel et al.,1999*). ADP-ribosylation factor (ARF) is a GTP binding protein required for the budding of clathrin-coated and COP I-coated vesicles from the Golgi complex. ARF is expressed in *G. lamblia*trophozoites and has been used to complement ARF function in *S. cerevisiae* (*Lee et al.,1992, Murtagh et al.,1992*). Antibody to ARF and to b-COP (from the COP I coat) label perinuclear vesicles in a manner that is inhibited by brefeldin A (*Shant et al., 2004*), also suggesting that *G. lamblia*trophozoites may have a form of Golgi complex. However, further investigation is required to further confirm the presence of a Golgi complex in trophozoites and to determine its characteristics. Specific importation of proteins into the nuclei has also been documented by using the simian virus 40 nuclear localization signal to direct green fluorescent protein (GFP) to the nuclei (*Nash et al.,1992*). Ran is a nuclear GTP binding protein that is involved in the import of proteins in the nuclei of *Xenopus* oocytes and participates in cell cycle progression in yeast (*sppi*1). It has been cloned and characterized in *G. lamblia*, but its cellular location and function have not yet been determined (*Chen et al.,1994*).

2.13.Encystation

2.13.1.Promoting and inhibiting factors

As trophozoites replicate and colonize the intestinal surface, some encyst in the jejunum after exposure to biliary secretions. Encystation has been performed in vitro by exposing trophozoites to an environment that mimics that of the jejunum (*Gillin et al.,1987*;*Schupp et al.,1988*; *Lujan et al.,1996*). Specific conditions that promoted encystation included a mildly alkalotic pH of 7.8 and conjugated bile salts plus fatty acids (*Gillin et al.,1987*). Other investigators reported that expression of cyst wall protein, a marker of the encystation

process, was detected 90 min after exposure to medium with lipoprotein-deficient serum (*Erlandsen et al.,1996*). Encystation was abolished when cholesterol was added, leading to the proposal that encystation results from cholesterol starvation. The relative importance of cholesterol starvation and addition of bile salts and fatty acids for the induction of encystation remains controversial.

2.13.2.Events of encystations

Encystation can be divided into two phases, early and late. The timetables determined by various studies have differed somewhat, probably because of the different approaches used. After the initiation of encystation conditions, the organisms do not all enter the encystation process at the same time. Therefore, morphologic studies that examine the time course of the first encysting organisms tend to give earlier times for the encystation events than studies that depend on pooled organisms. These morphologic studies indicate that the early phase is complete within about 10 h and the late phase is complete by 16 h (*Reiner et al.,1990*). For this discussion, we will assume that the early and late events from the various studies are comparable. The early phase consists of the intracellular synthesis and transport of cyst wall components. A Golgi-like stack of membranes become readily visible by EM and are probably the means of transporting cyst-specific molecules to the encystation- specific vesicles (ESV) (*Lujan et al.,1995*). The Golgi-like membrane labels with NBD-ceramide and protein secretion is inhibited by brefeldin A, as expected for the Golgi complex (*Lujan et al.,1996*). Expression of BiP, the molecular chaperone found in the ER, is increased during encystation (*Faubert et al.,1991*). The ESV become visible by light microscopy (*Reiner et al., 1989*) and are probably involved in the transport of building blocks to the nascent cyst wall. A set of proteins of 21 to 39 kDa are expressed in the early phase (*McCaffery et al., 1994*). A monoclonal antibody to several bands of 26 to 44 kDa initially labels the ESV and subsequently labels the cyst wall, indicating transport of these molecules to the cyst wall

(*Ward et al., 1990*, *Hehl et al., 2000*). The genes encoding two of the proteins incorporated into the cyst wall have been cloned and characterized, a 26-kDa CWP1 and a 39-kDa CWP2 (*Chatterjee et al., 2010*). They are related leucine-rich proteins and form a stable complex that is transported to the cyst wall by the ESV (*Lujan et al., 1995*, *Que et al.,1996*). Transfection studies performed with fusions of modified GFP and the CWP1 gene have demonstrated the importance of the N terminus for directing the protein to the ESV (*Lujan et al., 1995*). Localization to the cyst wall was dependent on the leucine-rich middle portion. A 110-bp 59- flanking region controlled encystation-specific expression, while the 39 flanking region down-regulated the level of steady RNA produced. Presumably, there is significant overlap between the 21- to 39-kDa and 26- to 44-kDa proteins and CWP1 and CWP2, respectively, but the exact relationship has not been determined. The late phase of encystation consists of the appearance on the trophozoite plasmalemma of sites for initiation of the assembly of cyst wall filaments followed by the assembly of the filamentous portion of the cyst wall. A set of larger (66-, 78-, 92-, and 103-kDa) proteins are expressed in addition to the lower molecular mass proteins (*McCaffery et al., 1994*). Perhaps this includes the 75-, 88-, and 102-kDa proteins found in the cyst wall (*Sauch et al., 1990*), but these larger proteins have not yet been characterized. One encystation-specific protein, enc6, has not been sequenced in its entirety, but its transcript size of 4.4 kb would be compatible with the 102/103-kDa protein (*Macechko et al., 1992*). By the time encystation is completed, motility disappears. The outer portion becomes rounded and filamentous, and the organisms are no longer attached to a surface. The ESV disappears, and the internal portion includes two trophozoites with four nuclei that have not yet completed cytokinesis.

2.13.3.Enzymatic pathways

The major cyst wall sugar, *N*-acetylgalactosamine, is produced by an enzymatic pathway that is induced during encystation. The activities of each of the enzymes in the proposed pathway have been documented (*Steimle et al., 1997*) and two of the enzymes have been cloned and characterized. In the first step, fructose-6-phosphate from the glucose metabolism pathway is converted to glucosamine- 6-phosphate by glucosamine-6-phosphate-isomerase (*van Keulen et al., 1998, Knodler et al., 1999*). One form of this enzyme (gpi2, or Gln6PI-A) is expressed constitutively at low levels during the entire life cycle. Transcription of the other form of this enzyme (gpi1, or Gln6PI-B) is greatly increased during encystation (*Bulik et al.,1998*, *van Keulen et al., 1998, Knodler et al., 1999*).This up-regulated gene has two transcripts with different initiation sites. The shorter transcript is expressed constitutively at low levels, while expression of the longer transcript with the 147 nucleotide 59 untranslated region (UTR) is markedly increased during encystation (*Knodler et al., 1999*). Another enzyme in this pathway, UDP-*N*-acetylglucosamine pyrophosphorylase, is allosterically regulated, with a sixfold increase in activity from activation by glucosamine-6-phosphate (*Bulik et al., 2000; Bingham et al., 1979*). During encystation, the uptake of oxygen and glucose decreases substantially so that glucose uptake is undetectable within 16 h of exposure to encystation conditions. However, aspartate uptake is unchanged, resulting in the suggestion that gluconeogenesis may result from the importation of amino acids.

2.14.Excystation

2.14.1.Promoting and inhibiting factors

In the mammalian host, excystation occurs with exposure to the contents of the proximal small intestine after passage through the acidic environment of the stomach. Excystation was first induced in vitro by exposure of animal- and human-derived cysts to an acidic pH (*Ward et al., 1997*). Optimum excystation occurred following exposure to a pH of 1.3 to 2.7.

Subsequently, excystation of *G. muris*cysts has been performed at pH 7.5 in phosphate buffer with bicarbonate, indicating that an acidic pH was not required for excystation. The inhibition of excystation by 4-49-diisothiocyanatostilbene- 2-29-disulfonic acid (DIDS) suggested that vacuolar acidification was required. Subsequent studies of cysts produced in vitro found an optimal pH of 4.0. Excystation was facilitated by pancreatic proteases and inhibited by a trypsin inhibitor, suggesting the importance of proteases in excystation. In addition to external proteases, a *G. lamblia*cysteine protease (CP2) of the cathepsin B family is required for excystation (*Meng et al., 1996*). Cysteine protease activity was found in the endosome-lysosome-like vacuoles. Inhibitors of cysteine proteases prevented excystation but did not affect trophozoite growth or replication. Certain calmodulin antagonists (TFP and W7, but not W5) inhibited excystation, suggesting that calmodulin may be involved in excystation. Excystation is inhibited by antibody to cyst wall and by wheat germ agglutinin (WGA), presumably by its reaction with one or more of the glycoproteins found in the cyst wall (*Coggins et al., 1984*).

2.14.2.Morphology of excystation

After exposure to conditions that promote excystation, the process of excystation is rapid, being completed within 10 min for *G. lamblia* or for *G. muris*(*Coggins et al.,1984, Coggins et al.,1986*), and appears to be similar for both species. Afterthe initiation of excystation, one or two pairs of cytoplasmicprotrusions (preventral flanges) appear to develop into theventral flange (*Hetsko et al.,1998*). The peritrophic space and the preventralflange enlarge as the emerging trophozoite separates from thecyst wall. Externally, the emergence of flagella through theopening cyst wall is followed by the entire trophozoite (*Buchel et al.,1987*).Karyokinesis occurs during encystation, so that the emergingtrophozoite has four nuclei but only the eight flagella of a singletrophozoite. The oval trophozoite becomes more

roundedand then undergoes cytokinesis within 15 to 30 min after theonset of excystation, so that two trophozoites are formed fromone cyst.

2.15.Biochemistry

2.15.1.Carbohydrate metabolism

Eukaryotic organisms usually thrive primarily on aerobic respiration for their energy production. Most of the eukaryotic protozoan parasites such as *Giardia, Trichomonas* spp. and *Entamoeba*do not contain any morphologically recognizable mitochondria so the chances of mitochondria mediated aerobic energy metabolism in this protozoan are negative.

Hence, they are dependent predominantly on anaerobic fermentative metabolism to maintain their energy and redox homeostasis.*Giardia,* as in most aerotolerant anaerobes, the Embden-Meyerhof pathway (EMP) and its brief extensions constitutes the basis of energy and redox homeostasis and dependent only on substrate level phosphorylation.Absence of the tricarboxylic acid (TCA) cycle in different stages of *Giardia* obviates the fact that glucose is not completely oxidized to CO_2 and H_2O but is incompletely metabolized to form acetate, ethanol, alanine and CO_2 *(Lindmark, 1980; Lindmark and Miller, 1988; Adam, 1991)* . The glucose concentration and oxygen tension in the medium controls the balance of the end product formation.

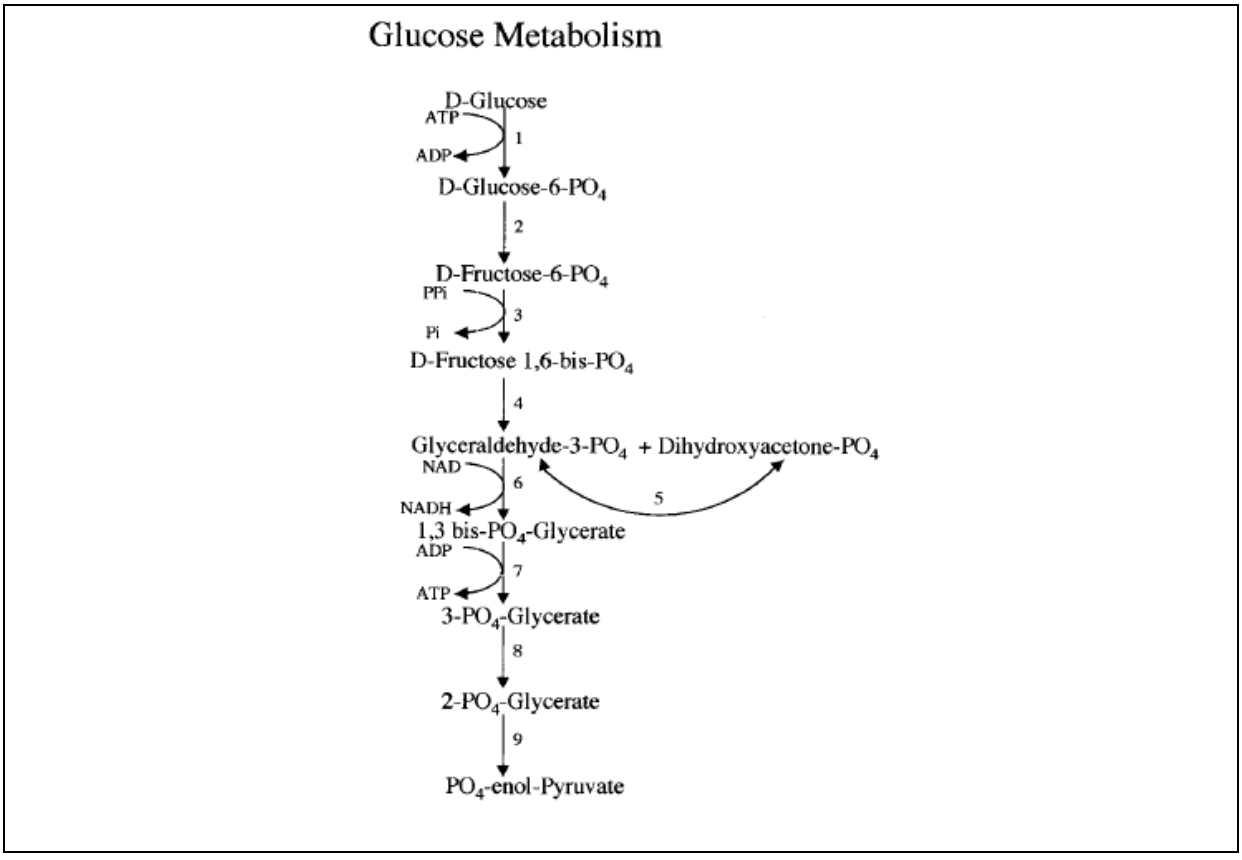
2.15.1.1. Embden-Meyerhof pathway

Using a classical EMP *Giardia* converts glucose or carbohydrates to phosphoenol pyruvate (PEP) and subsequently to pyruvate *(Lindmark, 1980; Lindmark and Miller, 1988)*. Two steps of this pathway are coupled with substrate level phosphorylations. The purpose of this coupling is to maintain the intracellular free NAD/NADH ratio at an optimal level. If the cytoplasmic redox ratio is too low, glyceraldehyde-3-phosphate dehydrogenase will not be

able to function in forward directionwhich will ultimately stop glycolysis *(Barrett, 1984)*.The key enzymes of the EMP in *Giardia duodenalis*and *Giardia muris*has been reported but ATP-dependent phosphofructokinase (PFK-1) and lactate dehydrogenase (LDH) is absent (Lindmark, 1980; Lindmark and Miller, 1988). This exemplifies the conflicting information on the occurrence and the regulatory properties of the EMP and related enzymes in *Giardia*. The major rate-controlling step of glycolysis is controlled by PFK-1 but until 1990 the presence of this important molecule in *Giardia* had not attempted *(Barrett and Beis, 1973).* The rate of glycolysis can be determined by the specific activity of PFK or from the combined rates of hexokinase (HK) and phosphorylase *(Crabtree and Newsholme, 1972).*

Though *Giardia* (Portland-1 strain) contains an active pyrophosphate : fructose-6-phosphate-1 -phosphotransferase (PPi-PFK), it is devoid of PFK-1 *(Mertens, 1990)*.*Giardia* extracts catalysed the ATP dependent formation of fructose- 1,6,-biphosphate (Fru- 1,6-P,), namely PFK-I-like activity, at only **3%** of the rate observed with PPi as phosphate donor (Mertens, 1990). It has been suggested (Mertens, 1990) that this PFK- 1-like activity is insufficient to account for the glycolytic flux, which was observed by Lindmark (1980).Glucose is ultimately converted to pyruvate by the Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways. The conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is irreversible and catalyzed by an ATP-dependent phosphofructokinase in most of the organisms. However, in case of *Giardia* this reaction is catalyzed by a pyrophosphate dependent phosphofructokinase which is a reversible reaction (*Mertens et al.,1990,1993*). Giardia (Portland- 1 strain) contained no traces of fructose-2,6,-biphosphate (Fru-2,6-P2) which is a potent stimulator of glycolysis and negative modulator of gluconeogenesis *(Mertens et al., 1990).* No traces of phosphofructo-2-kinase or its hypothetical PPi-linked counterpart has been detected in *Giardia* as well. Significantly Fru-2,6-P2 is notmodulatingPPi-PFK of Giardia nor on the organism's PK *(Mertens, 1990).* The potential

advantage of the presence of PPi-PFK is that it can significantly enhance the ATP yield of fermentative glycolysis using an otherwise useless metabolic byproduct (i.e. PPi). The possession ofPPi-PFK indicates convergent evolution of *Giardia* with some higher plants (Carnal and Black, 1979), and several important protozoan parasites (Mertens, 1990).Triosephosphate isomerise which catalyzes a reversible conversion between dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate is present in *Giardia* (*Mowatt et al.,1994*).



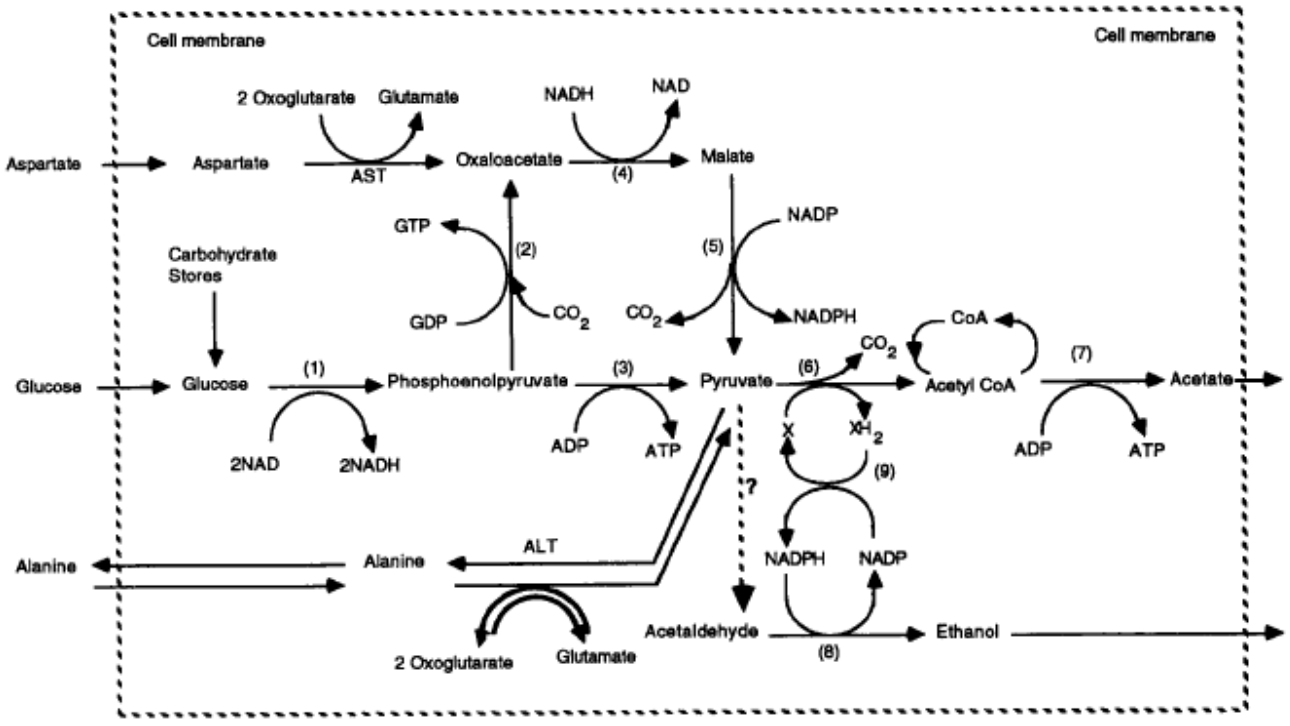
Source: *Adam, 2001*

Fig .8.: Metabolism of glucose to phosphoenolpyruvate. Many of the enzymes have been documented in terms of enzymatic activity, isolation of the enzyme, or cloning of the gene encoding the enzyme. The enzymes are labeled as follows: 1, hexokinase; 2, glucose phosphate isomerase (proposed); 3, pyrophosphate-dependent phosphofructokinase; 4, fructose bisphosphate aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde-3- phosphate dehydrogenase; 7, phosphoglycerate kinase (proposed); 8, phosphoglyceromutase (proposed);

9, enolase (proposed). Certain enzymes (for steps 2, 7, 8, and 9) are suggested on the basis of pathways in other organisms but have not yet been proved for *Giardia*.

2.15.1.2.Metabolism of phosphoenolpyruvate

Like, other eukaryotic parasites, depending on the level of Phosphoenolpyruvate (PEP), *Giardia* have a branched pathway ("metabolic loop"). Two enzymes, ATP-dependent pyruvate kinase, and pyrophosphate- dependent pyruvate phosphate dikinase (PPDK) is reported in the conversion of PEP to pyruvate (*Park et al.,1997; Hiltpold et al.,1999, Hrdy et al.,1993*). PPDK has potential energy advantage over ATP-dependent pyruvate kinase as it can generate two ATP molecules by a coordinated reaction involving PPDK and adenylate kinase and converts phosphoenolpyruvate to pyruvvate (*Rozario et al.,1995*).



Source: *Thomson et al., 1993*

Fig. 9.: Intermediary metabolic pathways operative in *Giurdia*, showing the points of entry for asparate and alanine. (I) Enzymes of the Embden-Meyerhof pathway; (2) phosphoenol pyruvate carboxykinase (GDP); (3) pyruvate kinase (ADP); (4) malate dehydrogenase (NAD); (5) malate dehydrogenase (decarboxylating) (NADP); (6) pyruvate

synthase (synonym, pyruvate ferredoxin oxidoreductase); (7) acetyl-CoA synthetase (ADP); (8) alcohol dehydrogenase (NADP); (9) NADPH oxidoreductase; AST: aspartate-2-oxoglutarate amino transferase; ALT: alanine-2-oxoglutarate amino transferase. Modified, with permission, from Mendis et al. (1992).

2.15.1.3. Metabolism of pyruvate

In eukaryotes two distinct enzymes may perform important role in the catalysis ofthe decarboxylation of pyruvate. The first one is ferredoxin oxidoreductase (PFOR) (= pyruvate synthase) which is dimeric or tetrameric protein (molecular mass 240 kDa) and considered asthe ancestral one.But the more advanced one is pyruvate dehydrogenase (PDH) complex (molecular mass > l0^3 kDa). The former is reversible and utilizes Fe-S protein (ferredoxin) as an electron acceptor while PDH complex is not. PDH and PFOR both are mutually exclusive in any given organism *(Kersscher and Oesterhelt, 1982)*whilePFOR is restricted to strictly anaerobic and nitrogen-fixing organisms.PFOR is functional in Giardia and the conversion of pyruvate to acetyl coenzyme A (acetyl- CoA) is catalyzed by it(*Lindmark et al.,1980; Edwards et al., 1989; Paget et al., 1990)*. The exact mechanism of reoxidation of the giardial ferredoxin which is reduced during these processes remains unknown. The possibility that it provides reducing equivalents for ethanol production *(Lindmark, 1980)* is plausible since the required alcohol dehydrogenase (ADH) and/or aldehyde reductase *(Schofield et al., 1991)* are dependent on nicotinamide adenine dinucleotide phosphate (NADP). Ethanol production was attenuated by the aldehyde reductase inhibitor valproate but not by pyrazole, an inhibitor of NAD-dependent ADH *(Schofield et al., 1991)*.

Acetate can be produced from Acetyl-CoA by acetyl-CoA synthetase, resulting in the production of ATP from ADP. Alternatively, acetyl-CoA is converted to ethanol, using acet-aldehyde as an intermediate, by the bifunctional enzyme alcohol dehydrogenase (*Sa´nchez et al.,1999*). Alcohol dehydrogenase has an acetaldehyde dehydrogenase activity in the amino

terminus that catalyzes the conversion of acetyl-CoA to acetaldehyde and an alcohol dehydrogenase activity in the carboxy terminus that converts the acetaldehyde to ethanol.

2.15.1.4.Generation of alanine

Alanine is one of the major end products which are generated by Giardia under anaerobic conditions *(Edwards et al., 1989)*. It is a common strategy to excrete alanine, employed by some parasitic protozoa and several helminths for maintaining a stable intracellular pH, while producing acidic end products such as acetate propionate and lactate*(MacKenzie et al., 1983; Darling et al., 1987; Barrett, 1984; Barrett et al., 1986). Giardia* produces equimolar CO_2, ethanol, alanine, and small amounts of acetate under strictly anoxic conditions but under aerobic condition, it attenuates its alanine and ethanol production and increases acetate and CO_2 production *(Edwards et al., 1989; Paget et al., 1990; Lindmark, 1980)*.In the gut of vertebrates, O_2 concentration fluctuates between 0 and 60 μM which gives rise a range of different end products in *Giardia* which makes it flexible in balancing its redox couples (*Paget et al.,1990).* There are three routs by which alanine is produced in *Giardia.* Firstly, alanine is formed directly from pyruvate by the catalysis of L-alanine dehydrogenase. Secondly, by the coupling action of alanine-2-oxoglutarate transaminase (ALT) and glutamate-dehydrogenase alanine is also formed in Giardia which is regulated by the NADP : NADPH ratio and finally via ALT *(Meloni et al., 1988a; Stranden and Kohler, 1991; Mendis et al., 1993)*.

2.15.2.Amino acids as putative energy sources

The main energy source for *Giardia* trophozoites is monosaccharides mainly glucose *(Lindmark, 1980)*. Though in “near zero” glucose concentration of growth media the growth rate of trophozoite has been reduced by 50%, the end products formation is similar to that of normal

glucose containing growth media *(Edwards et al., 1989; Schofield et al., 1991)*. Discounting CO_2, glucose utilization was sufficient to account for only 50% of the total carbon appearing in alanine, ethanol and acetate. This led to the suggestion that Giardia trophozoites may have alternative sources of energy, *(Edwards et al., 1989; Schofield et al., 1991)*. Certain helminth parasites can utilize amino acids like glutamine, and carboxylic acids in the form of citrate, malate and succinate, to fulfil their energy requirements *(Mendis et al., 1986, 1987)*. It has been shown that *T. vaginalis*uses the arginine-dihydrolase pathway to utilized arginine. Giardia also possesses the arginine dihydrolase pathway *(Schofield et al., 1990, 1992)*. The specific activities of the enzymes in *Giardia*of the above pathway were greater than those reported for T. vaginalis*(Schofield et al., 1990, 1992)*. When $^{14}C0_2$ liberation from 1-^{14}C] glucose was taken as a measure, It has been suggested that ATP yield is higher in this pathway than from glucose *(Schofield et al., 1992)*. It has been reported that *Giardia*trophozoites can uptake and convert L-[U-^{l4}C] aspartate and L-[U-^{14}C] alanine to $^{14}C0_2$. The uptake of aspartate, alanine, and arginine from extracellular medium, as well as the documentation of glucose independent metabolism, suggests the potential importance of amino acid metabolism for energy production in *Giardia* (*Mendis et al.1990*)

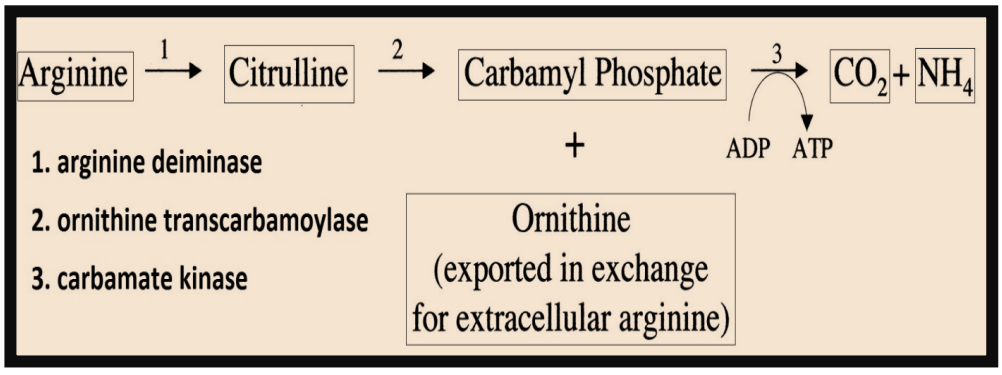


Fig.10. The Arginine dihydrolase Pathway

2.15.3.Lipid metabolism

Initially it has been suggested that bile has a role in the growth of *Giardia* trophozoites as it lies in the duodenum and jejunum and short-term axenic growth can be supported by bile.

This growth is supported by phosphatidylcholine, cholesterol nd the bile salts glycocholate and glycodeoxycholate (*Mendis et al.1990).* The trophozoites appear to satisfy their lipid requirements by obtaining cholesterol and phospholipids supplied by lipoproteins, b-cyclodextrins, and bile salts *(Mendis et al.1990).*

Glycerophospholipids (GPLs), the major structural components of biomembranes, play important roles in many important cellular processes *(Bogdanov et al., 1999; Fernandis et al., 2007)*. As an essential conserved cellular activity, GPL biosynthesis can be expected to retain some original features that can reflect *Giardia*'s primitivity if this organism is really an ancient eukaryote. In contrary, *Giardia* lives parasitically in the dietary lipid-rich intestine. Hence, it can consume GPLs directly from the environment without biosynthesize them, and thus its GPL biosynthesis pathways should have been subjected to parasitic reduction. Initially, it was thought that*Giardia* is unable to synthesize phospholipid *de novo*, but later it has been proved to be able to assimilate exogenous PC, PI and SM, and remodel them through Lands cycle and headgroup exchange reactions (*Jarroll et al., 1981; Stevens et al.,1997; Gibson et al., 1999; Subramanian et al., 2000*).The uptake, metabolism and intracellular localization of exogenous lipids have been demonstrated in *Giardia*using fluorescent conjugated and radiolabeled glycerophospholipid (GPL) and fatty acid analogs (*Stevens et al., 1997)*. Experiments in cultured trophozoites which are exposed to fluorescently conjugated analogs reveals that phosphatidylethanolamine (PtdEtn) is present in the inner leaflet of the plasma membrane as well as at intracellular locations, and phosphatidylcholine (PtdCho) is in the plasma and flagellar membranes, and phosphatidylglycerol (PtdGly) mostly in the perinuclear membrane and endoplasmic reticulum of Giardia*(Soltys et al., 1909)*. Radiolabeled lipids and fatty acids indicated that

*Giardia*is able to transport these molecules and incorporate them into cellular lipids (*Stevens et al., 1997; Gibson et al., 1999;Blair et al.,1987)*.

It has also been suggested that trophozoites bear a low level of endocytotic lipids (*Lujan et al.,1996*). The major fatty acids found in axenically grown trophozoites are palmitic acid, stearic acid, and oleic acid. Fatty acid desaturase activity, including desaturation of oleate to linoleate and linolenate, has been documented (*Ellis et al.1996*). Arachidonic acid is incorporated into neutral lipids, phospholipids, and a wide variety of cellular lipids (*Gibson et al.,1999*), while palmitic acid, myristic acid, and oleic acid are transesterified primarily into phospholipids, including cellular phospholipids, (e.g., phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) (*Jarroll et al.1981*). Interesterification also occurs with incorporation of conjugated fatty acids into phosphatidylglycerol. Isoprenoids, derived from mevalonate and commonly found in eukaryotic cells,are incorporated into proteins like the GTP-binding proteins by posttranslational modification. Incorporation of mevalonate and cell growth was inhibited in a reversible manner by competitive inhibitors of 3-hydroxy-3-methylglutoryl (HMG)-CoA reductase. Inhibitors of later steps of isoprenylation permanently inhibited cell growth.

2.16. Oxygen consumption of *Giardia*

Over a decade ago, it has been observed that *Giardia lamblia* trophozoites exhibited a high affinity foroxygen (*Weinbachet al., 1980; and Lindmark, 1980).* At 37°C the rate of oxygen consumption (E-QC) is observed to be 64 and 93 nmol min-’ mg-’ protein (Weinbach*etal.,* 1980; Lindmark,1980). Malate, glucose, ethanol and the electron donorcouple ascorbate/N,N,N’,N’-tetramethyl-p-phenylenediamine(TMPD) have stimulatory effect on the E-QO of intact *Giardia*trophozoites while pyruvate, lactate, succinate, glycerol-3-phosphate, L-glutamate, a-ketoglutarate,isocitrate has no effect *(Weinbach etal., 1980).* Apparent

K_2values for 0, suggest that trophozoites have a greateraffinity for 0, than cysts, indicating that cysts are more resistant to oxygen inducedtoxicity.The effects of temperature on the oxygen consumption (QO,) of cysts indicated that, below 7”C, little 0, consumption occurred. Since low temperatures also favour cyst viability *(Bingham et al.,1979),* low temperatures would be expected to extend the half-life of cysts, thus prolonging their infective period.

2.17. Antioxidant mechanisms

2.17.1. Glutathione/thiol cycle

In microaerobic or aerobic conditions most of the organisms uses glutathione (GSH)cycle which is one of the primary defence mechanisms to eradicate the toxic oxygen species (particularly peroxide). It is interesting that the endogenous GSH pool is not determined in *Giardia* but a considerable amount of thiol pool, estimate of 56-64 mmol GSHequivalents/ $l0^8$cells is detectable in this organism (*Smith et al., 1988*). However, in response to exogenous glutathione, *Giardia* exhibits glutathione peroxidase and glutathione reductase, which are mentioned as thiol-dependent peroxidase (TDP) and thiol-dependent reductase (TDR) *(Smith et al., 1988)*. It **is** possible that *Giardia*may possess an alternative thiol, analogous to, and isofunctional with, glutathione in other eukaryotes. Such an alternative, trypanothione, has been identified in trypanosomes *(Fairlamb etal., 1985)*. It has been indicated that *Giardia*can scavenge glutathione from an exogenous source (*Smith et al., 1988)*. Irrespective of GSH possessionorthiol metabolism in *Giardia* the specific activities of TDP and TDR is positively correlated with nitofuran tolerance among several isolates of *Giardia (Smith et al. 1988)*. This may have important implications with reference to furazolidone resistance in *Giardia.*

2.17.2. Superoxide dismutuse

Despite *Giardia* is classified as an aerotolerant anaerobe, it lack superoxide dismutase (SOD)which makes it unique among eukaryotes *(Smith et al., 1988)*. The assay for SOD using pyrogallol-autoxidation assay also generated negative result *(Marklund and Marklund, 1974; Smith et al., 1988)*. The negative results were also reported in several human and feline isolates of *Giardia (Meloni et al. 1988)*. Amore thorough investigation of the prevalence of SOD in *Giardia*spp. is imperative in order to clarify these equivocal reports.

2.17.3. Catalase

In contrast to SOD, the lack of catalase in *Giardia*is unequivocal *(Lindmark, 1980; Smith et al., 1988)*. Several other aerotolerant eukaryotes have also been shown to lack catalase *(Lindmark, 1980)*.

2.18. Oxidative stress

The oxidative stress is the imbalance between the production of reactive oxygen species (ROS) (free radicals) and its removal by antioxidant defence employed by the cell *(Halliwell, 1994)*. Many biochemical processes like reduction of molecular oxygen during aerobic respiration, oxidation of catecholamines and activation of the arachidonic acid cascade lead to the formation of large amounts free radicals as an unavoidable by-product. Free radicals can be also be generated in response to external electromagnetic radiation, which can split water to produce hydroxyl radicals. In this scenario if a cell is deficit in antioxidant defence system, damage may occur in a variety of tissues. The process of oxidative stress is as follows:

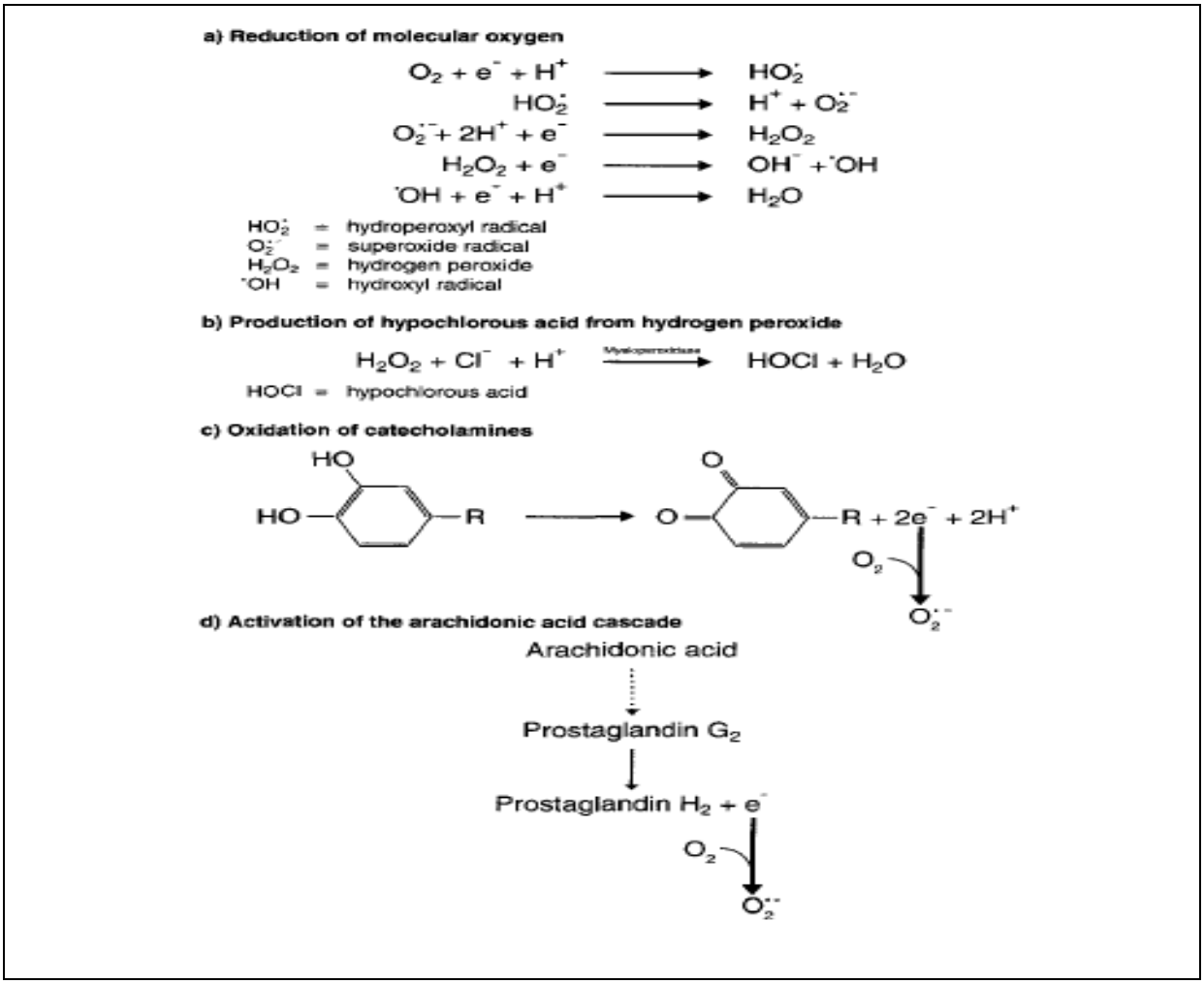
- An increase in oxidant generation.
- A decrease in antioxidant protection.
- Failure to repair oxidative balance.

2.18.1. Free radicals

Any chemical species which contains unpaired electrons can be defined as free radicals. The presence of unpaired electrons makes them inherently unstable molecules. Unpaired electrons increase the chemical reactivity of an atom or molecule. As a consequence they react locally to accept or donate electrons from adjacent molecules to achieve a more stable state. The hydroxyl radical (OH), nitric oxide (NO), superoxide anion (Oz-), transition metals such as iron and copper, and peroxynitrite (ONOO -) are the common examples of free radicals.The most potent oxidant, the hydroxyl radical, reacts at the site of its formation and has an extremely short half-life. It attacks most biological molecules resulting in the propagation of free radical chain reactions. Superoxide, formed when oxygen accepts an electron, is a weak oxidizing agent but has the potential to be a stronger reducing agent of iron complexes such as cytochrome C. It is likely to be more important as a source of hydroxyl radicals and hydrogen peroxide*(Betteridge, 2000)*.

2.18.2. Sites of free radicals generations

The main source of ROS in vivo is aerobic respiration, although ROS can also be produced by peroxysomal β- oxidation of fatty acids, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism and tissue specific enzymes.



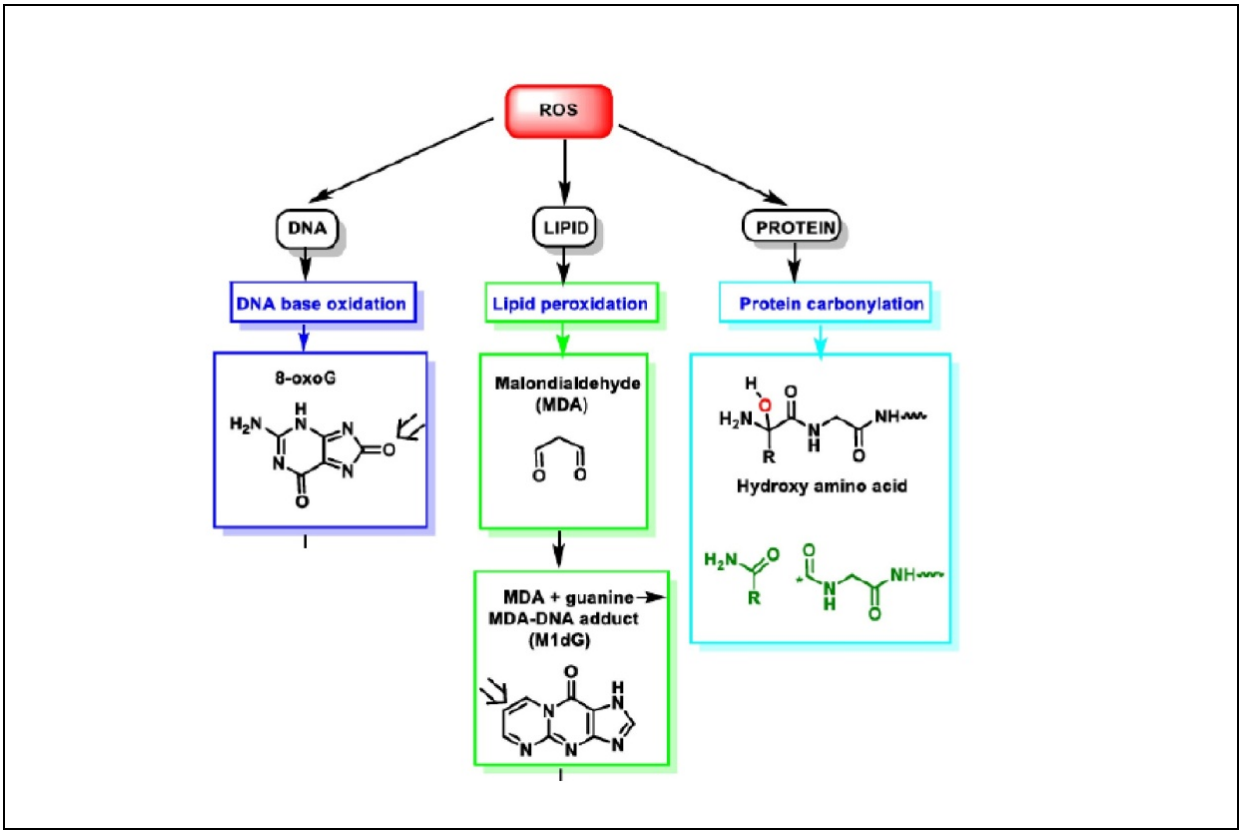
Source: *Betteridge, 2000*

Fig.11.: **Examples of the production of free radicals by different biochemical processes within the body.**

2.18.3. The consequences of oxidative stress

Due to the presence of unpaired electrons free radicals are inherently unstable molecules which make them highly reactive to achieve a more stable state. The free radicals react with nonradicals (all biological macromolecules, lipids, proteins, nucleic acids, and carbohydrates are possible targets) to start a free radical chain reaction and form new radicals to further react with macromolecules. Protein damage (addition of carbonyl groups or crosslinking or fragmentation), lipid peroxidation are important examples of oxidative stress. Carbonylation

of amino acid residues increases the protein susceptible to proteolysis, single- and double-strand breaks in DNA and protein/DNA crosslinks *(Stadtman, 1991; Spear et al.,1995; Hall et al., 1996; Halliwell et al., 1991)*. Lipid peroxidation is perhaps the most extensively studied consequence of free radical attack (Fig 12).



Source: Juan et al., 2021

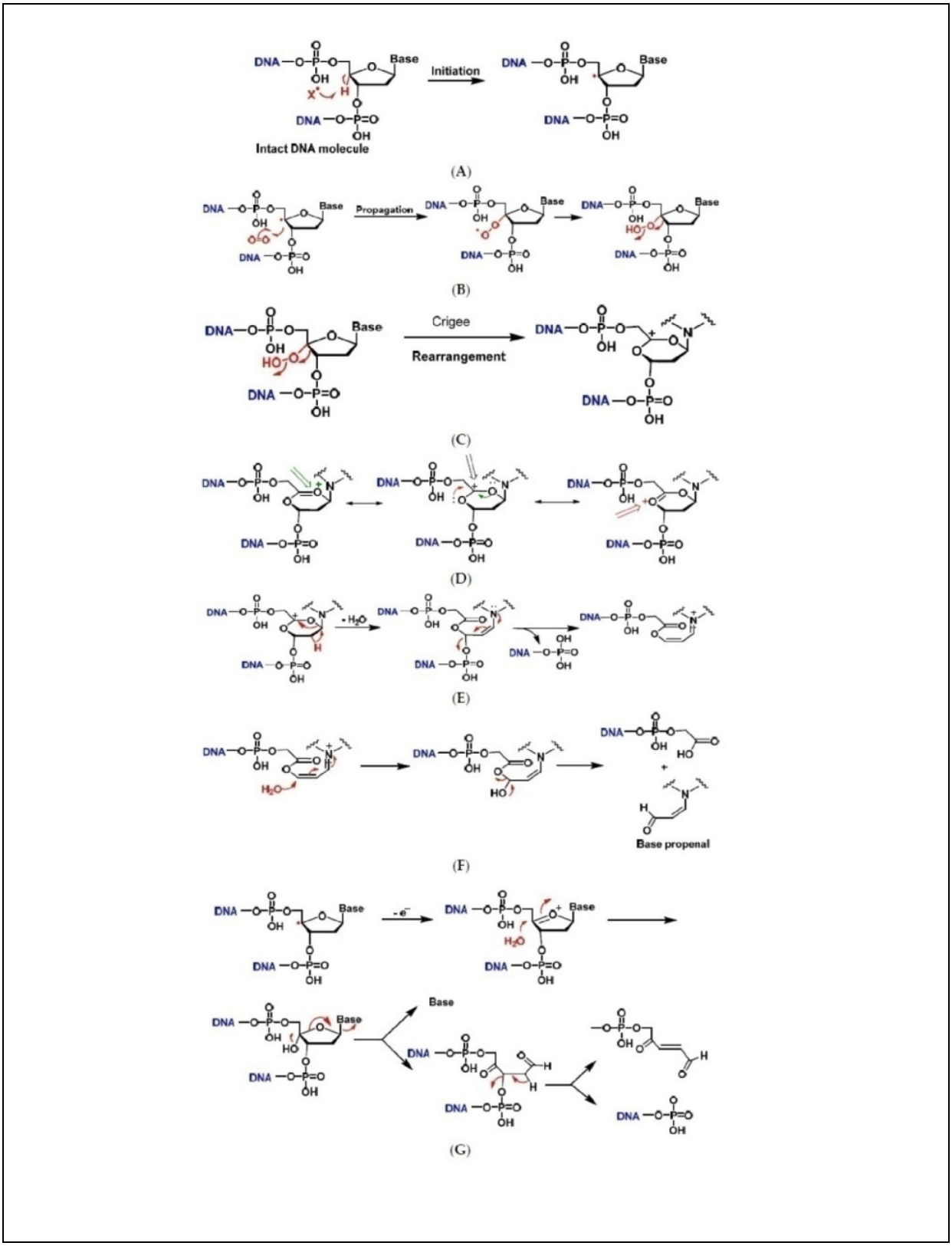
Fig.12.: Targets of ROS.ROS action on DNA, lipids and proteins lead to DNA base oxidation, lipid peroxidation and protein carbonylation, respectively. * Unpaired electron.

2.18.3.1. Oxidative damage to DNA

Numerous lesions in DNA can be induced by ROS which further leads to mutations, deletions and other lethal genetic effects throughout the DNA. The oxidation in the sugar and base moieties causes single strand breakage, base degradation and cross linkage to protein. Hydroxyl radicals oxidize the sugar moieties which lead to single strand breaks in DNA. The radical-induced proton abstraction from any position of the deoxyribose starts this process

and can result in many products.·OH radical easily leads abstraction of hydrogen (hydrogen atom transfer HAT) in deoxyribose forming different products and ribose fragments. Several steps of the oxidation of deoxyribose at the C-4 position leads to a radical carbon stabilised by resonance with the oxygen in the ring *(Balasubramanian et al., 1998)*.

Superoxides and peroxide alone cannot cause strand breaks under physiological conditions. The Fenton's reaction with the help of a metal catalyst causes the toxicity for the above mentioned oxidative agent. Cross linking of DNA to protein is often of order less abundant than single strand breaks, they are not as readily repaired, may be lethal if replication or transcription proceeds repair.



Source: Juan et al., 2021

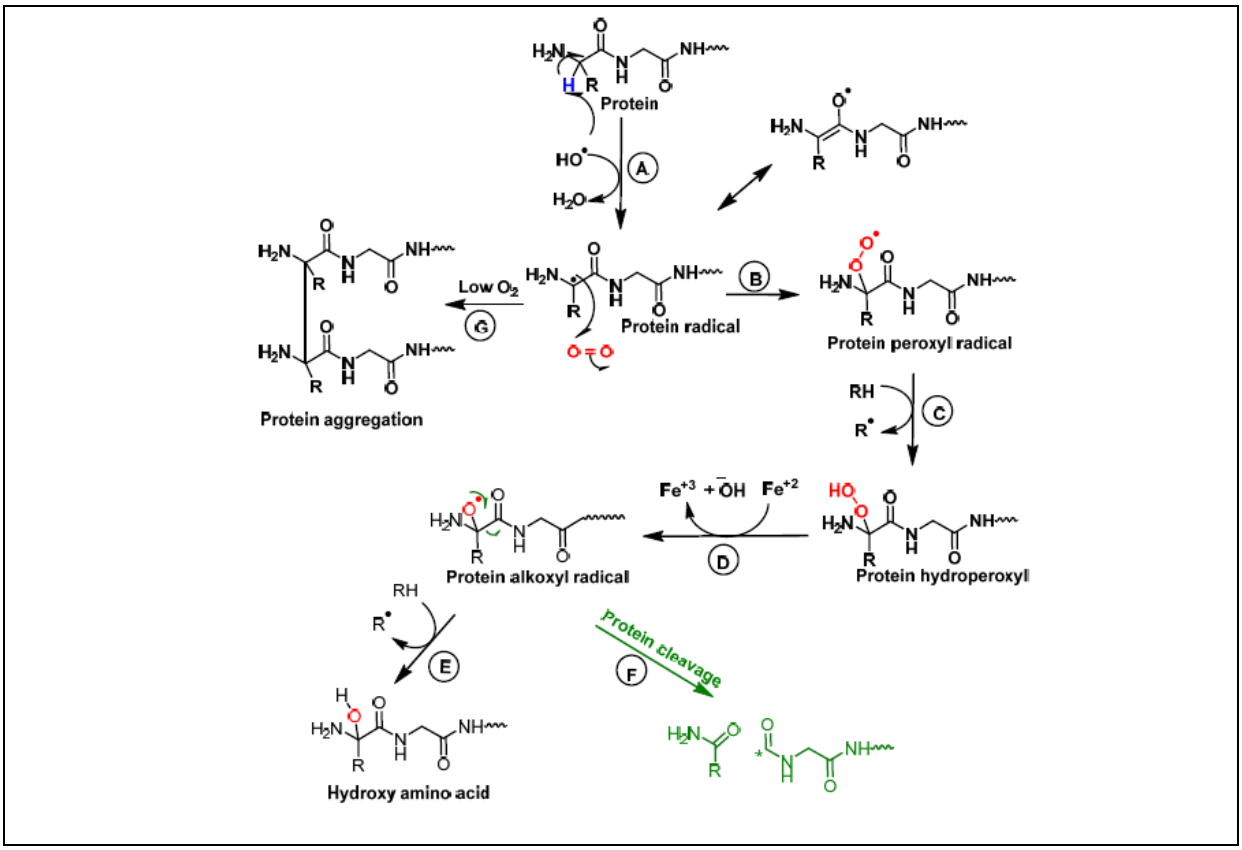
Fig. 13.:Mechanisms of oxidative damage to DNA-deoxyribose. The reaction with oxygen leads to several transposition reactions with expansion of the ring, which subsequently degrades to different products (**A**–**G**). (**A**) The reaction is initiated by abstraction of the

hydrogen on the C-4 of deoxyribose by the hydroxyl radical or any other radical present in the medium to form the radical on the carbon; (**B**) The carbon radical reacts with O2 present in the reaction medium and transforms into the peroxyl radical which evolves into the hydroperoxide derivative; (**C**) The alkyl hydroperoxide formed undergoes a rearrangement, i.e., a migration from one atom or group of atoms to another within the same molecule, in this case with ring expansion to a six-linked ring and formation of the carbocation; (**D**) The generated carbocation is stabilised by delocalisation of the positive charge with the two adjacent oxygens and formation of the oxonium cation; (**E**) Dehydration with ring opening to form the enamine derivative which evolves to the unsaturated imine by loss of the phosphate residue; (**F**) Addition of water on the carbon and formation of the hydroxy acetal derivative that fragments to generate the acrylaldehyde-derived base; (**G**) In low oxygen environments the radical evolves to the oxonium cation and nucleophilic attack by a water molecule, then decomposes into the free base and the various fragments.

2.18.3.2. Oxidative damage of protein

Oxidative attack on proteins results in site specific amino acid modification, fragmentation of the peptide chain aggregation of crosslinked reaction products, altered electrical charge and increased susceptibility to proteolysis. The various forms of activated oxygen differ in their potential reactivity. Sulfur containing amino acids and thiols group specifically are very susceptible sites. Activated oxygen can abstract H –atoms from cysteine residues to form a thiol radical that will crosslink to a second thiol radical to form disulfide bridge. Alternatively oxygen can add to a metheonine residue to form metheonine sulfoxide derivatives. Free radical attack on proteins is not reversible e.g., the oxidation of iron sulphur centres by superoxide destroys the enzymatic functions. Many amino acids undergo specific irreversible modification when a protein is oxidised. Tryptophan is readily crosslinked to form bityrosine product histidine, lysine, proline, arginine and serine from carbonyl groups on oxidation. The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. The metal then reacts with H_2O_2 in the Fenton's reaction to form a hydroxyl radical that rapidly oxidises amino acid residues at or near the cation binding site of protein. This site specific alteration of an amino acid usually inactivates

the enzyme by destruction of cation binding sites. The detailed process is explained in Fig. No. 14.



Source: *Juan et al., 2021*

Fig. 14.:Mechanism of protein oxidation. The abstraction of hydrogen from the protein by the hydroxyl radical generates the alkyl radical, stabilised by resonance with the carboxyl function (**A**). The alkyl radical reacts with oxygen to form the peroxide radical (**B**). The peroxide radical abstracts hydrogen from an adjacent protein and a hydroperoxide and an alkyl radical are formed (**C**). The hydroperoxide is reduced to an alkoxy radical in the presence of ferrous iron (**D**). Hydrogen abstraction from an adjacent protein by the alkoxyl radical forms hydroxy amino acid derivatives (**E**).The alkoxy radical upon cleavage generates different protein carboxy radicals and alkyl radicals (**F**). In the absence or at low oxygen levels the alkyl radicals form protein aggregates (**G**).

2.18.3.3. Oxidative damage to lipids

The hydroxyl radical is capable of abstracting a hydrogen atom (H) from a methylene group (--CH,--) of fatty acids and generating an unpaired electron on the carbon (--CH--).Polyunsaturated fatty acids are particularly prone to this attack as the presence of a double

bond weakens the carbon-hydrogen bond at the adjacent carbon atom. The rest carbon-centered radical undergoes molecular rearrangement foming a conjugated diene which can generate a peroxyl radical by combining with oxygen. This peroxyl radical further abstract hydrogen atom and begin a chain reaction. Though the resulting lipid peroxides are stable compounds, but transition metals and metal complex catalyzed decomposition of them produce alkoxyl and peroxyl radicals which further stimulate lipid peroxidation*(Betteridge, 2000)*.Lipid bilayer membrane is made of mixture of phospholipids and glycolipids that have a fatty acid chain attached to C_1 and C_2 of glycerol backbone by an ester linkage. The peroxidation of lipid involves the following 3 steps

1.	**Initiation**	$OH^{\bullet} + RH \rightarrow R^{\bullet} + H_2O$
2.	**Propagation**	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$ $ROO^{\bullet} + RH \rightarrow R^{\bullet} + ROOH$
3.	**Termination**	$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$ $R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$

It is of interest that disrupted tissue is more susceptible to lipid peroxidation. Lipid peroxidation can have profound effects on cellular function. Extensive peroxidation in cell membrane will result in changes in fluidity, increased permeability, a decrease in membrane potential, and eventually membrane rupture.

The hydroxyl radical in the initiation stage, oxidizes the organic substrate (RH) to form H_2O. An organic radical (R) the later product has a single unpaired electron and thus can react with

oxygen in triplet ground state. The addition of triplet oxygen to the carbon radical leads to the formation of a peroxyl radical ($ROO^{\bullet}$) which can readily abstract hydrogen from another organic molecule leading to the formation of a second carbon radical and by this way the reaction can propagate. The peroxidation reactions in the membrane lipids are terminated when the carbon or the peroxyl radical cross links to form conjugated products that are not radicals.

Toxic breakdown products such as hydroxynonenal are generated in lipid peroxidation. Once formed, the peroxy radical cycles to four-linked cyclic peroxide. The unsaturations of polyunsaturated fatty acid increase with the progression of peroxidation.Thus, when free radicals attack arachidonic acid (with four unsaturations) the initial abstraction of a hydrogen atom can occur at three points in the carbon chain of the fatty acid, because it has three methylene groups (-CH2-) attached to a carbon atom flanked by double bonds, thus increasing the complexity of the peroxidation reaction. The fatty acid undergoes a further reaction with oxygen followed by a break in the cycle to give hydroperoxynonenal, which is reduced to hydroxynonenal.

2.19. Effects of lipid peroxidation on biological membrane

The physicochemical properties and the chemical reactivity of the membrane bilayer are the two properties that can determine their susceptibility to oxidative damage damage (*Pamplona et al., 2002a,b,c, 2008; Hulbert et al., 2007)*. As oxygen and reactive species are more soluble in physicochemically more fluid lipid bilayer than aqueous cytosol they cause more damage in biological membrane *(Moller et al., 2005; Gamliel et al., 2008)*. Phospholipids are extremely sensitive to oxidation due to the more numbers of double bonds per fatty acid (PUFA) molecule *(Holman, 1954; Bielski et al., 1983)*. Consequently, PUFA side chains

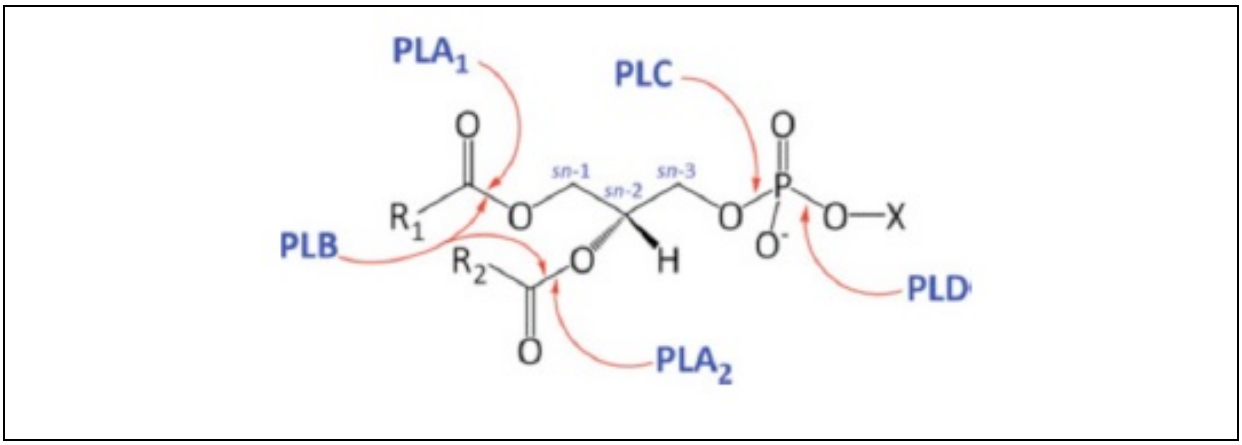
(with two or more double bonds) are much more easily attacked by radicals than are SFA (no double bonds) or MUFA (one double bond) side chains.

Hydroperoxides and endoperoxides, generated from lipid peroxidation, undergo fragmentation to produce reactive intermediates with three to nine carbons in length. Among these intermediates the most reactive are di-aldehydes [malondialdehyde(MDA)and glyoxal], keto-aldehydes (4- oxo-trans-2-nonenal) and α,β-unsaturatedaldehydes [4-hydroxy-trans-2-nonenal(HNE) and acrolein] (*Esterbauer et al., 1991; Catalá, 2009; Fritzand Petersen,2013*). The half-life of these reactive aldehydes is higher thn the ROS and its non- charged structure allowit's migrating easily through hydrophobic membranes and hydrophilic cytosolic media *(Pamplona, 2008).*However, these carbonyl compounds can have far reaching damaging effects on target sites within or outside membranes. These compounds can react with nucleophilic groups in macromolecules like DNA, proteins and aminophospholipids resulting in their chemical, nonenzymatic, and irreversible modification and formation of variety molecules *(West and Marnett, 2006; Thorpe and Baynes, 2003; Naudi et al., 2013; Pamplona, 2011)*.

Lipid peroxidation predominantly occurs at the sn-2 position of phospholipids, as most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. This position is also targeted by phospholipase A2 (PLA2) for cleavage*(Cummings et al., 2000)*. Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms*(Sapirstein et al., 2000)*. Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites*(Balboa et al., 2006, Lister et al., 1982)*.

2.20. Phoholipases and their role in phospholipid modification

Phospholipases, the lipolytic enzymes, can hydrolyze phospholipid substrates at different ester bonds *(Balboa et al., 2021)*. As a consequence of the cleavage of ester bonds within phospholipids a range of lipid products are generated which are able to control the cellular signalling. The widespread phospholipases has diverse roles from signal transduction to lipid mediator production in humans. Their structure, function, regulation, and mode of action vary considerably. Most of the phospholipases of the cells are as existed as cytosolic form, membrane associated form or secreted form. Depending on the cellular/tissue localizations and properties,the function of the phospholipases are diverse. Generally, the acylhydrolases and the phosphodiesterases – these two groups of phospholipases are present in cells. These enzymes are classified according to the cleavage site of the phospholipid substrates (Fig. No.-17). the acylhydrolase group is composed of Phospholipase A1 (PLA1), phospholipase A2 (PLA2), and phospholipase B (PLB) whereas the phosphodiesterase is composed of phospholipase C (PLC) and phospholipase D (PLD) *(Aloulou et al.,2018)*.



Source: *Aloulou et al.,2018*

Fig.15.: Typical glycerophospholipid structure and the site of action of various phospholipases. The glycerophospholipid molecule consists of a glycerol-3- phosphate esterified at its sn-1 and sn-2 positions with nonpolar fatty acids (R1 and R2, respectively) and at its phosphoryl group to a polar headgroup, containing alcohol, X (e.g., choline, ethanolamine, or inositol). The various sites of attack for hydrolytic cleavage of the various

phospholipase types A1, A2, B, C, and D are shown with arrows. The carbon atoms of the glycerol backbone of the glycerophospholipid are indicated according to the stereochemical numbering (sn-1, sn-2, and sn-3)

2.20.1. Phospholipases A1

Phospholipids are hydrolyzed at the sn-1 position by the catalysis of PLA1 enzyme to form 2-acyl-lysophospholipids and fatty acids. This enzyme is a potent component to produce bioactive lysophosphatidylinositol (lysoPI), lysophosphatidylserine (lysoPS) and lysophosphatidic acid (lysoPA) which are the lysophospholipid*(Ishii et al., 2004)*. After successful cloning and characterisation of several PLA1 it is reported that this enzyme posses the typical Ser-His-Asp catalytic triad *(Richmond et al., 2011; Aokiet al., 2007; Sato et al., 1997; Hiramatsu et al., 2003; Agarwal et al., 2015; Ishiguro et al.,2001; Carriere et al., 1998)*.It is also been reported that that PLA1 has an important role in virulence of bacterial and fungal pathogenesis *(Shimutaet al., 2009; Schmiel et al., 1998)*. The key to the PLA1 activity is dependent on a very short lid on its sequence and the deletion of the β9 loop (*Aokiet al., 2007*, *Carriere et al., 1998*). Some PLA1 like PI3P-PLA1s, PS-PLA1s and PA-PLA1s have strictly hydrolyze phospholipids, others like pancreatic PLA1 have broad substrate specificity and hydrolyze phospholipids, triglycerides, and galactolipids (*Aokiet al., 2007; Richmond et al., 2011; Carriere et al., 1998; Amara et al., 2012).*An open conformation of the crystal structure of this enzyme suggested that Lys295 and Ser356 are important for phospholipid binding and PLA1 activity *(Tiesinga et al., 2007).*

2.20.1. Phospholipases A2

The sn-2 position of the ester bond of membrane phospholipids, which frequently contains polyunsaturated FAs, is hydrolyzed by PLA2s, metabolizing various eicosanoids and related bioactive lipid mediators *(Burke et al., 2009; Murakami et al., 2011).*The PLA2 is a family of enzyme which is composed of six different and diverse types of enzymes such as cytosolic

PLA2 (cPLA2), calcium-independent PLA2 (iPLA2), lysosomal PLA2, adipose PLA2, platelet-activating factor acetylhydrolases (PAF-AHs) and several groups of secreted PLA2 (sPLA2). Based on the structural features, catalytic mechanisms, localizations and evolutionary relationships PLA2s are assigned to the certain groups *(Schaloske et al., 2006).* Most of these lipolytic enzymes have different regulatory and catalytic mechanisms and devoid of any structural similarity (*Burke et al., 2009; Dennis et al., 2011*). Biological roles of PLA2s have been investigated using PLA2 knockout (KO) or overexpressing transgenic mice, specific inhibitors, and information obtained from analysis of human diseases caused by mutations in PLA2 genes, along with lipidomics approaches to elucidate in vivo substrates and metabolites (*Murakami et al., 2011*).

Conventional sPLA2s (GI/II/V/X) are low-molecular-weight enzymes (14-19 kDa) and are closely related. The structural analysis revealed that they have a highly conserved Ca2+-binding loop and a His/Asp catalytic dyad as well as conserved disulfide bonds. Atypical sPLA2s (GIII/XII) are each classified into distinct classes *(Schaloske et al., 2006; Murakami et al.,2015; Valentin et al., 2000).*The sPLA2s are secretory and require millimolar Ca2+ for catalysis and thus target phospholipids in the extracellular space (Dennis et al., 2011; *Murakami et al.,2015;Lambeau et al., 2008).* They have distinct substrate specificity in terms of the polar head groups. Some sPLA2 isoforms (e.g., GIB/III/X) require cleavage at the N-terminus to be fully active as they are secreted as proenzymes *(Jemel et al., 2011)*. Individual sPLA2s play distinct biological roles by generating variety of lipid mediators, modifying extracellular phospholipids and promoting membrane remodelling (*Murakami et al.,2014*).

The sPLA2s are responsible for the release of arachidonic acid (AA) as well as unsaturated fatty acids, such as ω-3 fatty acids and lysophospholipids (*Murakami et al.,2015*). Despite of being secretary enzyme certain sPLA2s can also release AA by acting intracellularly and

some can hydrolyze cell surface phospholipids upon secretion *(Ni et al et al., 2006; Mounier et al., 2004; Singer et al., 2002;)*.

The cytosolic PLA2 family is comprised of six intracellular enzymes commonly referred to as (cPLA2)α, cPLA2-β, cPLA2-γ, cPLA2-δ, cPLA2-ε, and cPLA2-ζ, respectively and share only about 30% homology*(Ghosh et al., 2006; Leslie, 2015)*. They have characteristic Ser Asp catalytic dyad and except cPLA2-γ, all of them possess β-sandwiched N-terminal C2 domain which is attached to the C-terminal catalytic domain via a flexible linker (*Ghosh et al., 2006)*.Analysis of the interfacial kinetic and binding properties of the cPLA2 family showed that they exhibit very different relative lysophospholipase, PLA2, and PLA1 activities, inhibitor sensitivities, calcium dependence, and activation by anionic phospholipids, highlighting potential differences in regulation and function *(Ghosh et al., 2006; Leslie, 2015)*. New emerging studies shows members of the cPLA2 family play a role in regulating membrane trafficking which is devoid of the production of oxygenated metabolites of AA *(Leslie, 2015; Ohto et al., 2005; Ghosh et al., 2006a, Ghosh et al., 2007)*.cPLA2α is a highly conserved widely expressed enzyme in mammalian cells that promotes the production of lipid mediators derived from its products, AA and lysophospholipids, in response to extracellular stimuli (*Leslie, 2015)*. The regulation of cPLA2α is induced transcriptionally via number of signaling pathways and activated by posttranslational processes *(Bickford et al., 2013; Lee et al., 2011)*. These signalling pathways are activated in cells through engagement of many types of receptors, indicating that cPLA2α activation and AA release occur commonly in response to cell stimulation (*Leslie, 2010, 2015*). The preferential hydrolysis of sn-2 AA of cPLA2α and its role in initiating the release of AA for the production of lipid mediators is well documented *(Leslie, 2004; Rubin et al., 2005)*.

The Ca2+-independent PLA2s (iPLA2s) are intracellular enzymes which are expressed ubiquitously and its activity does not require Ca2+. This class is composed of seven members: iPLA2β, iPLA2γ, iPLA2δ, iPLA2ε, iPLA2ζ, and iPLA2η with similar catalytic site to cPLA2s. The enzyme manifests PLA2/PLA1, transacylase, lysophospholipase and thioesterase activities but show no substrate specificity *(Ramanadham et al., 2015;Jenkins et al., 2004; Carper et al., 2008)*. It can localize to different places like endoplasmic reticulum, mitochondrial membrane, and inner side of the cell membrane or cytosol*(Ramanadham et al., 2015)*. iPLA2s participate in a multitude of biological processes, including fat catabolism, cell differentiation, and maintenance of mitochondrial integrity, phospholipid remodeling, cell proliferation, signal transduction, and cell death *(Hermansson et al., 2016).* The extensive study report on iPLA2β shows that it is a regulated enzyme and has a specific role in phospholipid acyl remodelling by acting as a signalling molecule to mediate AA release *(Ramanadham et al., 2015; Balsinde et al., 2005).*

2.20.2. Phospholipases B

The enzymes which cleave both sn-1 and sn-2 positions of diacyl- or lysophospholipids are termed as PLB. As PLBs and lysophospholipases both possess a lysophospholipase activity, the distinction between them is difficult *(Matsumoto et al., 2013)*.Thus, there is some confusion in nomenclature for these enzymes *(Morgan et al., 2004)*. Synonyms including lysophospholipase and lysophospholipase-transacylase are used for PLB. The hydrolase activity allows the enzyme to cleave fatty acids from both phospholipids (PLB activity) and lysophospholipids (lysophospholipase activity), while the transacylase activity allows the enzyme to produce phospholipids by transferring a fatty acid to a lysophospholipid. PLB hydrolase and acyltransferase activities have been described in many microbial species, protozoa, and mammalian cells *(Morgan et al., 2004; Chen et l., 1997;Maury et al., 2002;*

Delagebeaudeuf et al., 1998). There is evidence that PLBs are an important part of the virulence repertoire of pathogenic fungi *(Djordjevic 2010).*

2.20.3. Phospholipases C

PLCs catalyze the cleavage of the phosphodiester bond present in between between the phosphate group and the glycerol backbone of glycerophospholipids and produce diacylglycerols and phosphorylated headgroups. Hence, it is termed as phosphodiesterases*(Goni et al., 2012)*. Based on the substrate specificities PLC is divided into PC-specific PLC that acts preferentially on PC and PI-specific PLC that hydrolyzes (almost) exclusively PI. Zn2+ and metal chelators play an important role in activation and inactivation of the enzyme respectively *(Titball, 1993)*. The structural study of PC-PLC from B. cereus presents the fact that the enzyme forms a twisted barrel structure with seven helices and three Zn2+ coordinate within the active site *(Hough et al., 1989).* Mammalian PC-PLCs are the key components of different cellular and physiological processes, such as apoptosis *(Cheng et al., 2006;Cifone et al., 1995)*. The molecular mass of PI-PLC ranges from 85 to 150 kDawith multi-domain organization*(Pokotylo et al., 2014;Kadamur et al., 2013).*PI-PLCs catalyze the hydrolysis of the proximal phosphodiester bond of PI-4,5-bisphosphate (PIP2), in a Ca2+-dependent manner, generating two second messengers1,2-diacylglycerol (DG) and inositol-1,4,5-triphosphate (IP3). DG is a neutral lipid that remains in the membrane and activates PKC.

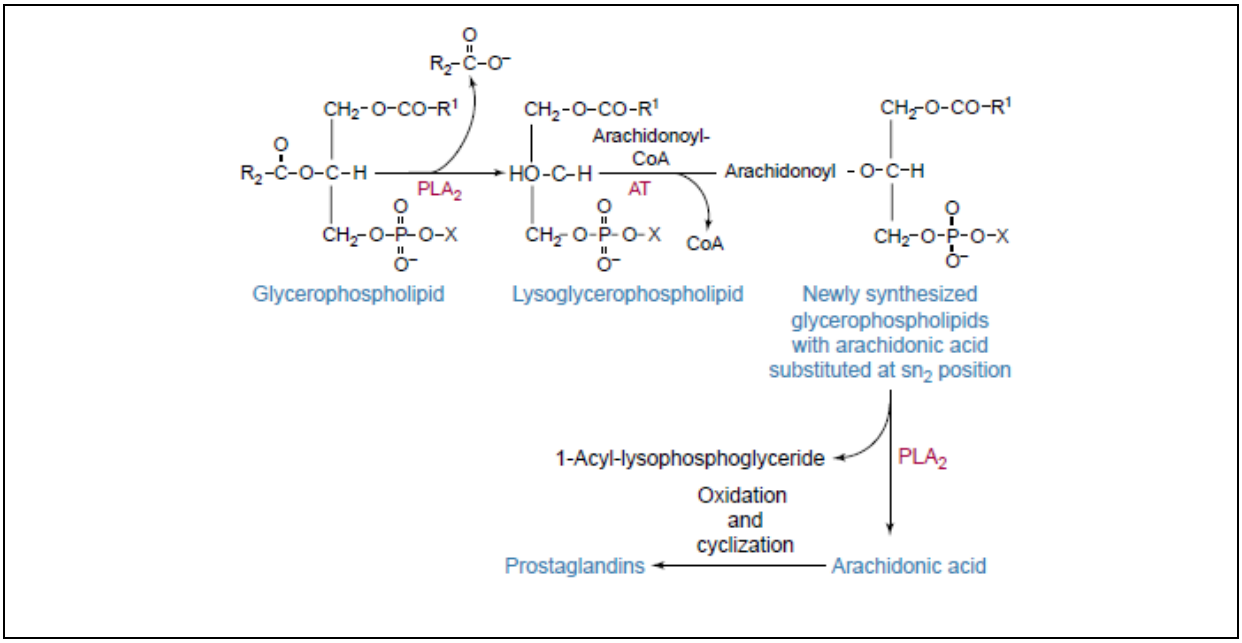
2.20.4. Phospholipases D

PLD hydrolyzes the distal phosphodiester bond of phospholipids releasing PA by the cleavage of the headgroup of the substrate. This enzyme is also able to switch the head groups of the substrate phospholipid to another head group and to produce a new phospholipid *(Yang et al., 1967)*. The later reaction competes with hydrolysis and involves a

nucleophilic acceptor (a primary alcohol) which is transferred to the phosphatidyl residue forming corresponding phosphatidylalcohol. PLD has been discovered initially, in plants showing catalytic activity in protein extracts. However first mammalian PLD has been from human PLD1 gene in 1995 *(Hammond et al., 1995)*. In mammalian cells PLD is represented as a key enzyme for cell signalling.This role of PLD is mainly attributed to the effects induced by PA, its reaction product, which is a lipid mediator involved in many cellular signalling pathways*(Shulga et al., 2013)*.

2.21. Arachidonic acid and Land's cycle

In response to oxidative stress, the PLA2 activity increases which also give rise to AA and AA- derived metabolites and lysophosphatidylcholine (lysoPC) *(Balboa et al., 2006)*. Arachidonic acid (AA) is a biologically active mediator, which is 20-carbon chain fatty acid with four methylene-interrupted cis double bonds and designated as all cis-5,8,11,14-eicosatetraenoic acid with usually a hairpin configuration*(Tallima et al., 2017)*.AA can be mobilized byreaculation process in which it is incorporated in lipid membrane or bydeacylation in which it further progress to eicosanoide production. The deacylation/ reacylation of cellular phospholipid is termed as the Lands cycle. In this cyclic process, lysophospholipids (LPLs) and free fatty acids are generated by the action of cellular phospholipases which can be recycled to produce new phospholipid and fatty acids. To check the accumulation of potentially toxic LPLs and fatty acids this cycle should be regulated properly*(Das et al., 2001)*. Under stimulation conditions, the rate of AA release exceeds that of reincorporation into phospholipids; hence net accumulation of AA occurs that is followed by its conversion into different oxygenated compounds, collectively called the eicosanoids.

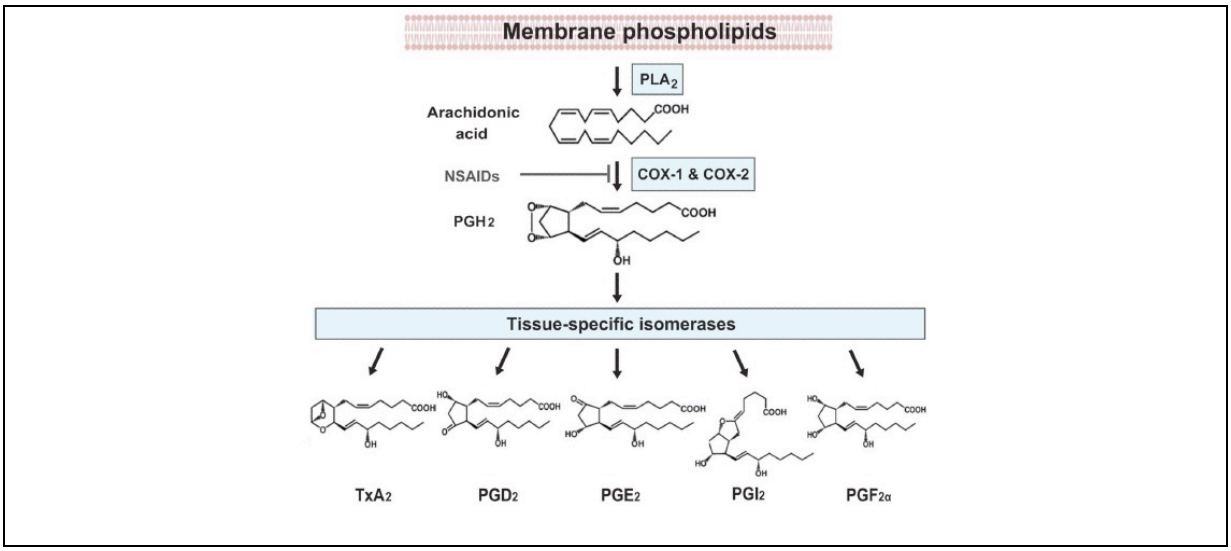


Source : *Das et al., 2001*

Fig.16.:Postaglandine production via arachidonic acid formation

AA can be mobilized either by Ca2+-dependent manner or by Ca2+-independent manner. While AA mobilization mediated by Ca2+-dependent enzymes generally appears to involve the activation of kinase cascades and intracellular calcium movements, AA mobilization by Ca2+-independent enzymes appears to require neither of these. This may suggest that Ca2+-dependent PLA2s, when involved in the response to oxidants, participate as signalling molecule on their own. On the contrary, involvement of Ca2+-independent phospholipase A2s may potentiate the damage in cells undergoing oxidative stress in the absence of clear regulated signalling.When AA is metabolized by the sequential actions of cyclooxygenase (COX), it generates prostaglandins. The four principal bioactive prostaglandins, generated *in vivo* are prostaglandin (PG) E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Usually each cell type generates one or two dominant prostanoides ubiquitously to serve autacrine and paracrine lipid mediators to maintain local homeostasis in the body*(Ricciotti et al., 2011)*. PGE_2 has been shown to be antiapoptotic in multiple cell types, including normal epithelial cells *(Tessner et al., 2004)*, cancer cells *(Sheng et al.,*

1998), T cells *(Weinlich et al., 2008)*, and adipocytes it is reported that PGE_2 promotes apoptosis of lung fibroblasts*(Huang et al., 2009)*.



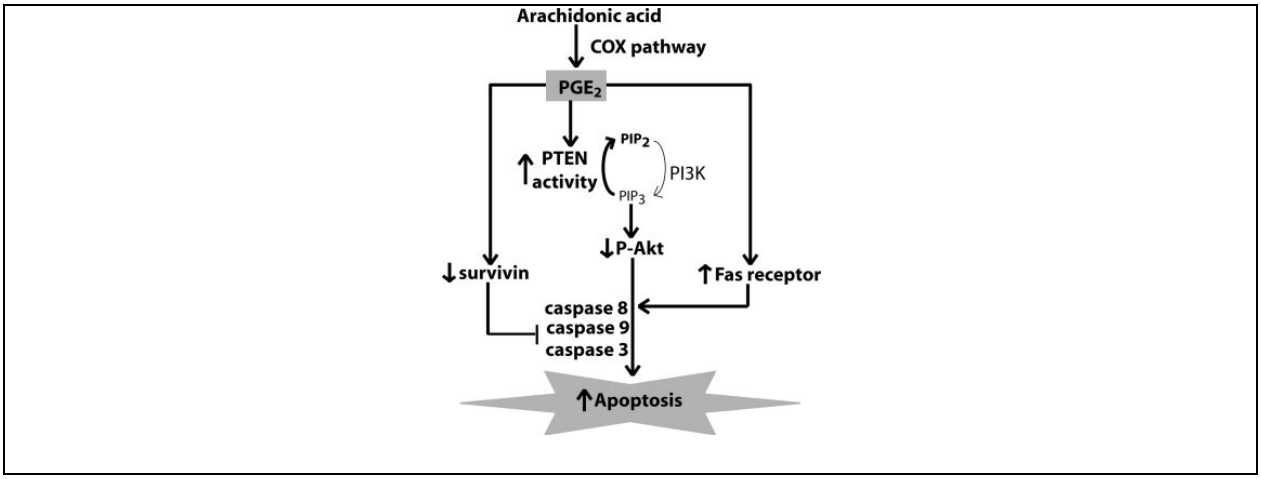
Source: *Ricciotti et al., 2011*

Fig.17.: Prostaglandin E_2 Formation via Arachidonic Acid

2.22. PGE2 induced cell death

Prostaglandin E2 (PGE2) is a lipid mediatorderived from the metabolism of arachidonic acidby cyclooxygenase. AA is oxygenated by cyclooxygenase (COX) enzymes to form prostaglandin endoperoxides. Specifically, prostaglandinG2 (PGG_2) is modified by the peroxidase moiety of the COX enzyme to produce prostaglandin H2 (PGH_2) which is then converted to PGE2 *(Hwa et al., 2017)*. Previously PGE2 has been demonstrated as the inhibitor of diverse fibrogenic factors in lung fibroblasts but recent study shows that PGE2 promotes fibroblast apoptosis via activation of multiple apoptotic pathways*(Huang et al., 2009)*.PGE2-induced fibroblast apoptotic mechanism is dependent on the activation of PTEN which results in down-regulation of prosurvival molecule Akt *(Huang et al., 2009)*. The participation of both caspase 8 and caspase 9 in PGE2-induced apoptosis is confirmed by the data showing increased activities of them and also the inactivation via selected inhibitors. The importance of both the intrinsic (as

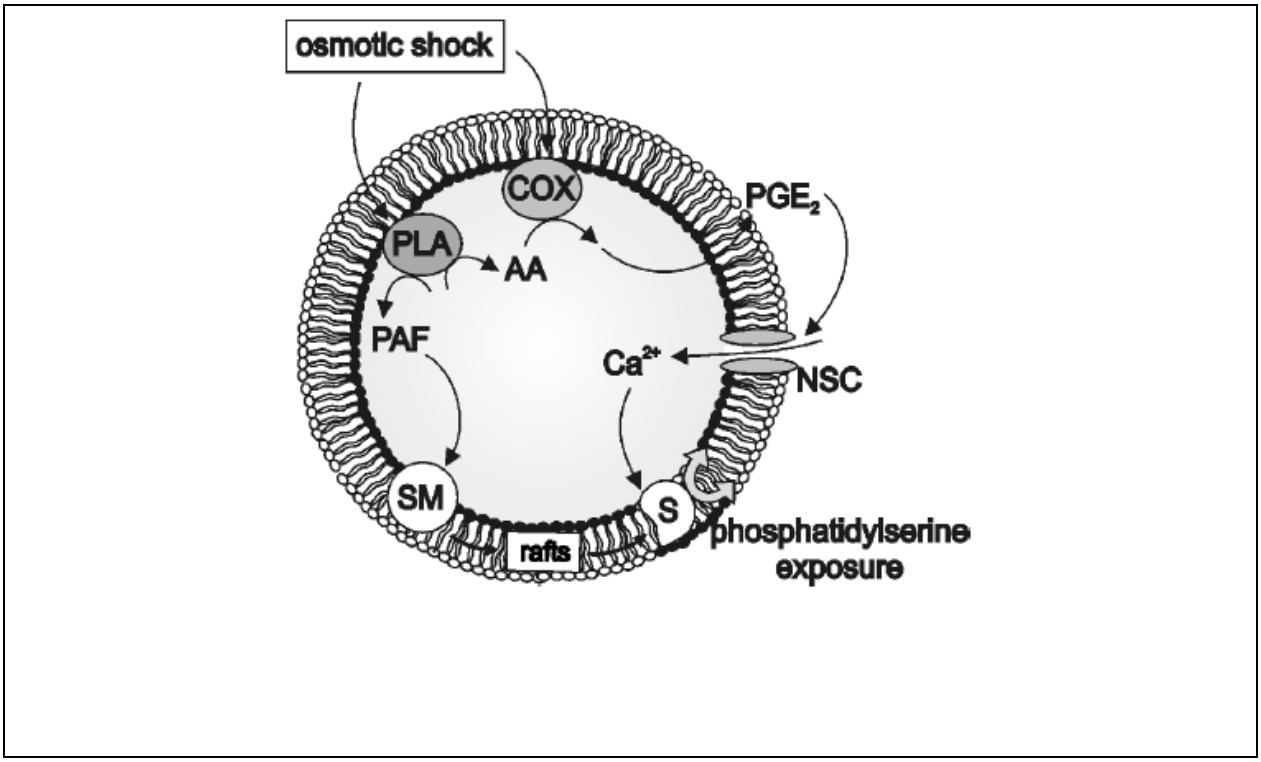
reflected by the role for caspase 9) and extrinsic (as reflected by the role for caspase 8) apoptosis pathways is not surprising, given their recognized capacity for crosstalk.PGE2 also decreases survivin and increases Fas receptor expression.



Source: *Huang et al., 2009*

Fig.18.:Schematic representation of PGE2-induced apoptosis in lung fibroblasts. PGE2, synthesized by the cyclooxygenase (COX) pathway, induces apoptosis by increasing PTEN activity and decreasing Akt activity. PGE2 also decreases survivin and increases Fas expression in lung fibroblasts. PGE2-induced apoptosis is mediated by ligation of the EP2 and EP4 receptors; relative contributions of EP2 and EP4 signaling to the 3 apoptosis pathways remain to be determined.

There is also evidential proof that prostaglandins play a role of in the regulation of eryptosis. Different studies show that the release of prostaglandin E2 (PGE2) activates the cation channels to increases the cytosolic Ca2+ concentration, which intern stimulates phosphatidylserine exposure at the erythrocyte surface *(Lang et al., 2005;Kaestner et al., 2002,2004)*. Oxidative stress or energy depletion leads to the activation of Ca2+ permeable cation channel which subsequently manages the entry of Ca2+ to the cytosol and PGE2 production. The subsequent entry of Ca2+ leads to activation of a Ca2+ sensitive scramblase which exposes phosphatidylserine at the surface of the erythrocyte cell membrane.



Source: Lang et al., 2005

Fig.19.Mechanisms involved in eryptosis. Note that there are Ca2+- and lipid- (ceramide)-mediated mechanisms. AA, arachidonic acid; COX: cyclooxygenase; NSC: nonselective cation channel; PAF, platelet activating factor; PGE2: prostaglandin E2; PLA, phospholipase A2; S, scramblase; SM, sphingomyelinase.

2.23. Cell Death

Though for a decade, cell death has been ignored by biologists but after the accumulation of huge evidential data it has been characterized as genetically encoded mechanisms for targeted elimination of damaged, and/or potentially harmful cells *(Conrad et al., 2016; Weinlich et al., 2017)*. Regulated cell death (RCD) is not only unique to multicellular life forms but also exists among unicellular eukaryotes and prokaryotes*(Cornillon et al., 1994;Büttner et al., 2006; Green et al., 2016)*. In contrast to accidental cell death (ACD), RCD is dependent on dedicated molecular machinery which can be delayed or accelerated *(Galluzzi et al., 2015,2016)*. RCD can occur in the absence of any exogenous environmental perturbation, hence operating as a built-in effector of physiological programs for development or tissue

turnover *(Fuchs et al., 2011;Conradt, 2009)*. This type of physiological forms of RCD is referred to as programmed cell death (PCD).PCD is well regulated cellular process that has been extensively characterized in multicellular organisms but inmany unicellular eukaryotes PCD is also observed. In general, there are three types of PCD, defined in large part by the appearance of the dying cell: apoptosis (also known as type I cell death), autophagic cell death (type II), and necrosis (type III) *(Galluzzi et al. 2007)*.

The characteristic feature of apoptosis is condensation of the chromatin (pyknosis), membrane blebbing and cell shrinkage (Kerr et al. 1972) and accompanied by the activation of caspase proteases *(Galluzzi et al. 2012)*.The death receptor (the extrinsic) pathway and the mitochondrial (the intrinsic) pathway are the two major signaling pathways that trigger apoptotic cell death. A classical ligand–cell-surface-receptor interaction is necessary for the extrinsic pathway.In contrast, the intrinsic pathway is initiated in a cell-autonomous manner. Due to cellular stresses like ROS induced DNA damage, ER stress damages cells beyond repair, then cell actively engage apoptosis.Large intracellular vesicles and engagement of the autophagy machinery are the characteristic features of autophagic cell death. Autophagy is mainly a survival process engaged in response to a metabolic crisis (e.g., low ATP levels and nutrient and amino acid deprivation) or to remove damaged organelles (e.g., mitochondria with low membrane potential) and protein aggregates. In most cases, autophagy accompanies rather than promotes cell death in a stress response most scenarios and represents as a failed survival attempt *(Shen et al. 2012)*.Necrosis is characterized by cell swelling and plasma membrane rupture, and a loss of organellar structure without chromatin condensation. Although necrosis can occur as a consequence of irreparable cell damage, at least one pathway of active necrosis exists. This form of cell death, sometimes called necroptosis, is engaged by several signaling pathways that all converge on the activation of receptorinteracting protein kinase 3 (RIP3). RIP3 is activated upon recruitment to

macromolecular complexes downstream from various cell-surface receptors: DRs, Toll-like receptors (TLRs), and the T-cell receptor (TCR). Additionally, DNA damage can directly induce the formation of a RIP3-activation platform, independently of cell-surface receptor ligation.

2.23. The mechanism of cell death in *Giardia*

Though PCD, a well regulated cellular process which has been extensively characterized in multicellular organisms but it is also reported inmany unicellular eukaryotes in which *Giardia* is the newly added amitochondrial eukaryote. It has been shown that the organisms use different pathways of PCD. Many studies indicate that *Giardia* undergoes PCD in response to various stresses via 2 non necrotic forms, apoptosis-like PCD and autophagy-like PCD. Our previous data with others suggests *Giardia* undergoes apoptotic like cell death via a mechanism independent of caspase *(Ghosh et al., 2009; Correa et al., 2009)*. Though the mechanism of PCD of amitochondriate *Giardia* is still unknown but the end results are similar to known pathway in other unicellular organisms. In case of *Giardia*, ROS can induce apoptosis-like PCD. So under induction of ROS we observed enhanced lipid peroxidation and oxidation of polyunsaturated fatty acid which results in formation of AA. Results identified AA triggers the production of numerous lipid mediators and represents an important step in signal transduction. The consequences of the production of AA and its possible relationship with the mode of apoptosis-like cell death in *Giardia* are discussed in this study.

2.23. Research bridge

Reactive oxygen species (ROS) production is an inevitable consequence of normal oxidative metabolism. Low levels of ROS can play a role in cell signalling by acting as direct cellular stimuli or as receptor-directed stimuli *(Balboa et al., 2006)*. When in high levels ROS exerts many direct and indirect effects on cell signalling pathways, which finally result in the

induction of apoptosis or necrosis*(England et al., 2005)*. One of the effects of ROS on cells is lipoperoxidation, i.e., the oxidative modification of membrane phospholipids. Lipid peroxidation predominantly occurs at the sn-2 position of phospholipids, as most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. This position is also targeted by phospholipase A2 (PLA2) for cleavage *(Cummings et al., 200)*. Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms*(Sapirstein et al., 2000)*. Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites*(Lister et al., 1989)*. In the case of *Trypanosoma,* PLA2 activity is stimulated by Ca2+, and the release of AA by the PLA2 enzyme provides positive feedback for eicosanoid metabolism*(Eintracht et al., 1998)*.

Despite the fact that PLA1 and PLA2 are essential for AA production in *Giardia lamblia*, either of these enzymes or their corresponding genes are absent in this parasite but their activities are present *(Villarreal et al., 2007)*. Studies on fungal and bacterial phospholipase B revealed that it has hydrolase and acyl transferase activities. A detailed study on cryptococcal Phospholipase B showed that the phospholipids of the host cell membrane are a potent site for its lipolytic activity*(Latouche et al., 2002)*.After the genome database (Giardiadb) survey, we could identify a unique enzyme called phospholipase B (PLB) which has the capability to catalyze the hydrolytic cleavage of both sn-1(like PLA1) & sn2 (like PLA2) acyl ester bonds of glycerophospholipids, forming free fatty acids and lysophospholipids*(Aloulou et al., 2018)*. We observed that exposing *Giardia* trophozoites to H2O2 led to an increase in lipid peroxidation compared to the control group. Additionally, we found a significant increase in the levels of intracellular arachidonic acid (AA) and prostaglandin E2 (PGE2) under oxidative stress conditions. Our study focuses on exploring the impact of *Giardia* PLB (gPLB) in generating AA in response to oxidative stress.

It is an enzyme which catalyzes hydrolytic cleavageof both the sn-1 & sn-2 ester bonds of glycerophopholipids, forming FFAs and lysophospholipids (*Matsumoto et al., 2011*). From the literature survey, it is known that Prostaglandine E_2 (PGE_2) activates the erythrocyte cation channels and thus lead to erythrocyte shrinkage andphosphatidylserin exposure (*Lang et al. 2005*), which intern is related to activation of a unique enzyme, Phospholipase B (PLB) hypothetically. This enzyme catalyzes hydrolytic cleavage of both sn-1 & sn2 ester bonds of glycerophospholipids, forming free fatty acids and lysophospholipids(*Matsumoto et al., 2011*). AA is an important metabolite of lipid peroxidation as it can actively participate in membrane reacylation and deacylation as well. It is a cyclical process termed as Land's cycle. In normal condition when cells are uninduced, the reacylation process is dominant but when the cells are in stress condition, the deacylationprocess takes over and proceeds to eicosanoid production. One of the major eicosanoid, PGE2 plays an important role in the ROS induced apoptosis-like death of *Giardia*. The major objective of this proposed study is to identify the signalling cascade by which the cell is committing death under oxidative stress and also study the lipid signaling mechanism involved in it.

CHAPTER 3

MATERIALS

3. Materials

3.1. Different growth mediums

3.1.1. Parasite growth medium

3.1.1.1. Modified TYIS-33 medium

Component	Amount (gm)
K_2HPO_2	1.0
KH_2PO_4	0.6
L-cysteine-HCl (monohydrate)	1.0
Ferric ammonium citrate	0.022
Sodium chloride	2.0
D-glucose	10.0
Ascorbic acid	0.2
Casitone	20.0
Yeast extract	10.0
Double distilled water upto	870 ml

The pH of the medium was adjusted to 6.8 with 1N NaOH. Then the medium was filtered with whatman no.1 filter paper to remove un-dissolved particulate matter and sterilized by passing through pre-autoclaved 0.22 μm membrane filter (Millipore, USA) system.

3.1.1.2. Vitamin-Tween mixture

3.1.1.2.1. Solution A

Vitamin mixture NCTC was prepared as originally described by Evans et al., 1956 for use in tissue culture medium.

3.1.1.2.2. Solution B

40 mg og vitamin B12 (Sigma) was dissolved in double distilled water and the final volume was made upto 100ml.

3.1.1.2.3. Solution C

100 mg of D-L-6,8 thioactic acid (Oxidized form) (Sigma) was dissolved in small volume of ethanol and after complete dissolution the volume of the solution was made upto 100 ml with double distilled water.

3.1.1.2.4. Solution D

50 gms (w/v) Tween-80 was dissolved in ethanol and finally the volume is made up to 100 ml by the addition of double distilled water.

To 1 litre of stock solution, 12 ml of solution B, 4 ml each of solutions C and D and 180 ml of double distilled water were added. The solution was then sterilized by passing through 0.22 µM membrane filters (Millipore, USA) and stored at -20°C.

3.1.1.3. Bovine serum

Bovine blood was collected freshly from slaughterhouse and allowed to clot by resting it at 37°C for 1 hour and subsequently at 4°C for overnight. The un-clotted material was centrifuged at 10,000 g for 10 mins to pellet down the red blood corpuscles (RBC). The clear serum was collected in a fresh container. Next the serum was decomplemented at 56°C for 30 mins. and sterilized by passing through 0.22 µM membrane filters and stored in aliquots at -20°C for further use.

3.1.1.4. Antibiotic solution

Penicillin (Benzylpenicillin Injection IP, Alembic Limited: 10,000,000 IU) and streptomycin (Ambistyrin S®, Sarabhai Piramal: 1 gm) were dissolved in 10 ml of sterile triple distilled water.

Complete Growth Medium

All the above mentioned components were mixed at specific concentration to prepare the complete growth medium for parasite culture. The composition of 100ml of complete TYIS-33 medium is –

Components	Volume (ml)
Autoclaved TYIS-33 growth medium	87
Sterilized heat inactivated bovine serum	10 ml
Complete vitamin mixture	3ml
Antibiotic solution	120 µl

The complete growth medium was aseptically dispensed in 9 ml volumes in a sterile screw capped bottle (15X125 mm Borosil, India). The medium was incubated at 37°C for overnight to ascertain the sterility of the medium.

3.1.2. Luria broth (LB) and agar (LA)

20 gm of luria broth mixture from DIFCO was measured and added to triple distilled water and the volume was made upto 1 litre. 15 gm of agar was added per litre of LB was added to produce Luria agar.

3.1.2. SOC medium

The composition of SOC medium is as follows-

2% trytone

0.5% yeast extract

10 mM Nacl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20mM glucose

3.2. Solutions and buffers

3.2.1. Solutions for genomic DNA isolation

3.2.1.1. Lysis buffer

The concentration of the used lysis buffer components are as follows-

20mM Tris-HCl (pH-8)

50 mM EDTA solution

0.5% Sarcosyl

500 µg/ml Proteinase K

3.2.1.2. CTAB/NaCl solution

4.1 gm of NaCl was dissolved in 80 ml of double distilled water. 10 mg of CTAB (Hexadecy-ltrimethylammonium bromide) was added and dissolved with constant stirring and heating at 65°C.

3.2.1.3. Phenol: Chloroform: Isoamyl alcohol

The solution is composed of a mixture comprising of 50 mM Tris-HCl (pH-8), saturated phenol, chloroform and isoamyl alcohol in a ratio of 25:24:1 respectively.

3.2.1.4. 3M Sodium acetate

24.6 gm sodium acetate was dissolved in minimum volume of triple distilled water and the pH was adjusted with the addition of glacial acetic acid. Finally, the volume was adjusted.

3.2.1.5. RNase A

10 mg/ml of RNase A solution was prepared followingthe manufacturer's protocol.

3.2.2. Solutions for plasmid DNA isolation

3.2.2.1. Solution I (Glucose-Tris-EDTA)

The concentration of the components of this solution was as follows-

50mM Glucose

25mM Tris-HCl (pH-8)

1mM EDTA

3.2.2.2. Solution II (SDS-NaOH)

The composition of this solution was

0.2 M NaOH

1% SDS

3.2.2.3. Solution III (Sodium acetate)

24.6 gm sodium acetate was dissolved in a minimum volume of triple distilled water and the pH was adjusted with the addition of glacial acetic acid. Finally, the volume was adjusted.

3.2.2.3. TE buffer

This buffer consists of –

10mM Tris-HCl

1mM EDTA (pH-8)

3.2.2.3. RNase A and Phenol: Chloroform: Isoamyl alcohol solution

Similar to the solution described in sections 3.2.1.5. and 3.2.1.3.

3.2.3. Solutions and buffers for gel electrophoresis

3.2.3.1. TBE for agarose gel electrophoresis of DNA

The concentration of the components of 10X TBE was –

89 mM Tris

89 mM borate

2 mM EDTA (pH-8)

3.2.3.2. Agarose solution

The required amount of agarose powder was suspended in 0.5 X TBE, melted and cooled to 60°C and finally poured into casting block.

3.2.3.2. Ethidium bromide (EtBr) solution

To prepare this solution 0.1 gm of EtBr was dissolved in 10 ml of triple distilled water.

3.2.3.2. Buffers for polyacrylamide gel electrophoresis

3.2.3.2.1.10X PBS (Phosphate-buffered saline, 100 mM)

0.58 M Na_2HPO_4

0.17 M NaH_2PO_4

0.69 M NaCl

3.2.3.2.2. Cell lysis buffer

The lysis buffer was prepared by dissolving the following components in triple distilled water

-

1OOmM NaCl

10mM Tris-HCl (pH-8)

10mM EDTA (pH-8)

3.2.3.2.3. 100mg/ml proteinaseK

100mgproteinaseK

1 mldH_20

3.2.3.2.4. Shearing buffer

10 mM Tris-Cl

1mM EDTA (pH8.0)

20% Glycerol

3.2.3.2.5.6X gel-loading buffer

0.25%bromophenolblue

40% (w/v)sucrose in water

3.2.3.2.6. 1X Protein sample buffer

2% SDS

5% 2-mercaptoethanol

10% Glycerol

0.2% Bromophenol blue

50mM Tris-HCl (pH-6.8)

3.2.3.2.7. 10X SDS running buffer

25 mMTris base

250 mMGlycine

0.1%SDS

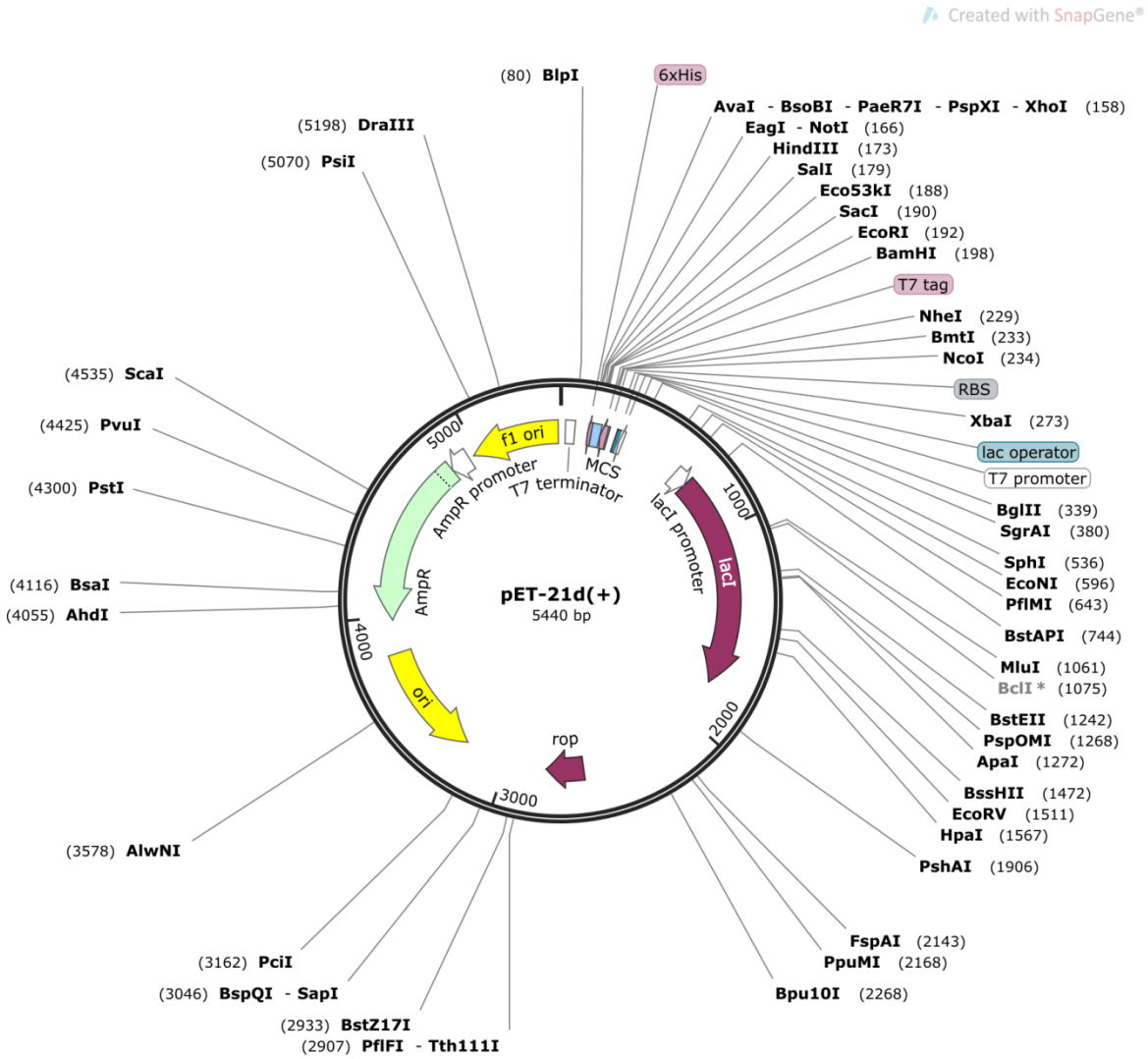
3.2.3.2.8. Transfer buffer

25mM Tris-HCl

192 mM Glycine

20% (V/V) ethanol

3.3. PET 21d vector for cloning and expression



CHAPTER 4

INCREASED Ca^{2+} CONCENTRATION AND ITS EFFECT UNDER OXIDATIVE STRESS IN Giardia

4.1. Introduction

The *Giardia lamblia* is a flagellated protozoan parasite which colonizes in the O_2 -limited mucosa of the gastrointestinal tract of humans to cause the water-borne disease called giardiasis *(Adam, 1991)*. The carbohydrate and amino acid fermentation is the main source of energy demand in this parasite *(Brown et al., 1998)*. Though the organism lacks mitochondria but it has the affinity for O_2 consumption comparable to other aerobic protozoa *(Paget et al., 1993)*. The consumption of O_2 has been shown to rise linearly with the surrounding O_2 tension, up to a threshold level (30–100 μM O_2, depending on the species), above which consumption is arrested due to the formation of reactive oxygen species (ROS) (Paget et al., 1993b; Biagini et al., 1997; Lloyd et al., 2000).The detail mechanism of ROS detoxification in *Giardia* is unclear. *Giardialambia* is also devoid of the conventional mechanisms of the oxidative stress management system, including superoxide dismutase, catalase, peroxidase, and glutathione cycling, which are present in most eukaryotes*(Brown et al., 1995)*. Oxidative stress triggers a range of physiological and adaptive responses in cells either as result of cellular damage or through specific signalling molecule.One of the effects of ROS on cells is lipoperoxidation, i.e., the oxidative modification of membrane phospholipids. Lipid peroxidation predominantly occurs at the sn-2 position of phospholipids, as most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. Both isolated polyunsaturated fatty acids (PUFAs) (especially arachidonate) and those incorporated into lipids are readily attacked by free radicals, becoming oxidized into lipid peroxides.

The ROS can damage cell which is initiated by up regulation of Ca^{2+}. It is one of the most important signalling agents in mammalian cells, and has the ability to control diverse cellular

functions. Intracellular free Ca^{2+} acts as a messenger to regulate growth, differentiation, and also cell death. There are close connections between oxidative stress and organellar Ca^{2+} homeostasis. Coordination of all these signalling functions requires precise regulation of intracellular free Ca^{2+} levels. A sustained high intracellular free Ca^{2+} level may initiate a cascade of signals leading to activation of phospholipases, endonucleases, and Ca^{2+}-dependent proteases which threaten cell survival *(Trump et al., 1995)*.

One of the various targets of intracellular$Ca2^{+}$ is phospholipases which is the key molecule for further generation of signalling molecule. To study the consequence of oxidative stress we have used H2O2and metronidazole.H2O2is awell known agent to generate free oxygen radicals *(Lindley et al., 1988)*, metronidazole involves a free nitro radical generator *(Brown et al., 1998)*, cysteine is a thiol blocking agent and ascorbic acid protect trophozoites under high PO_2 condition. Using above mentioned agents oxidative stress was generated in the*Giardia* trophozoites. Initially, both the agents were used to calculate the LC50 value for further study.

4.2. Materials and methods

4.2.1. Maintenance of cultures

Axenic cultures of Portland1 strain (ATCC 30888^{TM}) of *Giardia lamblia* were routinely maintained in Diamond's complete TYI-S-33 medium, supplemented with 10% decomplemented bovine serum and 3% NCTC-107 vitamin mix. The cells were sub cultured every 48 hours. Briefly, tubes containing *G. lamblia* in the exponential growth phase were chilled in an ice bath for 5-10 minutes, which dislodged the trophozoites adhering to the glass surface. The content of each mother tube was distributed into three fresh media tubes containing complete TYI-S-33 medium inside a BSL-2 cabinet. Tubes were incubated at

35.5ºC in a slanting position and allowed to grow for 48 hours before the next sub-culture. The growth and condition of cultures were routinely checked under a microscope.

4.2.2. Generation of oxidative stress in *Giardia* trophozoites

Dose and time kinetics of the different oxidative stress generating condition has been standardized following the IC_{50} values according to the references *(Lindley et al., 1988)*. Ultimately, from the standardized data, 0.1 µM H2O2and 1µg/ml metronidazole has been administered to all the experiments mentioned below.

4.2.3. Imaging of ROS burst in Giardia using confocal microscopy

H2DCFDA is a non-fluorescent, cell-permeable molecule that, upon entering the cytosol, is converted to fluorescent dichlorofluorescein by the esterase activity of the cell. The fluorescence intensity of the dye is proportional to the state of oxidation by ROS. So, we evaluated the formation of intracellular reactive oxygen intermediates resulting from H2O2and MTZ treatment using the fluorescent probe dichlorodihydrofluoresceine diacetate (H2DCFDA), following the methodology described by (*Raj et al. 2015*). In this study, we incubated treated and untreated cells with H2DCFDA (1.5 µM) for 15 minutes at 37°C. Since H_2DCFDA is permeable to cell membranes, no permeabilizing agent was used. Afterwards, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.2) and fixed with 2% paraformaldehyde. The prepared slides were then observed under a confocal microscope (LSM510, Meta; Carl Zeiss, Thornwood, NY, USA).

4.2.4. Total ROS measurement in Giardia trophozoites using a spectrofluorometer

We measured intracellular ROS production by estimating the fluorescence of dichlorofluorescein, which is the product of H_2DCFDA oxidation. H2O2and MTZtreated and untreated cells (approx.$4x10^6$ cells/ml) were harvested by centrifugation at 2000 rpm. for 10

mins. The cells were then washed and re-suspended in PBS. Finally, 1.5 µM H_2DCFDA was added to the cell suspension. Fluorescence was measured by spectrofluorometer (QuantaMaster30,Photon Technology International) at ex. 488nm. and em. 530 nm.

4.2.5. Measurement of cytosolic free calcium by spectrofluorometric analysis

Intracellular Ca^{2+} concentration and its change with respect to ROS were measured with the fluorescent probe Fura-2/AM according to the method described by Ruben et el., 1991. Treated and untreated cells which were incubated for 0-9 hours were harvested. Harvested cells were then incubated with Fura-2/AM and lysed with 0.1% triton X-100 and prepared for Flourosence measurement at ex.- 340 nm and em.-510nm. The minimum fluorescence signal was obtained by the addition of 30mM trizma base and 4mM EGTA and the maximum fluorescence was obtained upon addition of 10mM $CaCl_2$.

4.2.5. Localization of cytosolic calcium by confocal microscopy

Treated and untreated trophozoites (2 x 10^6 cells/ml) were harvested and washed with ice cold PBS and preciptated at 2000 rpm for 5mins at 4°C. The supernatant was discarded and Fura 2AM, a membrane permeable calcium indicator was added to the precipitated trophozoites and re-suspended at PBS. Inside the cell acetoxymethyl group of Fura 2AM is removed and fluorescent calcium indicator Fura 2 is formed. The calcium localization is captured using LSM 510,Meta; Carl Zeiss confocal microscope.

4.2.6. Measurement of Arachidonic acid under stressed condition

Treated and untreated *Giardia* trophozoites (4x10^7 cells/ml) were harvested, washed, and homogenized in ice-cold PBS in a proportion of 4x10^6 cells/ml. Homogenates were centrifuged at 10000g for 15mins, and the collected supernatant was mixed with 125µl of 20% trichloroacetic acid. The mixture was then centrifuged at 15000g for 10mins. The

supernatant was collected for Fluorescence measurement using ex.340nm em.390nm. Arachidonic acid (10µl) dissolved in 1 ml methanol was used as the reference standard.

4.2.7.Malondialdehyde (MDA) measurement by Spectrophotometer

Lipid peroxidation leads to the formation of MDA, which is a later product in the sequence of this process. To assess the level of lipid peroxidation, we measured the concentration of MDA in the homogenates of *Giardia* trophozoites. To conduct this study, we harvested treated and untreated cells ($4x10^6$ cells/ml) and washed them with PBS. Next, we homogenized the cells in 1 ml of ice-cold PBS. The homogenates were then centrifuged for 15 minutes at 4°C and 10,000g. After centrifugation, 125µl of 20% trichloroacetic acid was added and mixed thoroughly to the collected supernatants. The mixed solutions were centrifuged again at 15,000g for 10 minutes at 4°C. Next, 200 µl of 0.8% thiobarbituric acid (TBA) was added to the supernatant and incubated at 100°C for 60 minutes. Finally, the mixture was kept at room temperature and the absorbance of the chromophore was measured at 535 nm.

4.3. Results

4.3.1. Standardization of in vitro oxidative stress generation in *Giardia* trophozoite

The oxidative stress generation and ROS production have been standardized using H2O2 and metronidazole treatment. According to the data, with increasing concentrations of H2O2 and metronidazole the rate of cell death also increases (Fig.4.1.A, 4.1.B). The time kinetics study was also performed where the concentration (IC_{50}) of the oxidative agents was kept constant. This study revealed that with increasing time the rate of cell death is also increasing (Fig.4.1.C, 4.1.D)

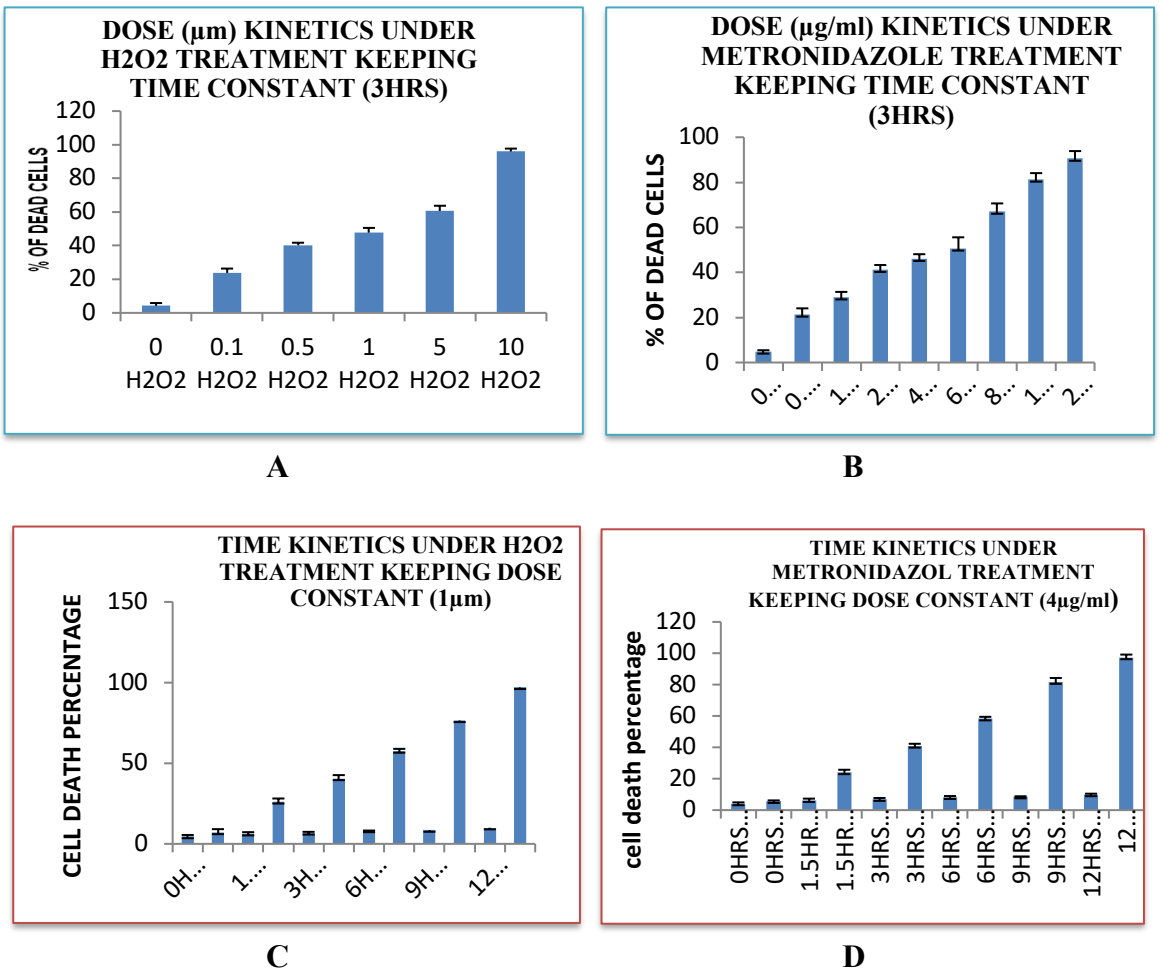


Fig.4.1. Standardization of in vitro stress generation in *Giardia* trophozoites: A. DoseKinetics of *Giardia* trophozoites under H2O2 stress condition keeping time constant. B. DoseKinetics of *Giardia* trophozoites under metronidazole stress condition keeping time constant. C.Time Kinetics of *Giardia* trophozoites under H2O2 stress condition keeping dose constant. D. Time Kinetics of Giardia trophozoites under metronidazole stress condition keeping dose constant.

4.3.2. Imaging of intracellular ROS

The H2DCFDA is a non-fluorescent molecule which can enter cells. Upon entering the cytosol of the cells, H2DCFDA is converted by cell esterase activity to fluorescent dichlorofluoresceine. The fluorescence intensity of it is proportional to the rate of oxidation by the ROS. To observe the produced cellular fluorescence in the trophozoites, they were examined by using confocal microscopy under different stress conditions. Our results suggest

that H2O2, metranidazole and CAD can potentially generate intracellular ROS in *Giardia* trophozoites.The data indicates that the green fluorescence is higher in treated cells, suggesting higher ROS generation compared to untreated cells (Fig.4.2.).

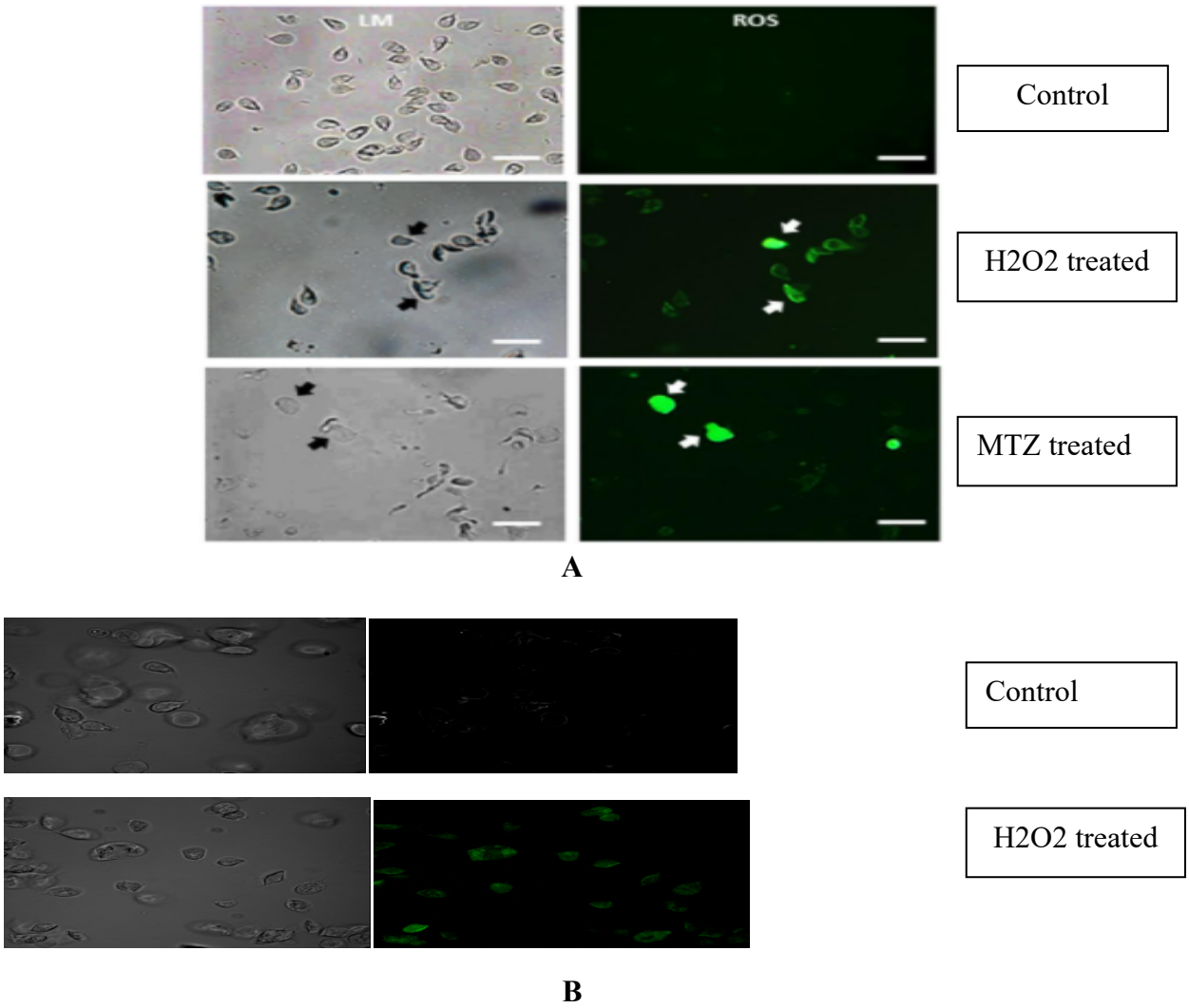
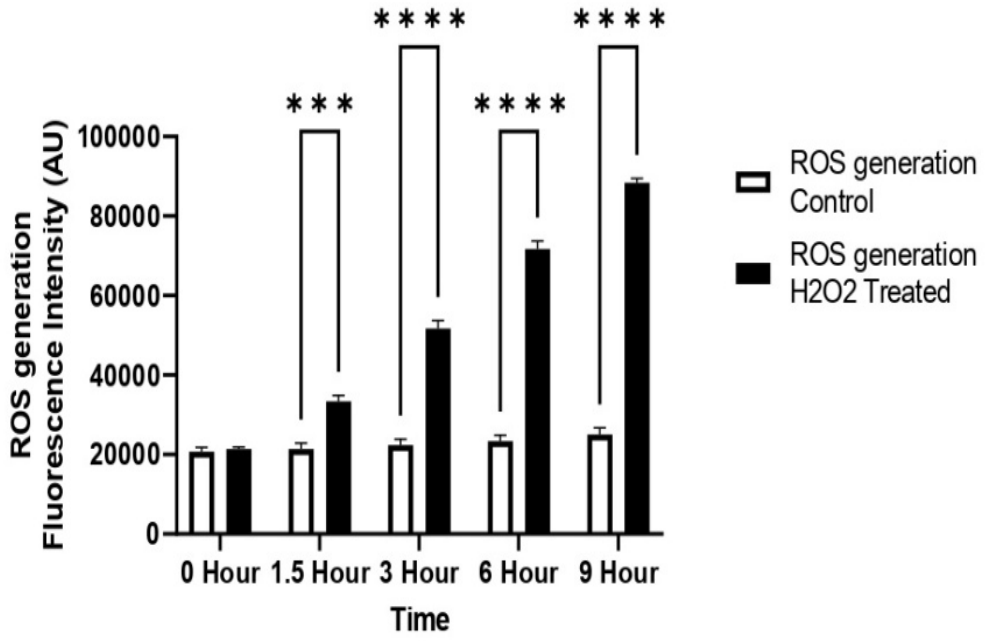


Fig.4.2. Trophozoite of *Giardia* incubated with H_2DCFDA and fluorescence was monitored from suspensions of live cells where the non fluorescent H_2DCFDA is converted to fluorescent DCF by the effect of generated intracellular ROS. A. the cells at 1.5 hours of stress incubation. B. The cells at 6 hours incubation. In both the cases stressed cells showed more green fluroscence than control one which indicate the treated ones generated more ROS than the control one.

4.3.3. Oxidative agents induce ROS generation in *Giardia* trophozoites

To quantify the level of ROS production, we utilized a spectrofluorometer to measure the fluorescence intensity of the dye DCF, as the intensity of fluorescence is directly proportional

to the generation of ROS. The spectrofluorometric data showed that the level of ROS was significantly increased by the addition of H2O2 compared to the controlled trophozoites (Fig. 4.3.A.).Simultaneously, the MTZ treatment also significantly increases the ROS production in trophozoites compared to control cells (Fig. 4.3.B.).



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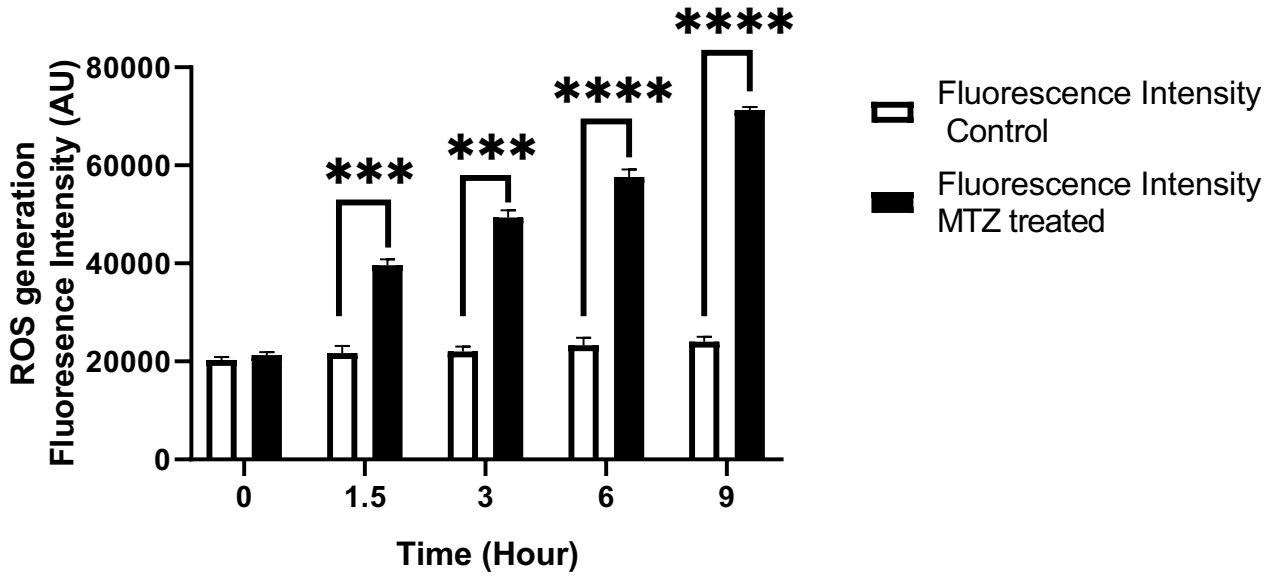


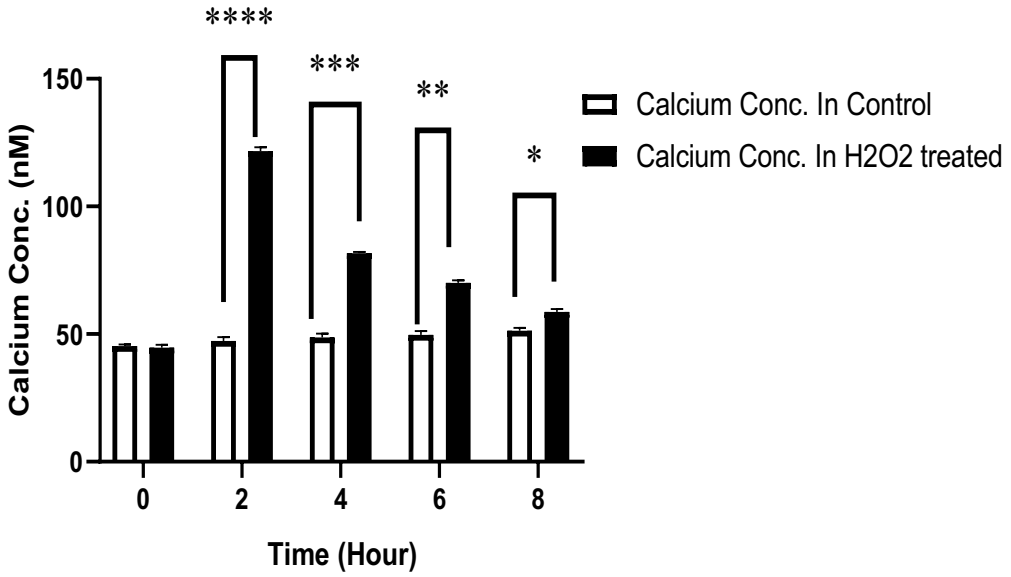
B

Fig.4.3. Comparison of ROS Generation in Treated and Untreated Cells.A. To investigate the effect of H2O2 exposure on *Giardia* trophozoites, cells were subjected to

increasing levels of oxidative stress (0.1 μM H2O2) for different time periods ranging from 0 to 9 hours. The level of reactive oxygen species (ROS) generation was measured using a spectrofluorometer with H2DCFDA as the fluorescent probe. Results showed that ROS generation increased significantly over time compared to the control untreated cells, as determined by the fluorescence intensity of dichlorofluorescein. The data represent the mean of three independent experiments, each performed in triplicate.P value of less than 0.05 was considered significant (***P=0.00002, ****P=0.000018). B. Due to the effect of MTZ treatment in trophozoites ROS is generated and level of reactive oxygen species (ROS) generation was measured using a spectrofluorometer. Results showed that ROS generation increased significantly over time compared to the control untreated cells, as determined by the fluorescence intensity of dichlorofluorescein.

4.3.4.Effect of ROS in intracellular calcium concentration

One of the crucial effects of ROS generation is intracellular calcium concentration up-regulation. This up-regulation is the indication of apoptotic like death in *Giardia*. ROS affects cellular arachidonic acid (AA) production which is dependent on phospholipases. The activation of this cellular phopholipases is dependent on calcium ion concentration. The increased calcium concentration activates cellular phospholipase which further produce AA. In our study we observed that after 2 hours of incubation with the oxidative agents (H2O2 and MTZ) the calcium concentration increased but after that it gradually decreased whereas in controlled cells where no oxidative agents were no such increase in calcium concentration was encountered (Fig. 4.4.A. and 4.4.B.). The data suggests that in the initial 2 hours of treatment the calcium ion accumulated in cytosol which then bind to phospholipase and activate it. When the purpose,the activation of phospholipase, was served the concentration of calcium ion gradually decreased.



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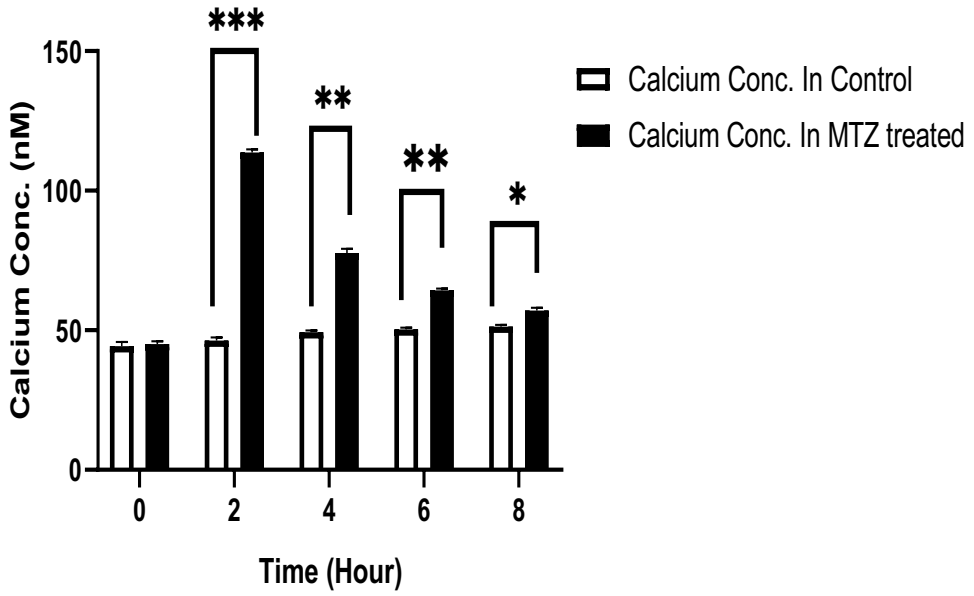


B

Fig. 4.4. Determination of calcium concentration in trophozoites under oxidative stress. A. The intracellular calcium concentration was measured using Fura-2/AM fluorescent dye. The data showed that the calcium ion concentration was increased till 2 hours of H2O2 induction with compared to control cells. After that it gradually decreased. The data represent the mean of three independent experiments, each performed in triplicate. A P value of less than 0.05 was considered significant (*P=0.0015, **P=0.000043, ***P=0.000004, ****P <0.000001). B. In case of MTZ treatment similar phenomenon occurred. The data represent the mean of three independent experiments, each performed in triplicate. A P value of less than 0.05 was considered significant (*P=0.0012, **P=0.000008, ***P <0.000001). This data

supports the fact that the increased calcium concentration activated phospholipase which produce arachidonic acid.

4.3.5. Localization of intracellular Calcium ion under stress condition

To view the location of accumulated intracellular calcium ions, *Giardia* trophozoites were imaged under a confocal microscope. For the purpose of localization study trophozoites were loaded with Fura-2/AM fluorescent dye and slides were prepared to study it under a confocal microscope. The imaging analysis revealed that calcium ions were located at the periphery of the trophozoites under various stress conditions.

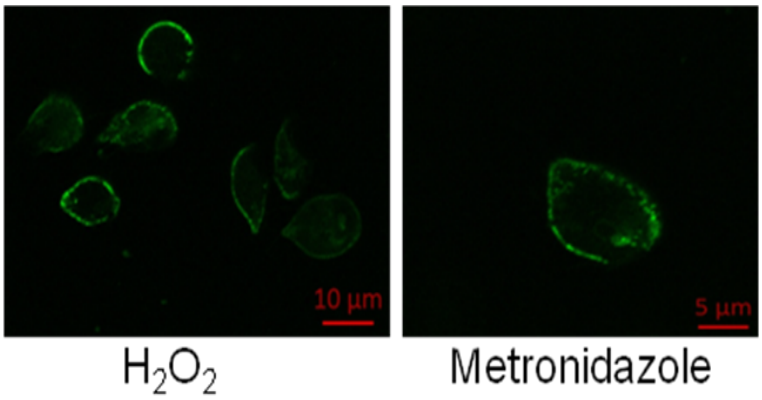
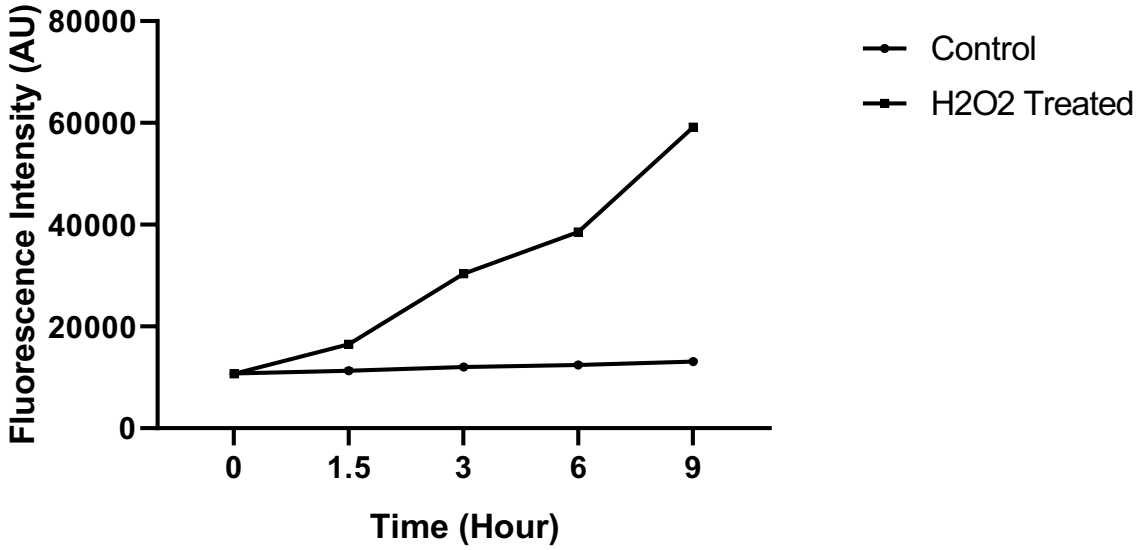


Fig.4.5. Under different stress condition the accumulated calcium ions were localized to the periphery of the *Giardia* trophozoites.

4.3.6. Induced production of Arachidonic acid under oxidative stress

The fluorescence intensity resulting from the production of arachidonic acid in *Giardia*, when exposed to H2O2and MTZ, had been measured spectroflourometrically. After three hours of incubation, the fluorescence intensity of the sample extracted from *Giardia* trophozoites under stress increased significantly compared to the control. Increased Fluorescence intensity suggests increasing production of arachidonic acid in the trophozoites (Fig. 4.6.). The data

suggested that increased intracellular calcium concentration induces arachidonic acid production in trophozoites.



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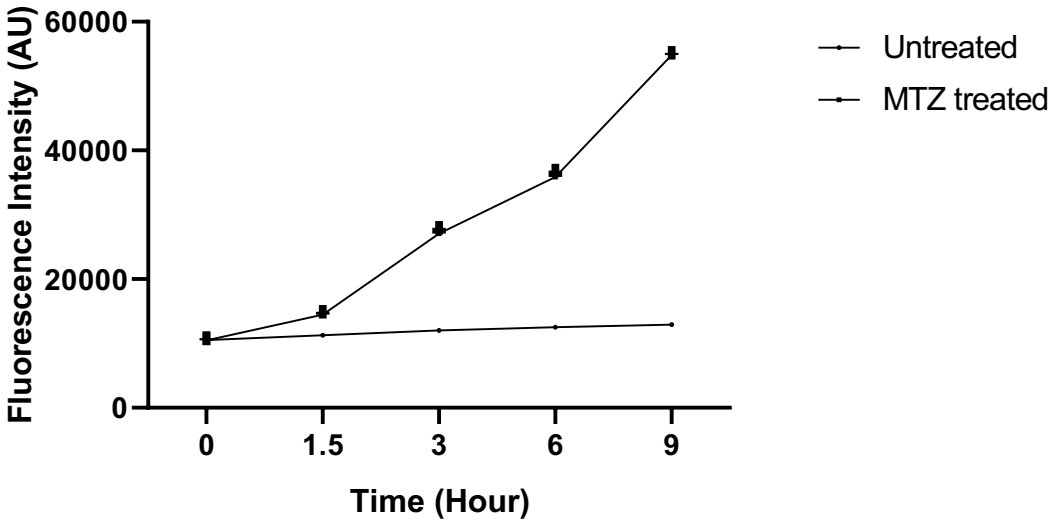
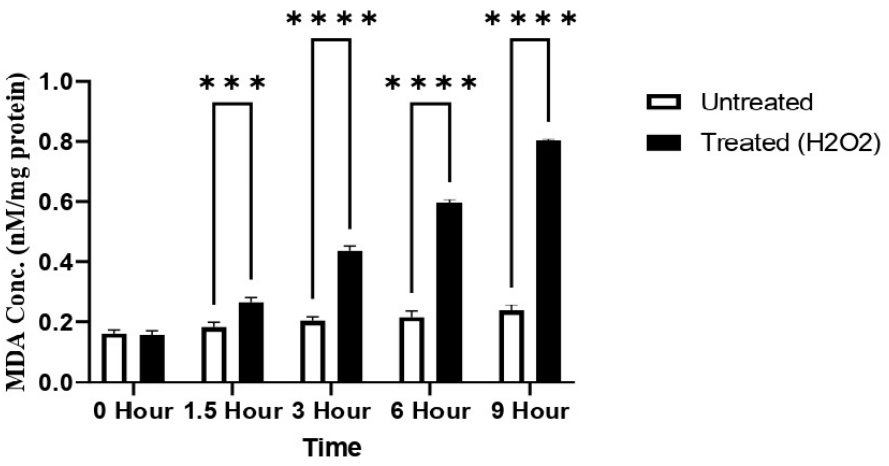


B

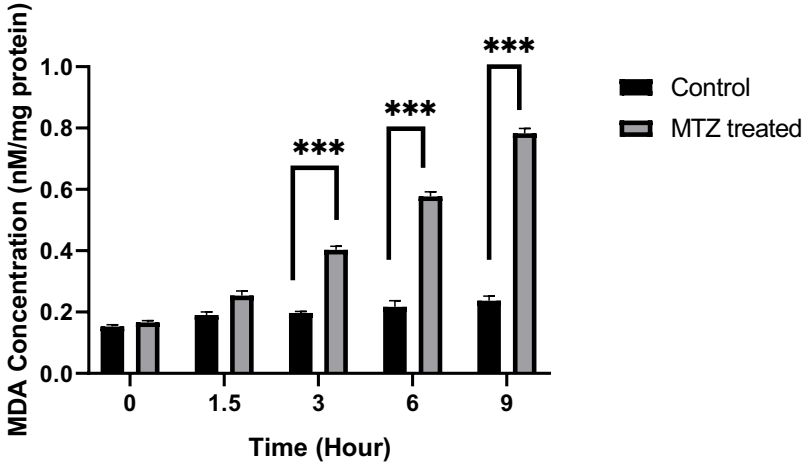
Fig. 4.6.Measurement of Arachidonic acid (AA) production under oxidative stress condition. A. The AA production was measured under H2O2 stress. The concentration of arachidonic acid was measured by a spectrofluorometer at excitation wavelength 340nm and emission wavelength 390nm. The fluorescence intensity is proportional to the amount of AA produced in *Giardia* trophozoites. B. This graph depicts the effect of metronidazole (MTZ) stress in AA production in trophozoites. Both the graph shows that the fluorescence intensity increases with the duration of H2O2and MTZ stress, indicating an increase in AA production. The data represent the mean of three independent experiments, each performed in triplicate. A P value of less than 0.05 was considered significant (****P < 0.000001).

4.3.4. Increased lipid peroxidation in oxidative stress induced Giardia trophozoites

MDA is a by-product of the process of lipid peroxidation. The degree of lipid peroxidation was measured based on the formation of MDA, which served as an index of lipid peroxidation. We found that lipid peroxidation was significantly increased with time in trophozoites under H2O2and MTZ treatment compared to controlled cells (Fig.4.7.A & 4.7.A).The production of intracellular MDA is proportionate to the increase of intracellular arachidonic acid. Introduction of extracellular AA at different concentration (50-500 µM) showed constant increase in intarcellular MDA concentration (Fig. 4.7.C).



A



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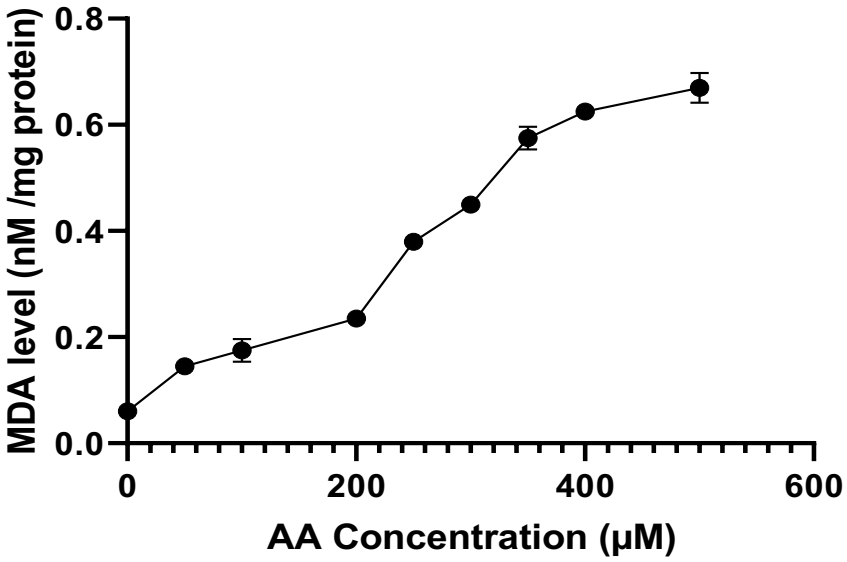


C

Fig.4.7.Measurement of lipid peroxidation level in *Giardia* trophozoites in stressed condition. The formation of malondialdehyde (MDA) was used as a marker for lipid peroxidation. The concentration of MDA was measured spectrophotometrically at different time points in H2O2-treated and untreated trophozoites. A.The graph represents the mean of three independent experiments, each with triplicate values. Statistical analysis was performed using a two-tailed t-test, and a P value less than 0.05 was considered significant. The levels of significance are denoted by asterisks as follows: ***P=0.00002, and ****P=0.000001, indicating significant differences between H2O2-treated and untreated cells. B. The graph shows that MDA production increases with time under the MTZ induction. The data represented is the mean of three independent experiments, each with triplicate values. Statistical analysis was performed using a two-tailed t-test, and a P value less than 0.05 was considered significant. The levels of significance are denoted by asterisks as follows: ***P=0.0001. C. Data showed the effect of AA on the level of lipid peroxidation in *Giardia* trophozoites. MDA concentration in Giardia trophozoites were measured upon AA exposure after 8 hours incubation. The data was analysed by regression analysis method and p value less than 0.05 was considered significant.

4.4. Discussion

Giardia lamblia, a biflagellate diplomonad which causes giardiasis that affects 280 million people worldwide *(Ortega, 1977)*. *Giardia,* the microaerophillic organism, usually is unable to tolerate elevated O_2 level (not higher than 50µM). The oxygen consumption is arrested due to the ROS formation (*Biagini et al., 1997*; *Lloyd et al., 2000*).Oxidative stress triggers a range of physiological, pathophysiological and adaptive responses in cells either as a result of

cellular damage or through specific signalling molecules. These responses ultimately modulate transcriptional outputs to influence cell fate and disease process. To thoroughly understand the role of oxidative stress in *Giardia* trophozoites we created stressed conditions in cultured trophozoite using H2O2 and metronidazole. Hydrogen peroxide (H2O2) has long been recognised as a destructive molecule. The well-established roles they have inthe phagosome and genomic instability have led to the characterisation of these molecules as non-specific agents of destruction. High H2O2concentrations in cellslead to injury bydamaging key cellular molecules such as DNA and lipids (*Plaine, 1955*). It is now proved that at lower physiological concentrations, H2O2 can act as a classical intracellular signalling molecule regulating various cellular processes. Proper quantification and visualization of H2O2 have been the rate-limiting factors in ROS cell signalling research.The most common way to image biological ROS is by using fluorescent redox-sensitive dyes like 2,7-dichlorofluorescein diacetate (DCFDA) or similar dyes such as dihydroethidium, dihydrorhodamine, MitoSox Red and Amplex Red.*Giardia* trophozoites were killed by 2h-incubation with H2O2≥50μM (Hill et al.,1987) and damaged by 1h-incubation with sub-lethal H2O2 concentrations(*Crouch et al.,1991*), but the mechanisms underlying H2O2toxicity are not fully understood yet (*Lloyd et al., 2000).*The most common and effective drug against giardiasis, metronidazole was used to induce stress in trophozoites involving free nitro radical generation.

In our study we were able to detect significant amount of intracellular ROS generation in suspension of *Giardia* trophozoites using H2DCFDA under both conditions. As the time of the treatment increases the ROS generation also increases, which induces different signalling molecule generation in trophozoites. One of the key molecules that can trigger ROS signalling in cells is arachidonic acid (AA). Increased concentration of free AA in cells plays a crucial role to induce further signalling and increased lipid peroxidation. As our data

suggest increased induction of AA in cells increased the MDA concentration in trophozoites.We also observed increased MDA production, significantly by 37.5% in trophozoites treated with H2O2and 28.3% in MTZ treatment compared to control ones. MDA production is the marker for intracellular lipid peroxidation. The data suggested increased ROS generationtriggered AA production which further induced the lipid peroxidation in *Giardia.*

The formation of AA needs a potent cellular phospholipase usually phospholipase A2 (PLA2). Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites *(María et al.,2006; Mark et al., 1989)*. In the case of *Trypanosoma,* PLA2 activity is stimulated by $Ca^{2+}$and the release of AA. As our study revealed AA formation in trophozoite under oxidative stress condition we further investigated if the parasite shows increased intracellular calcium ion (Ca^{2+}) concentration. Our study showed the concentration of intracellular $Ca^{2+}$was increased till 2 hours of both treatment but then gradually decreased and simultaneously after that from 3hours the AA production increased significantly. This type of pattern indicates that Ca^{2+}act as inducer molecule to activate intracellular enzyme which can further produce AA from phospholipids. The accumulation of $Ca^{2+}$was visible throughout the periphery of the trophozoites which indicate the enzyme is effective on membrane phospholipids.

CHAPTER 5

CLONING AND CHARACERIZATION OF ARACHIDONIC ACID PRODUCING NOVEL ENZYME PHOSPHOLIPASEB

5.1. Intorduction

Normal oxidative metabolism leaves an inevitable consequence, which is Reactive oxygen species (ROS). ROS participates in cell signalling by acting as direct cellular stimuli or as receptor-directed stimuli *(Balboa et al., 2006)*. However, high ROS level exerts many direct and indirect effects on cell signalling pathways, which finally result in the induction of apoptosis or necrosis *(England et al., 2005)*. One of the effects of excess ROS generation on cells is lipoperoxidation, i.e., the oxidative modification of membrane phospholipids. The sn-2 position of phospholipids most vulnerable site for lipid peroxidation because most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. This position is targeted by phospholipase A2 (PLA2) for cleavage as well *(Cummings et al., 2000)*. Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms *(Sapirstein et al., 2000)*. One of the several biologically active mediators generated by increased PLA2 activity in cells under oxidative stress is arachidonic acid (AA) and associated metabolites (*Balboa et al., 2006*; *Lister et al., 1989)*.

Despite the fact that PLA1 and PLA2 are essential for AA production, either of these enzymes or their corresponding genes are absent in *Giardia lamblia* but their activities are present*(Vargas-Villarreal et al., 2007).* After the genome database (Giardiadb) survey, we could identify a unique enzyme called phospholipase B (PLB) which has the capability to catalyze the hydrolytic cleavage of both sn-1(like PLA1) & sn2 (like PLA2) acyl ester bonds of glycerophospholipids, forming free fatty acids and lysophospholipids*(Aloulou et al., 2018)*. A detailed study on cryptococcal Phospholipase B showed that the phospholipids of the host cell membrane are a potent site for its lipolytic activity*(Latouche et al., 2002)*.

We observed that exposing *Giardia* trophozoites to H2O2 led to an increase in lipid peroxidation compared to the control group. Additionally, we found a significant increase in the levels of intracellular arachidonic acid (AA) and prostaglandin E2 (PGE2) under oxidative stress conditions. Our study focuses on exploring the impact of *Giardia* PLB (gPLB) in generating AA in response to oxidative stress. Our rationale for investigating this is based on literature indicating that PLB can hydrolyze the sn-2 position of membrane phospholipids, such as PLA2, resulting in the release of AA.To comprehensively investigate this enzyme, we cloned the PLB gene from *Giardia lamblia*, obtained the recombinant protein through purification, and performed biochemical and bioinformatic characterization on it.

5.2. Materials Methods

5.2.1. RNA isolation cDNA formation and Real-time PCR

The trophozoites, both treated with H2O2 and untreated, were collected and their total RNA was extracted using the TRIZOL (Invitrogen) method based on the protocol outlined by Raj et al. 2013*(Raj et al., 2014)*. Our investigation into the PGE2 pathway in *Giardia* focused on the analysis of the gene expression of PLB when under oxidative stress. PLB is the first critical enzyme responsible for converting membrane lipids into arachidonic acid. We have performed a real-time PCR assay to study PLB gene expression using F - 5’ 5’CACCGTGTATGGACACACGCAAACAAA3’ and R- 5’TTACATATCAGAGTGCCAATAAACCGG 3’ primers. Here, the beta-actin gene was used as control gene F- 5’ ACATATGAGCTGCCAGATGG3’ and R- 5’ TCGGGGAGGCCTGCAAAC3’. The experiment was conducted three times with triplicate samples, utilizing the Roche RT-PCR system. The RT-PCR was carried out with the

following conditions: denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

5.2.2. Isolation and PCR of genomic DNA of *Giardia lamblia*

Genomic DNA from *G. lamblia* Portland1 strain (ATCCR 30888TM) was isolated usingQIAamp® DNA Mini Kit (QIAGEN, USA). RNase digestion was performed for 30 mins at 37°C to remove RNA contamination. The primers were designed to amplify the Phospholipase B gene from the Portland1 strain of *G. lamblia*, using the sequences of *Giardia* PLB-like genes obtained from GiardiaDB as a reference. Specifically, the reference sequence used for primer design was GiardiaDB entry GL50803_93548.PCR amplification was carried out using Expand Long Template PCR system by Roche (Cat. No. 1681834), following PCR conditions - Initial denaturation - 94°c 5mins, denaturation - 94°c 30sec., annealing - 50°c 1.5 mins., elongation - 72°c 3mins, final extension - 72°c 7mins. For cloning of the *Giardia* PLB gene in the pET21d expression vector, the forward primer used for the PCR was - FP-5’ GGATCCGAATTCATGCTTCTGGCCAT 3’ (with ECORI restriction site), and the reverse was RP- 5’ AAGCTTTTACACAAAGAGGGAGTGTG 3’ (with BamHIrestriction site).

5.2.3. Cloning and Purification of PLB

A PCR-based approach was used to clone the Phospholipase B gene of *Giardia lamblia*. PCR amplified DNA was digested with BamHI and HindIII, and the desired PLB gene was inserted in between the restriction site of the pET21d vector under the T7 promoter. The clone was transformed into BL21(DE3) cell. The cloning was confirmed by sequencing the cloned product. The transformed colonies were screened on LB medium supplemented with

ampicillin and cultured overnight at 37°C. From the seed culture, 1ml was transferred to a fresh medium and cultured until O.D reached 0.6-0.8 and then induced with 0.5 mM IPTG at 20°C for 16 hours. The protein expression level was detected at 10% polyacrylamide gel according to the standard protocol. For the purification of the desired protein, the induced cells were harvested and disrupted by sonication in Tris-HCL buffer (pH- 7.0). The supernatant was collected by centrifugation at 12,000g for 20 mins at 4°C. The protein was purified by Ni-NTA column (Invitrogen ProBond Resin, USA). A gravity-flow column was prepared by adding Ni-NTA resin slurry (50% slurry in 20% ethanol) and pre-equilibrating it with equilibration buffer (7M urea, 25 mM Tris, 100 mM NaCl). The soluble protein was then transferred into the equilibrated resin and incubated for 1.5 hours at 4°C with shaking. During incubation, both ends of the column were tightly capped to prevent any leakage. The column was then held upright to allow the resin to settle, and the caps were opened to capture the flow-through. The column was washed three times with wash buffer (50 mM sodium phosphate buffer, 500 mM sodium chloride, 0.5% Triton X 100, 20 mM imidazole) using twice the volume of the resin, and the flow-through was collected. The protein was eluted twice with elution buffer (7M urea, 25 mM Tris, 100 mM NaCl, 250 mM imidazole), and each elution was collected separately. Finally, all the flow-through was analysed by SDS-PAGE.

5.2.4. Development of anti-phospholipase B antiserum (raised in rabbit)

To generate anti phospholipase B antisera the rabbits were injected purified protein with adjuvant subcutaneously. For this purpose a male rabbit, aged 200 days, weighted 1000 gm and a female rabbit, aged 160 days, weighted 1100 gm, were selected. They were given shots in every 14 days for 5 times. The final sera were collected after 5^{th} booster dosage of immunogesic injection. For the quality assessment we had performed western blot by using varying amount of purified protein. Increasing amounts of pure protein (0.5, 1, 5, 10, 20, 30,

50, 100 ng) were loaded into the well and electrophoresed and blotted with 1:5000 primary antisera and 1:5000 secondary antibody.

Immunization record

Stage	Immunization	1st Booster	2nd Booster	3rd Booster	4th Booster	5th Booster
Antigen dosage (µg)	250	150	150	150	150	150
Type of Adjuvent	FCA	FIA	FIA	FIA	FIA	FIA
Antigen: Adjuvent	1:1	1:1	1:1	1:1	1:1	1:1
Vol. Injected (µl) X No. of sites	250X2	100X2	100X2	100X2	100X2	100X2

5.2.5. Sequencing and Sequence alignment

Amplified DNA (using FP-5'GGATCCGAATTCATGCTTCTGGCCAT3'RP-5'AAGCTTTTACACAAAGAGGGAGTGTG3' respectively) was run on horizontal agarose gel, and amplicon of specific band size was extracted and processed using manufacturer's protocol (Gel extraction kit Roche) and sequenced bidirectionally (using Thermo Fisher Scientific - BigDye Terminator v3.1 cycle sequencing kit) in ABI PRISM^{R}3100 Genetic Analyzer(Applied BioSystems, Waltham, Massachusetts, USA) sequencer. The sequence was aligned with ClustalW (NCBI online) method, and similarity was assessed by BLASTN and BLASTP programs.

5.2.6. Primer construction

For the construction of primers of the gPLB gene product GiardiaDB entry GL50803_93548 gene sequence was considered as template sequence and Primer3 software was used to

prepare the primer sequences. The sequences were further checked for any probability of primer dimer formation or loop formation in the primer sequence.

5.2.7. 3D Homology modelling using predicted amino acid sequence

SWISS Model online modelling servers were used to generate the homology model of the protein *(Waterhouse et al., 2018)*. For homology modelling and other structural models the lysomalphospholipase b like protein (3FBX – PDB japan) of mouse was used as template and our deduced amino acid sequence was used as query sequence. Verif3D software programs were used to validate the generated model; the fittest model was selected from the analysis of this software. The secondary structure was analyzed by the Phyre2 tool of the 3DLigandsite website *(Wass et al., 2010)*. Further, the motifs on the protein were predicted by the Prosite tool of Expasy and molecular weight was deduced using ProtParam*(Sigrist et al., 2013; Wilkins et al., 1999)*. The NetNGlyc tool detected potential N glycosylated sites *(Gupta et al., 2002)*.

5.2.8. Phylogenetic tree construction

A phylogenic tree was constructed by the neighbor-joining method using the computer software Figtree. Sequence of Moraxella bovis (AAK53448.1), E.Coli.(pdb|1T16|B), Lactobacillus johnsonii (AEB92860.1), Acidobacterium capsulatum (ACO32224.1), Human (sp|Q6P4A8.2), Dictyosteliumdiscoideum (AAN03644.1), Saccharomyces cerevisiae (AAB38425.1), Stenotrophomonas maltophilia (CAQ46340.1), Cavia porcellus (AAC40129.1), Rattus norvegicus (BAA23813.1), Candida albicans (AAC72296.1), Anopheles darlingi (ETN62840.1) proteins were used to construct this phylogenetic tree.

5.2.9. Lipase activity

To detect the lipase activity of phospholipase B a photo-metric assay was performed where p-nitrophenyl palmitate dissolved in 2.5 ml isopropanol was mixed with 22.5 ml of 0.05 M So¨rensen phosphate buffer, pH 8.0, containing 207 mg sodium deoxycholate and 100 mg of Arabic gum (Sigma) was used as substrate. In each well, 240µl freshly prepared of this substrate solution and10µl of enzyme sample were added to a 96-well microtiter plate. Incubated at 37℃ for 60 mins, the absorbance was measured at 410 nm. One enzyme unit was equal to 1 nmol of p-nitrophenol enzymatically released from the substrate per minute. The enzymatic activity of the samples was calculated as activity units per mg protein. An OD at 410nm of 1.0 is equivalent to 9.7 nmol of p-nitrophenol released from p-NPP per min, i.e., 0.161 nkat*(Winkler et al., 1979; Stuer et al., 1986)*.

5.2.10. SDS-PAGE

SDS-PAGE is the most commonly used gel electrophoretic system for analyzing proteins. This method is based on the separation of proteins according to size and can also be used to determine the relative molecular mass of proteins. SDS is an anionic detergent which binds strongly to and denatures proteins to produce linear polypeptide chains. On average one SDS molecule will be present for every two aminoacids. The presence of β-mercaptoethanol assists in protein denaturation by reducing all disulfide bonds. The detergent binds to the hydrophobic region of the denatured protein in a constant ratio of about 1.4g of SDS/gm of protein. The protein-SDS complex carries net negative chargesand hence moves towards the anode and the separation is based on the size of the proteins. SDS-PAGE gels were cast with a molar ratio of Bisacrylamide:Acrylamide of 1:29 which had been shown empirically to be capable of resolving polypeptides that differ in size by as little as 3%. The Polyacrylamide gel was cast as a separating gelbuffered with Tris-cl buffer at pH 6.8 and topped stacking gel with high percentage of acrylamide and was cast in Tris-cl buffer at pH 8.8. Protein resolved in the gel was stained with Coomaassie brilliant blue. Coomassie blue are electrostatically

attracted to charged groups on the protein, forming strong dye: protein complexes that are further augmented by vanderwaals forces, hydrogen bonding and hydrophobic bonding. Next the proteins were transferred to the nitrocellulose membrane. The membrane was overlaid with a primary antibody for a specific PLB and then with a secondary antibody labelled.

5.2.11. Intracellular arachidonic acid measurement

Giardia trophozoites ($4x10^7$ cells/ml) were harvested, washed, and homogenized in ice-cold PBS in a proportion of $4x10^6$ cells/ml. Homogenates were centrifuged at 10000g for 15 minutes, and the collected supernatant was mixed with 125µl of 20% trichloroacetic acid. The mixture was then centrifuged at 15000g for 10 minutes. PLB-specific inhibitor alexidine dihydrochloride (0.25 µM) was used to assess the role of PLB in arachidonic acid production. The trophozoites were pre-incubated with alexidine dihydrochloride for 30 mins at 35°C, and then H2O2 (0.1 µM) and MTZ were administered separately to the trophozoites and incubated for time course analysis. The supernatant was collected for Fluorescence measurement using ex.340nm em.390nm. Arachidonic acid (10µl) dissolved in 1 ml methanol was used as the reference standard.

5.2.12. Localisation study of gPLB

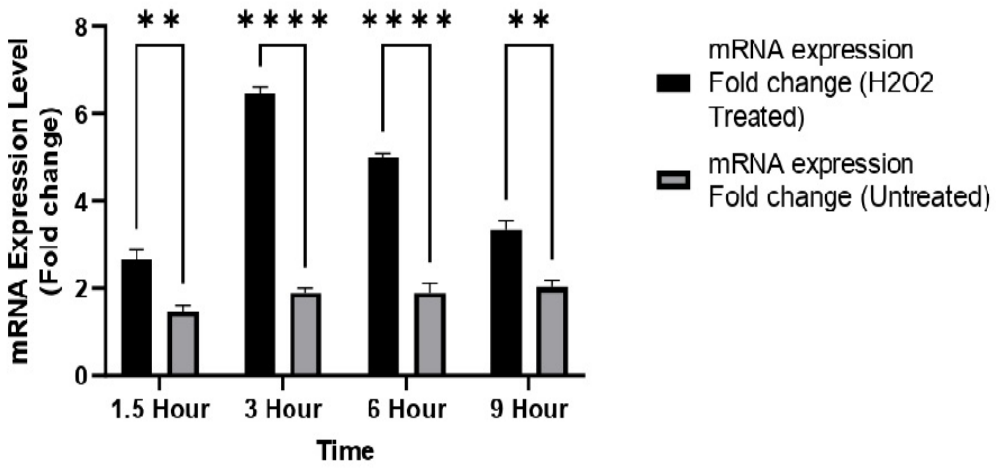
$1.4x10^7$ cells/ml of *Giardia* trophozoites were harvested, washed, and homogenized in ice-cold PBS in a proportion of $4x10^6$ cells/ml. The samples were incubated for 10 min with PBS containing either 0.1–0.25% Triton X-100. It is the most popular detergent for improving the penetration of the antibody. Next, cells were washed in PBS three times for 5 mins each. The cells were then incubated with 1% BSA, in PBST (PBS+ 0.1% Tween 20) for 30 min to block the unspecific binding of the antibodies and then incubated in the diluted primary antibody (1:2500) in 1% BSA in PBST in a humidified chamber for 1 h at room temperature. After washing with PBS three times, trophozoites were incubated with a secondary antibody

(dilution 1:5000) for 1 hour at room temperature. The secondary antibody solution was decanted and washed three times with PBS for 5 min each in the dark. The slides were mounted with a drop of a mounting medium and coverslip.Coverslips were sealed with nail polish to prevent drying and movement under a microscope. Finally, the slides were checked in a confocal microscope.

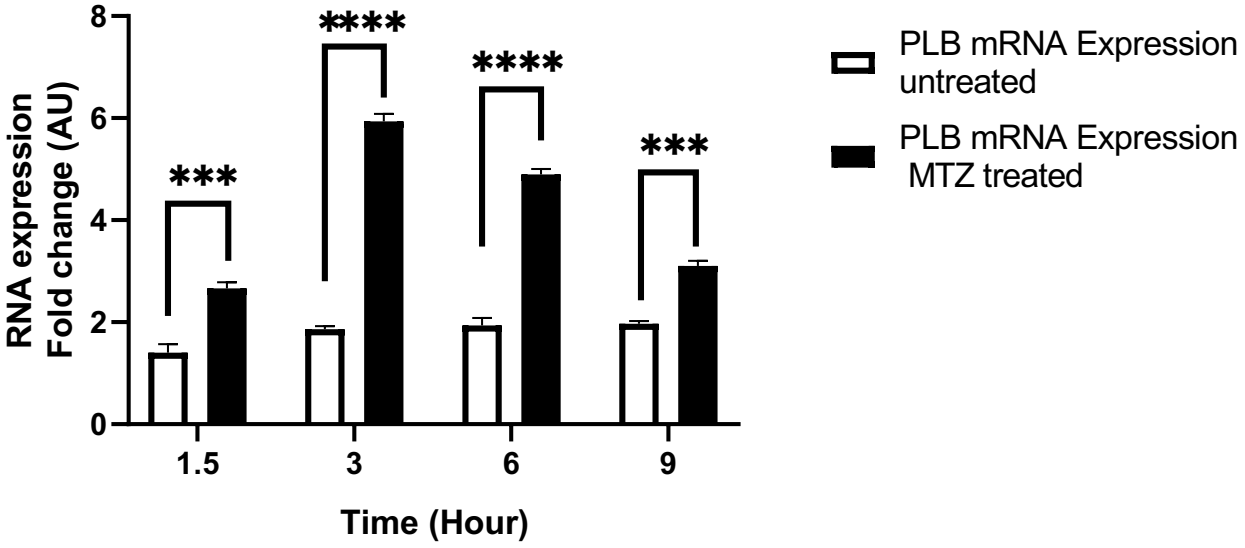
5.3. Results

5.3.1. Upregulation of PLB gene expression under oxidative stress

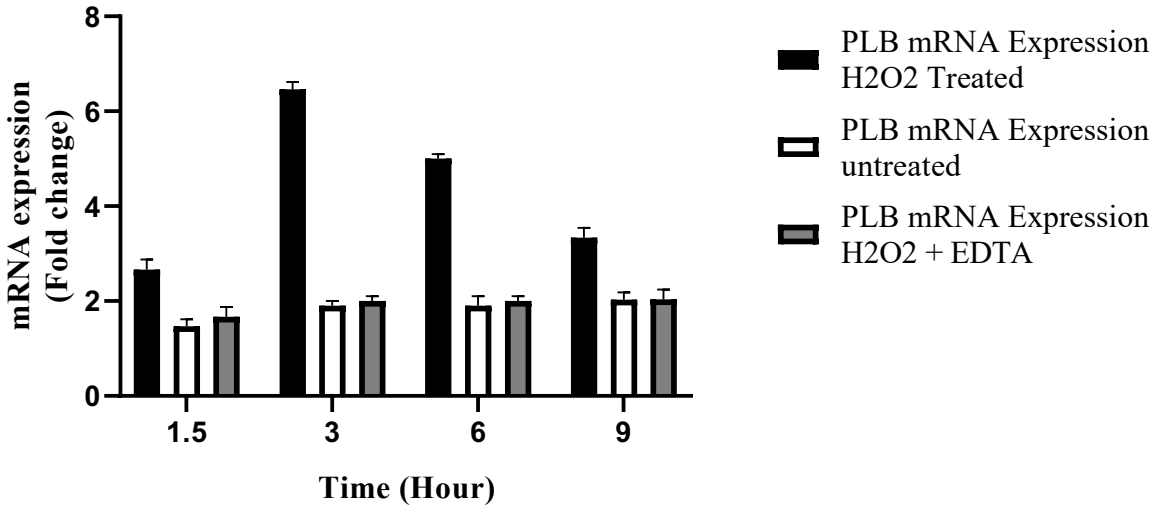
To understand the effect of oxidative stress with H2O2 in the transcriptional regulation of PLB gene expression, we performed a time course analysis of the PLB gene using the quantitative RT-PCR method. In the present study, we observed that when trophozoites were induced by oxidative stress (H2O2and MTZ), the expression of PLB level started to up-regulate. The gene was upregulated with maximum induction of 6-fold at 3 hours of incubation and remained up-regulated till 9 hours post-exposure with H2O2 and MTZ (Fig.5.1.A and 5.1.B.). This result correlates with the increased levels of intracellular Ca^{2+}andArachidonic acid under oxidative stress, as discussed previously. We further investigated this phenomenon by using Ca^{2+}chelater, EDTA. The previously EDTA treated trophozoites did not show any significant increase in gPLB upregulation.


mRNA Expression Level (Fold change)
8
6
4
2
0
**
****
****
**
1.5 Hour
3 Hour
6 Hour
9 Hour
Time
mRNA expression Fold change (H2O2 Treated)
mRNA expression Fold change (Untreated)

A


RNA expression Fold change (AU)
8
6
4
2
0
***
****
****
***
1.5
3
6
9
Time (Hour)
PLB mRNA Expression untreated
PLB mRNA Expression MTZ treated

B



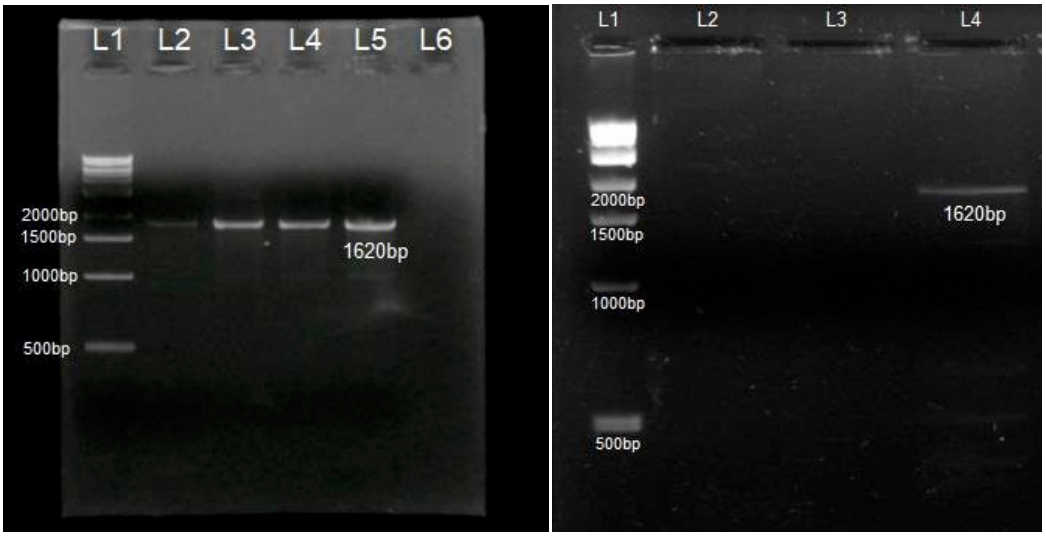
C

Fig.5.1.Effect of oxidative stress on mRNA expression level of *Giardia* PLB.A.The mRNA expression level of Giardia PLB was analyzed using the Real-time PCR method under oxidative stress conditions. The data represent the mean of three independent experiments. The PLB expression level was upregulated throughout the induction time (0-9 hours) and reached its peak at 3 hours of incubation before starting to decline. B. this Graph represents the expression of PLB is upregulated after 3 hours of MTZ induction and then gradually decreases.The statistical analysis showed that the upregulation of PLB expression was significant (***P<0.001 and ****P<0.0001) compared to the control group. C. The Ca ion chelater, EDTA decreased the gPLB mRNA expression level suggesting that it played an important role in gPLB increased expression.

5.3.2. Cloning and expression of PLB homolog from *Giardia lamblia*

A 1620-bp fragment was obtained as the primary product of PCR amplification using *Giardia* genomic DNA as a template and primers FP and RP (Fig.5.2.A). The gPLB gene was incorporated into the pET21d vector after undergoing restriction digestion and ligation. The vector was then transfected into BL21(DE3) *E.coli* strain with ampicillin selection. PCR was performed on both the transformed colony and the non-transfected untransformed BL21(DE3) colony. The transformed colony PCR showed a clear band around 1620 bp (lane no 4, Fig.5.2.B), while no band was detected in the untransformed colony (lane no 2, Fig.5.2.B). This result indicates that the sequence originated from outside of *E. coli* and was

transferred to it through transfection and that the transformed *E. coli* colonies contained the gPLB gene.The PCR product from the transformed colonies was sequenced.The DNA sequence that was obtained has been submitted to the NCBI GenBank and assigned the accession number OM939681.The PLB gene was then over-expressed by IPTG induction and an induced band was observed near 56 kDa (lanes 1 and 4, Fig.5.2.C). Moreover, no such band was observed in the case of an uninduced one (lane L3, Fig.5.2.C). 1.6kbp PCR product was used to determine the nucleotide sequence of the *Giardia* PLB gene. After being subjected to Ni-NTA column purification, the protein was effectively purified. Analysis of the eluted sample via SDS-PAGE demonstrated a notable presence of a band(Fig.5.2.D).



A **B**

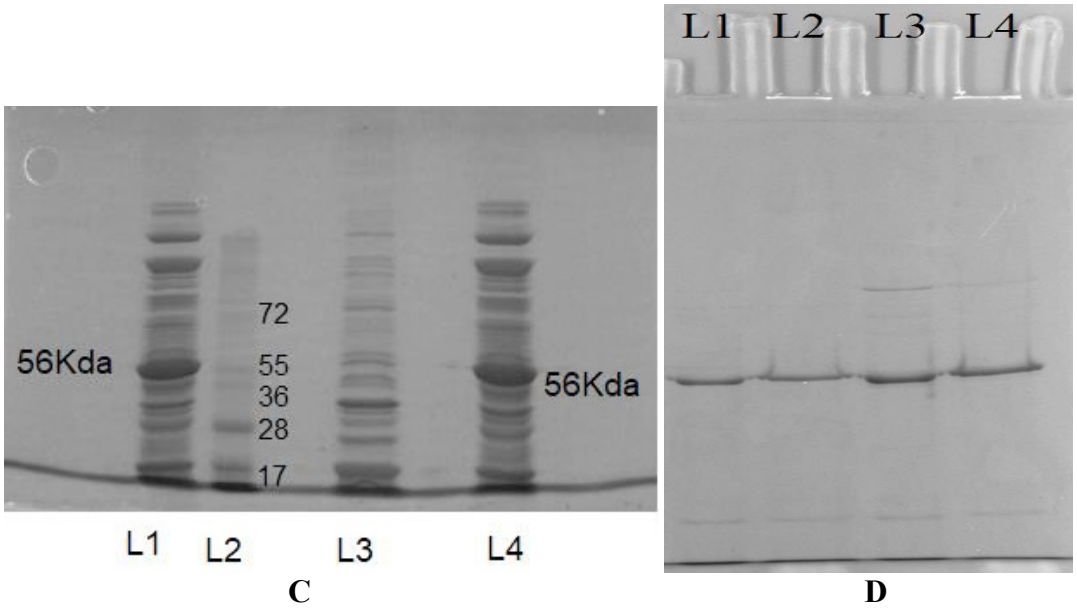


Fig.5.2. Cloning of *Giardia* PLB in *E.coli*.A.PCR amplified *Giardia* DNA using Phospholipase B (PLB) specific forward and reverse primer. Lane no 1 (L1) was the marker DNA and lanes 2-5 were the amplified product of the PLB gene. Lane L6 contains negative control(The reaction mixture did not contain any template DNA from *Giardia*). B. After cloning, the inserted gene product was confirmed by colony PCR. Lane 1 was maker DNA, lane 2 was the PCR product from the untransformed colony and lane 4 was the PCR product of the transformed Colony. L3 contain negative control(The reaction mixture did not contain any template DNA from *Giardia*). C. After successful cloning PLB protein was induced by IPTG to express. Lanes 1 and 4 were the proteins from 0.5 mM IPTG-induced cells (20°c 16hrs.) and lane 3 was the protein from uninduced cells. Lane 2 was the protein marker. Here in lanes 1 and 4, the induced cells show a strong band near 56kda which is equivalent to the PLB protein and in lane 2 no such band was seen. D. SDS-PAGE for purified protein showed single band suggesting pure protein is eluted. L1, L2,L3,L4 contain elution1, elution2, elution3 and elution4 respectively.

5.3.3. Sequence and its alignment

The cloned product of PLB gene was sequenced and the derived sequence (Fig.5.3.A) was aligned with ClustalW (NCBI online) method. The alignment analysis showed that the derived sequence from the clone was 90%similar with *Giardia* Phospholipase B. The sequence was submitted to Genbank (accession no. -OM939681).The sequence from clone was used as the template to predict protein sequence (Fig.5.3.B).

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A

ATG	CTT	CTG	GCC	ATT	CTT	GCC	CCC	CTC	CTC	GGG	GCC	GAG	GTT	CGA	CAG	TGC	GTT	TAC	TAC	GAA	AAC	GAC	AGC	TTT	ACT
M	L	L	A	I	L	A	P	L	L	G	A	E	V	R	Q	C	V	Y	Y	E	N	D	S	F	T
CTT	GTA	CCT	CTC	GAG	GAC	AAG	GGC	CAC	AAC	TGC	GTC	GCC	ACT	ACC	ATT	TCT	GAC	ATC	GAG	GTC	TTC	AAC	GAG	ACG	GGC
L	V	P	L	E	D	K	G	H	N	C	V	A	T	T	I	S	D	I	E	V	F	N	E	T	G
TGG	ATG	CGC	TTC	AAC	GTC	ACG	ACG	AGC	GCA	AGC	TAT	CCA	GAC	TTC	ATC	CAG	ATG	GCC	GCA	GTC	GGA	TTC	GCT	GAG	GGC
W	M	R	F	N	V	T	T	S	A	S	Y	P	D	F	I	Q	M	A	A	V	G	F	A	E	G
AAG	ATC	TTC	CAG	CAC	CAC	ATC	TTC	AAC	CAT	TAT	CAT	AAT	CTC	AAG	GAC	TGG	TTC	CTC	TTT	CGA	TAC	CTT	CAC	GCA	GAC
K	I	F	Q	H	H	I	F	N	H	Y	H	N	L	K	D	W	F	L	F	R	T	L	H	A	D
GAC	TAC	CCC	CAA	GAG	CTC	TAC	GAC	TTT	CTT	CAG	GAG	AAC	CTT	GAC	TGG	ACC	CGT	AGC	ACT	GCC	TAC	AAG	TAC	GCC	GAG
D	Y	P	Q	E	L	Y	D	F	L	Q	E	N	L	D	W	T	R	S	T	A	Y	K	Y	A	E
GTT	GAC	AGC	TTC	TGG	CGT	CAG	GTC	GCC	TAC	ACA	CTC	GAT	CAC	TTC	GAC	GGG	CTG	GTA	GAG	GCG	TAT	CGG	GCA	ACG	GCT
V	D	S	F	W	R	Q	V	A	Y	T	L	D	H	F	D	G	L	V	E	A	Y	R	A	T	A
CCG	CAG	GAG	GAG	ATG	CTT	TCA	GAG	CTG	GAC	CTC	TGG	ACG	TAC	CTA	TCG	AGC	GGC	GAT	CTT	CTC	GAT	ATC	GTA	AAC	TTT
P	Q	E	E	M	L	S	E	L	D	L	W	T	Y	L	S	S	G	D	L	L	D	I	V	N	F
GCA	GTG	CCA	TCC	ACA	CGG	ATG	GAC	TTC	TCA	AAG	ATG	ACG	CGC	GAG	GAA	ATC	TCC	GAC	TAT	CTC	GCT	TTT	AGA	AGC	CAT
A	V	P	S	T	R	M	D	F	S	K	M	T	R	E	E	I	S	D	Y	L	A	F	R	S	H
TGC	AGC	GGC	CTT	GTT	TAC	TAC	AAT	GGC	GAC	ACC	GTC	TAC	ATT	GGC	CAA	ACA	GCC	TGG	TTC	TTC	TAT	GGT	GGC	ATG	ACT
C	S	G	L	V	Y	Y	N	G	D	T	V	Y	I	G	Q	T	A	W	F	F	Y	G	G	M	T
CGT	GTC	TTC	AAG	GGA	TAC	ACT	CTC	AAC	CTT	AAC	GAT	GTG	TCT	AAT	GCA	GCC	GAA	ACG	TCC	ACG	TTT	AGC	TCA	TAC	CCA
R	V	F	K	G	Y	T	L	N	L	N	D	V	S	N	A	A	E	T	S	T	F	S	S	Y	P
GGC	TTC	TCC	TAC	TCA	TTT	GAC	GAC	TGG	ACA	GTG	ACC	TCG	CAA	GGA	CTT	GTT	ATC	ATT	GAA	ACG	ACA	GCC	AAC	GTT	CTA
G	F	S	Y	S	F	D	D	W	T	V	T	S	Q	G	L	V	I	I	E	T	T	A	N	V	L
AAC	ACC	AGC	CTG	TAT	GAT	AAC	TGC	ACG	CCT	AGG	TCT	GTT	CTC	ACG	TGG	ATT	CGC	TCC	CAG	GTG	GCC	TCC	CGG	ATG	GCA
N	T	S	L	Y	D	N	C	T	P	R	S	V	L	T	W	I	R	S	Q	V	A	S	R	M	A
GTC	CTA	GGC	GCC	GGC	TGG	CAT	CAA	GCC	AAC	AGT	CAG	TAC	AAC	TCT	GGA	ACG	TAT	AAC	AAC	CAG	TGG	ATT	GTC	GTC	GAC
V	L	G	A	G	W	H	Q	A	N	S	Q	Y	N	S	G	T	Y	N	N	Q	W	I	V	V	D
CTT	AAG	CAA	ATT	GAG	AGT	ATG	ACC	GTG	TAC	AAG	AAC	AAC	TTT	GTT	TGG	GTT	AGC	GAG	CAG	ATG	CCC	GGA	TAT	ATC	GTT
L	K	Q	I	E	S	M	T	V	Y	K	N	N	F	V	W	V	S	E	Q	M	P	G	Y	I	V
TCG	GAA	GAT	GTC	GCC	CAT	GAT	ATG	ACT	TAT	GGC	GGA	TAC	TAC	TTC	CCA	TCT	TAC	AAC	ACG	CCC	TTC	TTC	GAT	GAG	CTC
S	E	D	V	A	H	D	M	T	Y	G	G	Y	Y	F	P	S	Y	N	T	P	F	F	D	E	L
TGG	ATT	GTC	GCT	GGC	TAT	CCC	AGC	GTC	CCA	AAG	GGG	GAT	GCC	AGC	TAT	CGT	TAT	GAC	AAG	AAT	CCT	CGG	GGA	CTT	ATG
W	I	V	A	G	Y	P	S	V	P	K	G	D	A	S	Y	R	Y	D	K	N	P	R	G	L	M
TTT	GCA	GCC	AAT	GTT	CCA	AAA	ATT	ACT	GGC	TTC	GAC	GAA	TTG	AAA	AGG	GTC	ATG	CCC	TAT	AAA	AGG	TGG	CAA	AAA	GAT
F	A	A	N	V	P	K	I	T	G	F	D	E	L	K	R	V	M	P	Y	K	R	W	Q	K	D
CCG	TTT	GGC	ATT	TCC	GAG	GGC	CAA	AGG	GAA	CCC	AAC	CCG	GGA	AAC	GGG	ATT	CCT	GCC	AGG	TAC	AAT	TTG	CCC	CCT	AAT
P	F	G	I	S	E	G	Q	R	E	P	N	P	G	N	G	I	P	A	R	Y	N	L	P	P	N
GAA	TCC	CTC	CAT	AAG	GGT	TTC	CGA	GGT	TAC	GAG	ACA	AGA	ATT	CTT	AAT	AAT	AAT	GGC	CAA	AAC	AGG	AGA	ATG	TAT	CTC
E	S	L	H	K	G	F	R	G	Y	E	T	R	I	L	N	N	N	G	Q	N	R	R	M	Y	L
CTT	TTT	AAT	TTC	TGG	GCC	CCC	ACA	AAA	AAA	TCA	AAA	AAC	ATG	GGA	TTC	CTC	CAG	AGA	ACA	CCC	TCT	TTT	TTT	CCC	ATA
L	F	N	F	W	A	P	T	K	K	S	K	N	M	G	F	L	Q	R	T	P	S	F	F	P	I
AAG	GGA	TTA	AGG	AGG	AGC	CCC	CCC	AAG	ATA	GGT	GGG	AAA	ACA	CAC	TCC	CTC	TTT	GTG	TAA						
K	G	L	R	R	S	P	P	K	I	G	G	K	T	H	S	L	F	V							

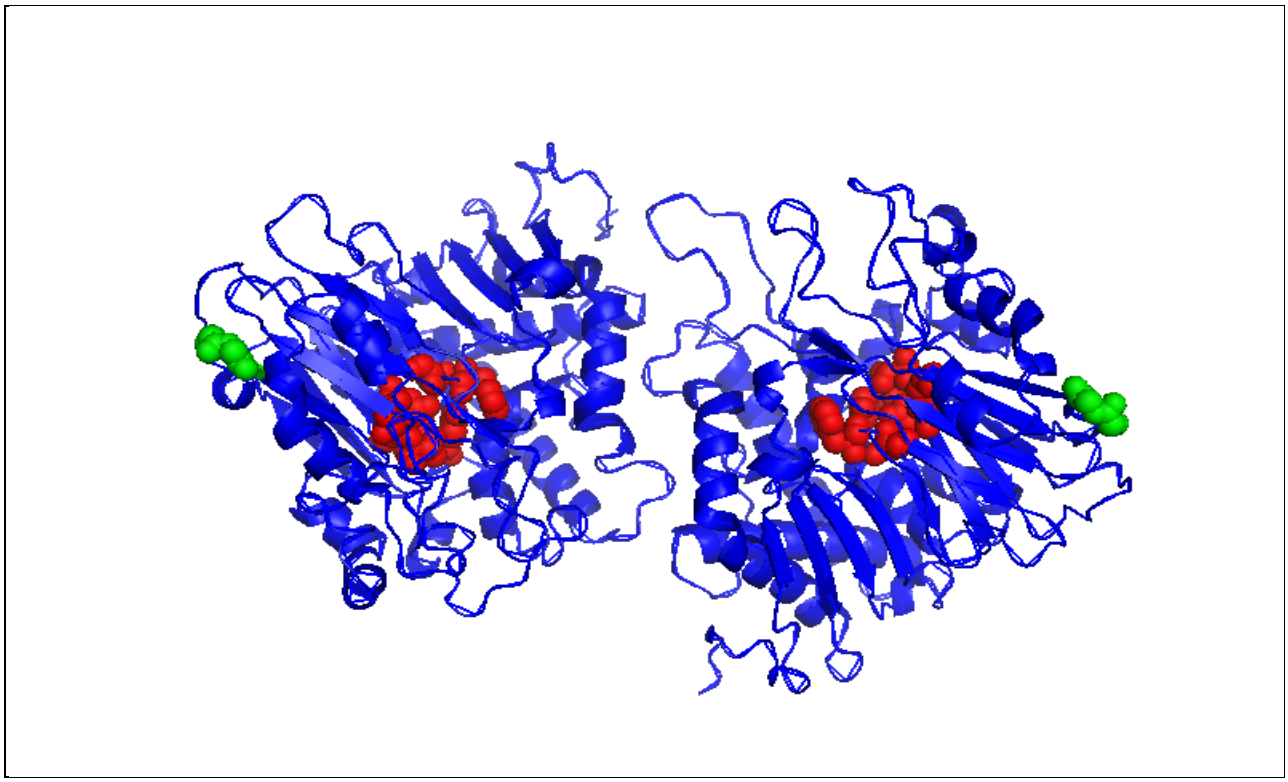
B

Fig.5.3. Sequence of gPLB. A. The gPLB DNA sequence derived from the cloned product of this enzyme. B. Using the derived sequence and expasy online tool protein sequence was constructed.

5.3.4. Homology modelling

The 3D homology model of *Giardia* PLB was constructed (Fig.5.4.A) using the SWISS-MODEL tool of Expasy. After its construction, the protein structure model was subjected to validation using the Verif3D tool. This tool employs a structural protein database to predict various stereochemical parameters of the protein structure, such as loops, sheets, alpha-helices, and the Ramachandran plot. The Ramachandran plot is used to predict the structural stereochemical properties of a protein. The results showed that our model had a Ramachandran favoured percentage of 88.08%, indicating that 88.08% of the model's residues were in the most favoured region of the Ramachandran plot(Fig. 5.5.B & 5.5.C). According to the secondary structural analysis, the protein consisted of 32% alpha helix and 22% β sheets (supplementary file 2). The ligand binding site was formed in cluster1 with 266 PHE, 268ASP, 280GLU, 281THR,282 THR, 330TYR,332ASN (Fig. 5.5.A) and in cluster 2 with residues 375 GLY,376GLY (Fig. 5.5.A). Based on the signal peptide prediction, it was determined that the protein existed in a dimeric form. Additionally, the initial sequence of the protein contained a putative signal peptide cleavage site after the first 15 residues. The protein had also 20 possible mutation sites (Fig.5.6). According to Prosite analysis, the desired protein had four full Protein kinase C phosphorylation sites (residue 186, 295,405,502) (Fig.5.7.A). In *Giardia* PLB, four specific N-myristoylation sites (at residues 147-152: GLveAY, 328-333: GTynNQ, 414-419: GLmfAA, and 445-450: GIseGQ) were identified (Fig.5.7.B).Myristoylation enhances localization and protein-protein interaction in subcellular locations, allowing it to interact with its signalling partner. The 539-amino acid sequence was found to potentially contain four N-Glycosylation sites located at residue numbers 22, 49, 57, and 468 (Fig. 5.8). ThePredicted amino acid sequence did not show the GxSxG sequence or GDSL motif present in rabbits, rats, and guinea pigs and *M. bovis and M.*

catarrhalis, respectively. Sequence analysis revealed that *Giardia* PLB was different from existing PLBs found in bacteria, and fungi, suggesting that it was a member of a new gene family of PLBs.The phylogenetic tree showed that PLBs and potential PLB analogues are contained in a large cluster of the PLB family. gPLBs is more closely related to *Acidobacterium capsulatum* PLB and *Dictyostelium* PLB than those of *E.coli*and *Candida*(Fig.5.9).



A

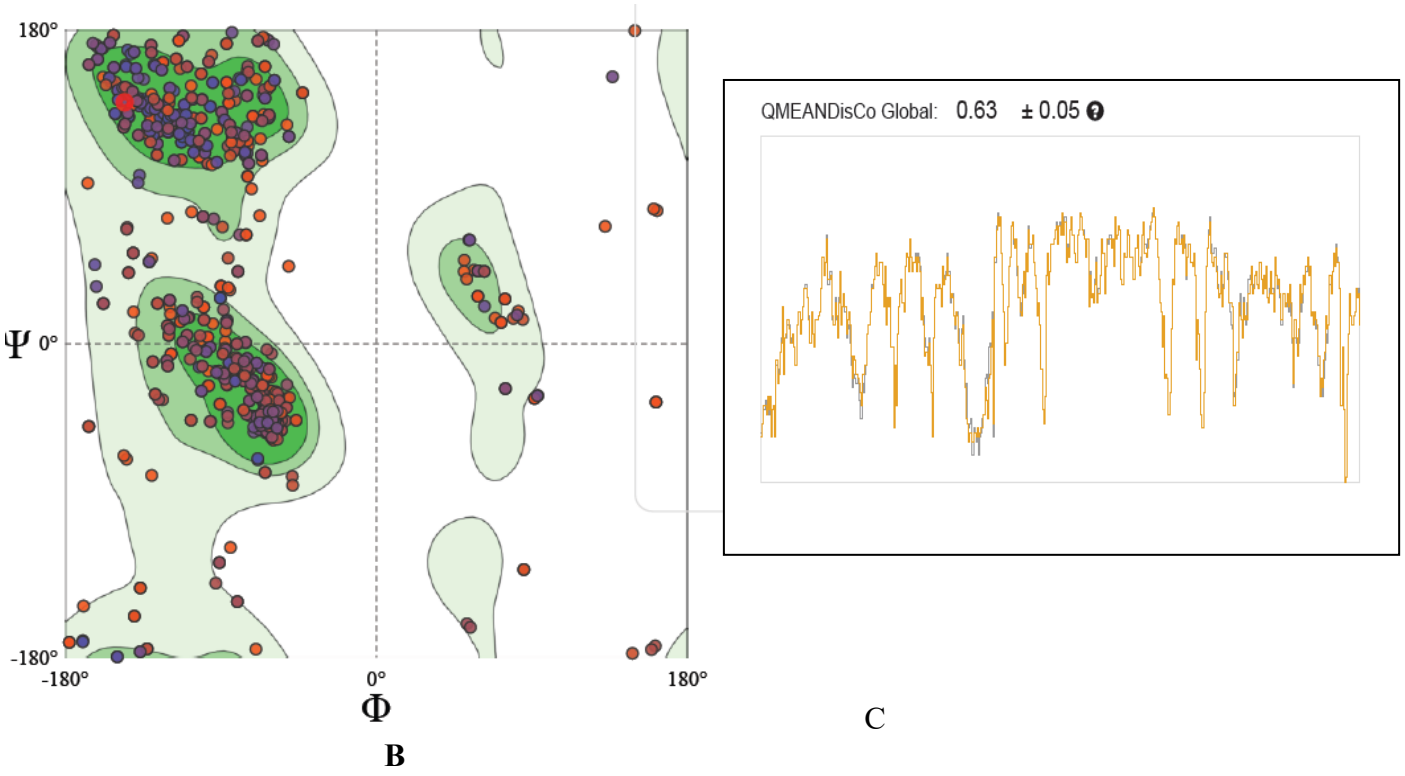


Fig.5.5.Predicted homology model of *Giardia lamblia* PLB.A.The protein existed in homodimeric form. The model was 88.08% Ramachandran favoured. Ligand binding site 1 in PLB was shown in red colored globular form. The figure shows the ligand binding site clustering in PLB comprising the residues 266 PHE, 268ASP, 280GLU, 281THR,282 THR, 330TYR, and 332ASN. Ligand binding site 2 in PLB was shown in green coloured globular form. The figure shows the ligand binding site clustering in PLB comprising the residues 375 GLY,376GLY. B-C. The results showed the analysis of constructed protein structure. The quality of the structure was assessed by ramachandran plot. It was constructed MolProbity version 4.4.

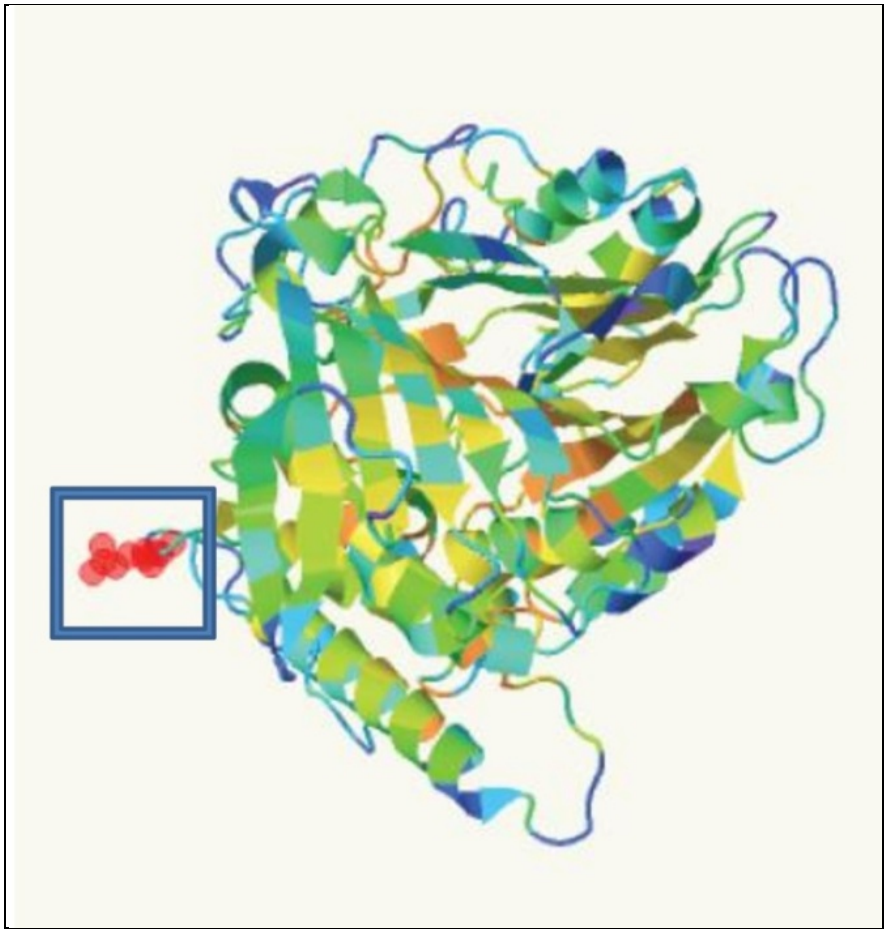
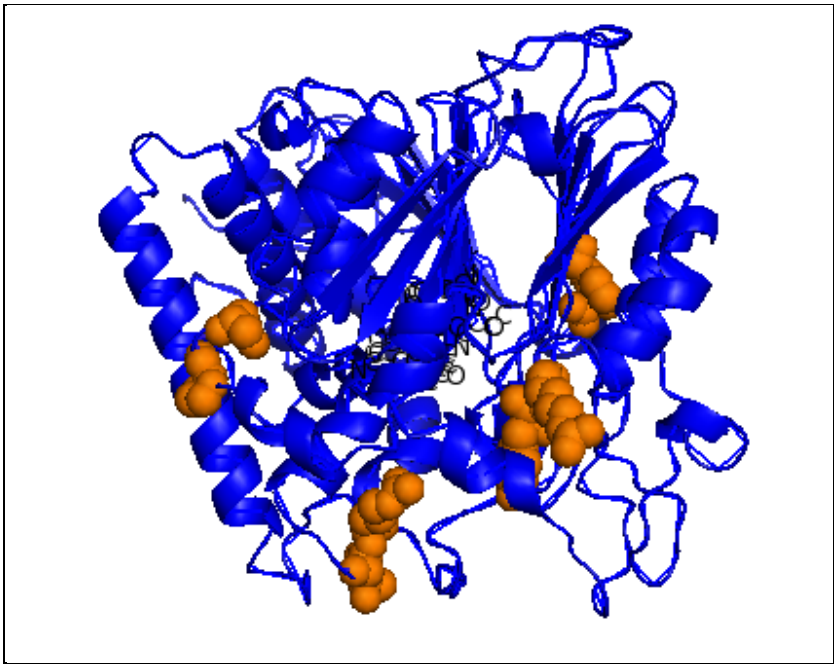


Fig.5.6.The tool SuSPect has been used to predict whether missense mutations in the sequence of the protein are likely to have afunctional/phenotypic effect. SuSPect uses sequence-, structure- and systems biology-based features as inputto a machine learning approach and shows balanced accuracy >80% on a large benchmark test set. Positions were coloured according to the average effect of the 20 possible mutations at that position.



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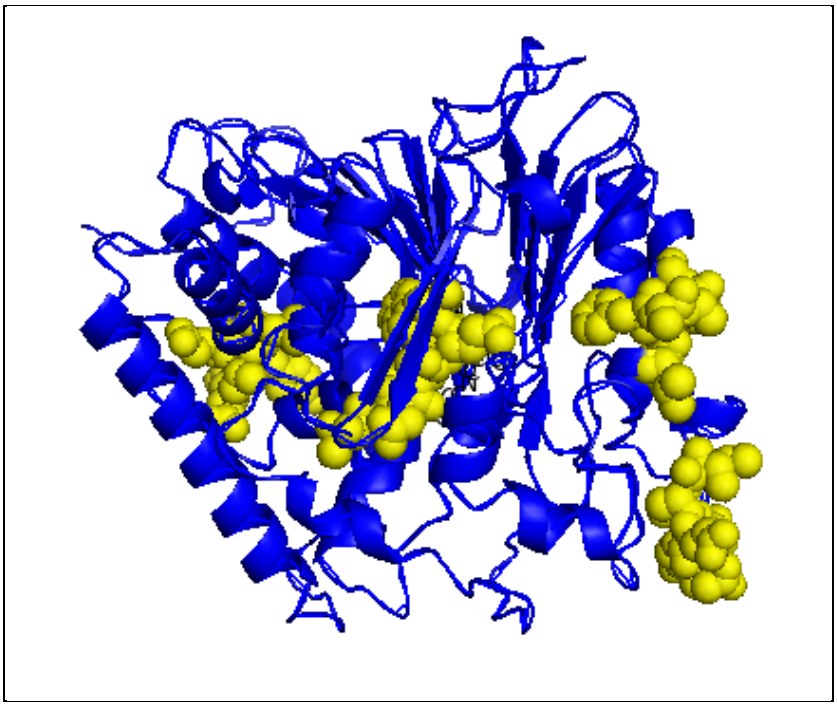
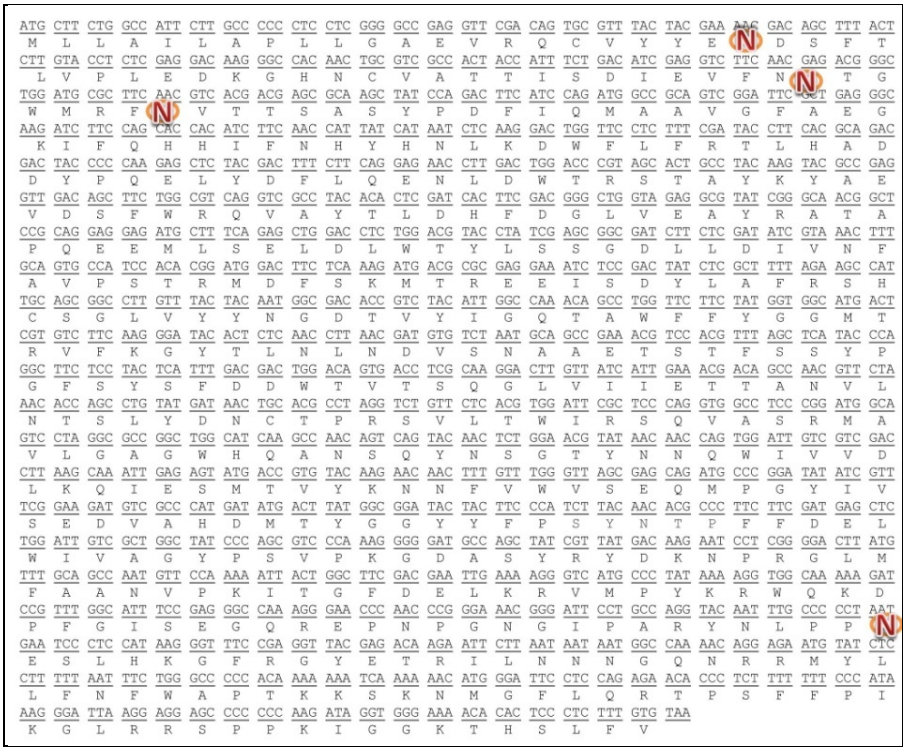
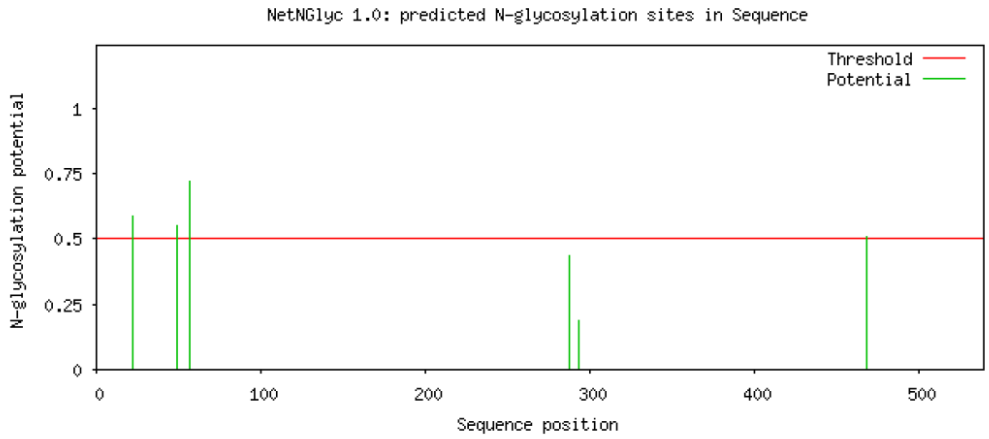


B

Fig.5.7. Presence of different sites in gPLB.A.ThegPLB has four protein kinase C phosphorylation sites. According to the Prosite tool the residue no. 186, 295,405,502 contains phosphoserin and phosphothreonine residues. The orange coloured spheres in the diagram, are the four sites. B. The diagram represented the four specific N-myristoylation sites at residues 147-152: GLveAY, 328-333: GTynNQ, 414-419: GLmfAA, and 445-450: GIseGQ in gPLB.Both the diagram was constructed using PyMol graphic system version 1.7.4.5.





4

SeqName	Position	Potential	Jury agreement	N-Glyc result
Sequence	22 NDSF	0.5881	(6/9)	+
Sequence	49 NETG	0.5509	(7/9)	+
Sequence	57 NVTT	0.7222	(9/9)	++
Sequence	287 NTSL	0.4337	(6/9)	-
Sequence	293 NCTP	0.1901	(9/9)	---
Sequence	468 NESL	0.5099	(6/9)	+

Fig. 5.8. Analysis of NetNGlyc tool showed that the protein had four potent N glycation sites which resided at residue no. 22, 49, 57, and 68

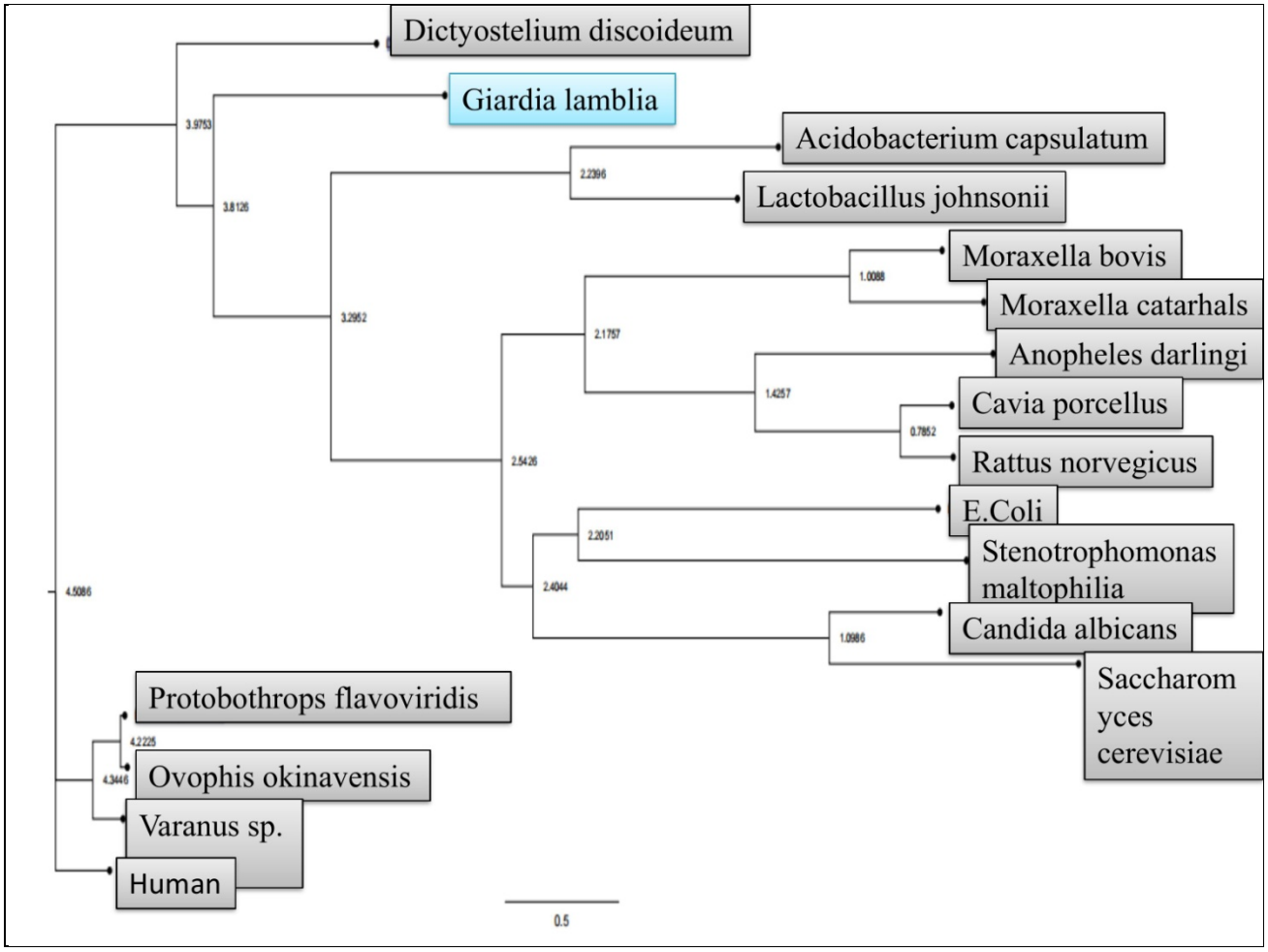


Fig.5.9. Phylogenetic tree of gPLB protein. The gPLBs is more closely related to Acidobacterium capsulatum PLB and Dictyostelium PLB than those of E.coliand Candida.

5.3.5.Lipase activity

The cloned pure protein was isolated and subjected to analyze lipase activity of it. The gPLB showed a potent lipase activity by converting p-nitrophenylpalmitate to p-nitrophenol. The enzyme showedactivity at pH ranging from 4.5 to 10.The enzyme exhibited the highest level of activity at a pH of 7.5, reaching a value of 0.32 nkat. The gPLB has a broad temperature range which lies between 25° C- 50°C but was highly active at 35°Cwith an activity of 0.259 nkat(Fig.5.9).

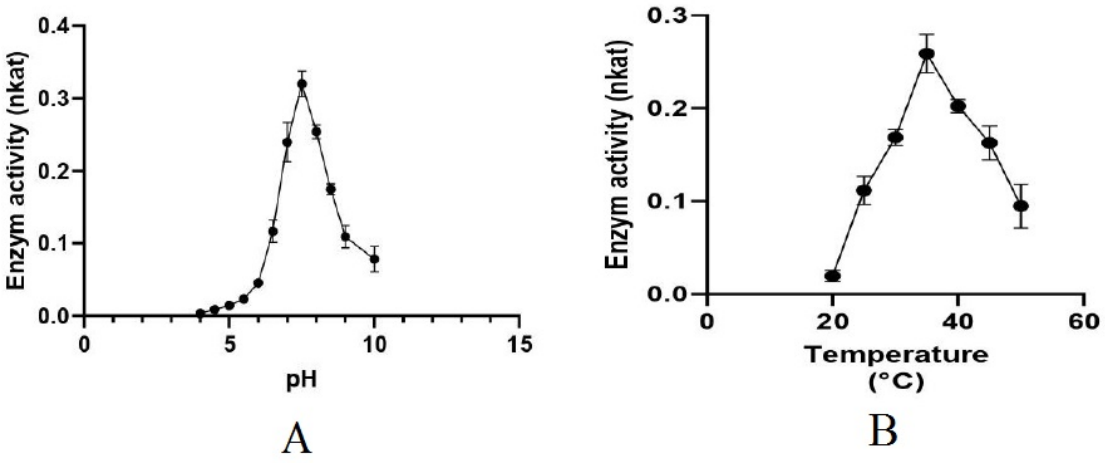


Fig. 5.9. The enzyme, PLB, exhibits activity across a wide range of pH and temperatures. A.The graph depicts the enzyme activity at various pH conditions, demonstrating that it remains active from a pH of 7.5 to as high as 10, with its highest activity observed at pH 7.5. B. The graph shows the PLB activity at different temperatures, indicating that the optimal temperature of the enzyme is 35.5°C, after which its activity declines gradually.

5.3.6. PLB protein expression under oxidative stress condition

The protein expressions of gPLB under oxidative stress conditions were illustrated in fig no.5.10. Repeated experiments showed essentially the same pattern of PLA2 expression. The gPLB expression under H2O2 induction showed an increased pattern. The expression was highest after 3 hours of incubation then it gradually decreased (Fig.5.10.A.). In case of MTZ induction the highest expression was seen after 6 hours of incubation (Fig.5.10.B.), which suggested that H2O2 was more potent in initiate intracellular signalling cascade for further downstream process. Previously, in fig. we also had seen that under oxidative stress the Ca^{2+} was accumulated increasingly after 2 hours of oxidative stress which then drastically decreased. This data suggested intracellular Ca^{2+}accumulation is responsible for gPLB activation.

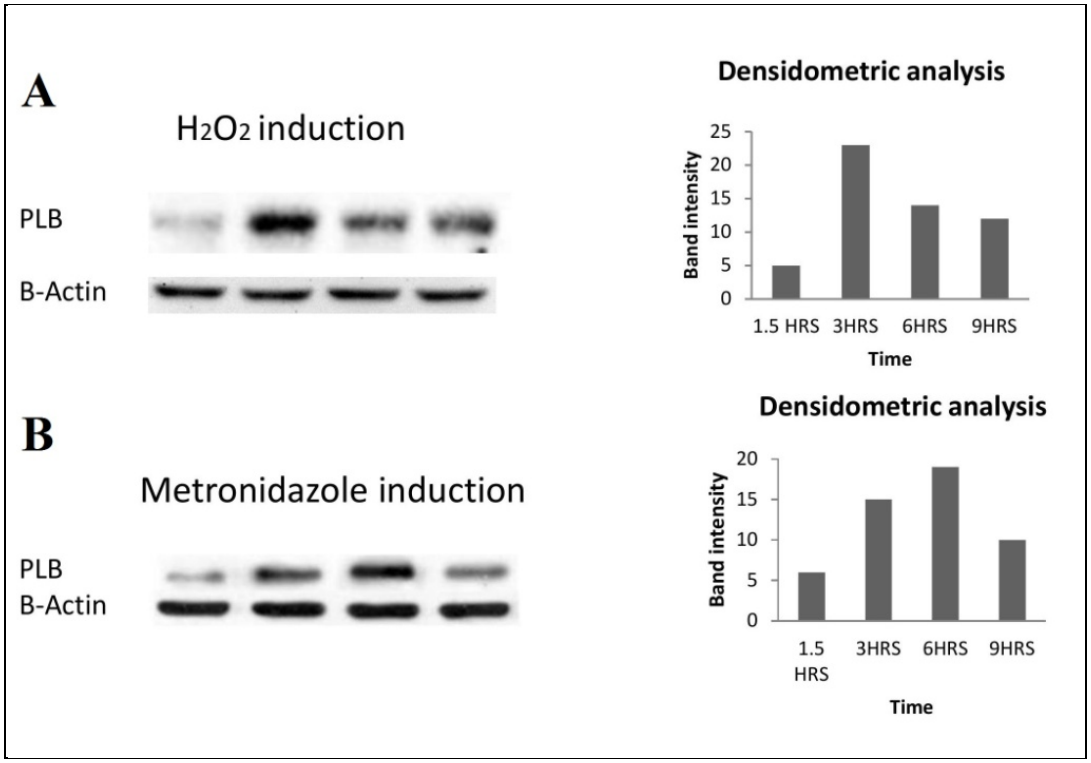
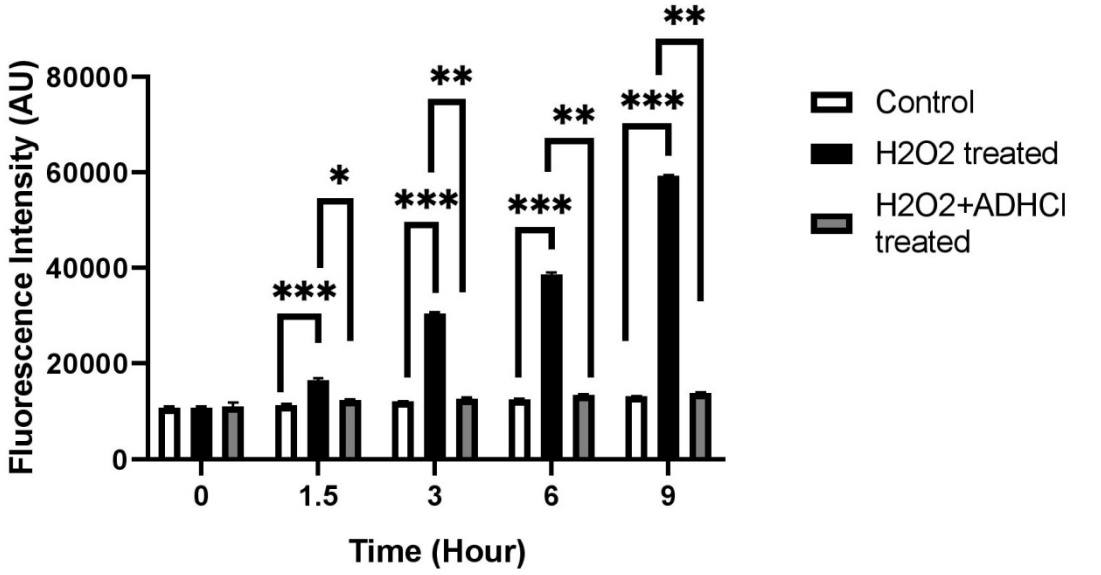


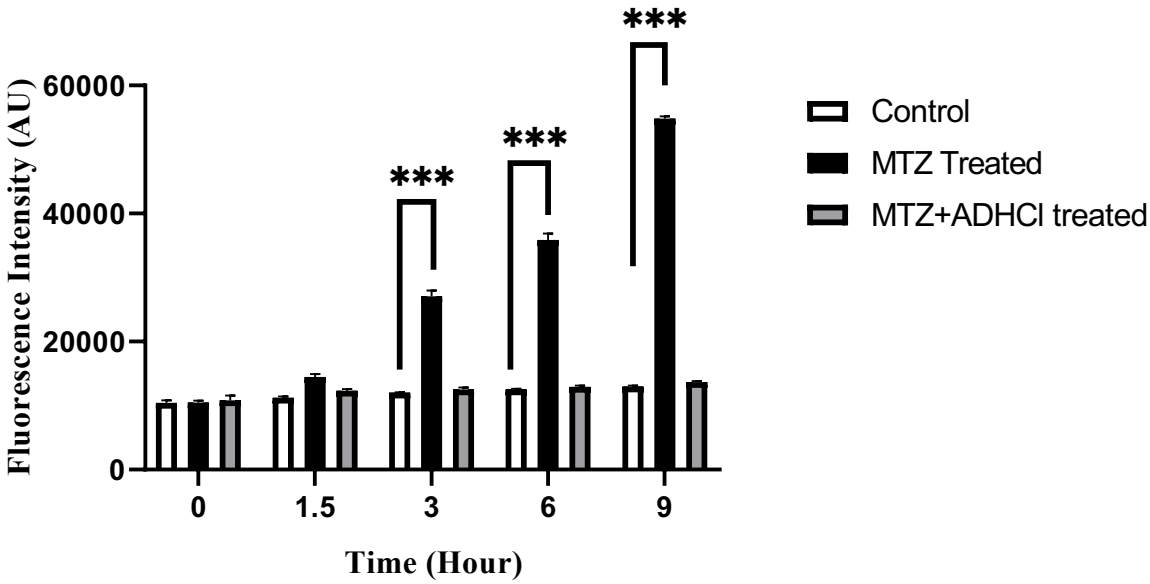
Fig.5.10. The expressions of gPLB under oxidative stress condition. A. The PLB expression was highest after 3 hours of incubation with H2O2 and then it gradually decreased. B. In case of MTZ induction the highest expression was seen after 6 hours of incubation.

5.3.7.Induced production of Arachidonic acid under oxidative stress

The fluorescence intensity resulting from the production of arachidonic acid in *Giardia*, when exposed to H2O2and MTZ, has been measuredspectroflourometrically. After three hours of incubation, the fluorescence intensity of the sample extracted from *Giardia* trophozoites under stress increased significantly compared to the control. Increased Fluorescence intensity suggests increasing production of arachidonic acid in the trophozoites. When the trophozoites were pre-treated with a specific PLB inhibitor, alexidine dihydrochloride (0.25 μM), the arachidonic acid production reduced significantly (Fig. 5.11.A and 5.11.B). Thus inducing expression of the PLB gene coupled with increased levels of arachidonic acid suggests the potential role of PLB in arachidonic acid formation under oxidative stress.



A



B

Fig.5.11. Measurement of Arachidonic acid (AA) production under oxidative stress condition. The concentration of arachidonic acid was measured by a spectrofluorometer at excitation wavelength 340nm and emission wavelength 390nm. The fluorescence intensity is proportional to the amount of AA in *Giardia* trophozoites. A.The graph shows that the fluorescence intensity increases with the duration of H2O2 stress, indicating an increase in AA production. Trophozoites pre-incubated with the PLB inhibitor, alexidine dihydrochloride (0.25µM) for 30 minutes, did not show a significant increase in AA production compared to treated trophozoites under H2O2 stress condition (***$P < 0.00001$). B. The data showed that MTZ induction increases intracellular free arachidonic acid production with time but the PLB blocker affected the arrachidonic acid production significantly.

PLB protein of *Giardia*upheldfour potent PKC phosphorylation site. In these four sites the protein can be phosphorylated by PKC and activated to produce AA. For this purpose we treated trophozoites with calphostinC, a potent PKC blocker, and prior to the oxidative stress induction. Then we measured intracellular free AA production in treated and untreated trophozoites. Our data indicated that without PKC blocker, calphostinC the AA production increased drastically over the time under oxidative stress. The calphostinC treated cells showed reduced level of intracellular free AA production (Fig.5.12.). This data suggested that PKC played important role in production of intracellular AA via gPLB activation as the inhibition of PKC ceased the AA production.

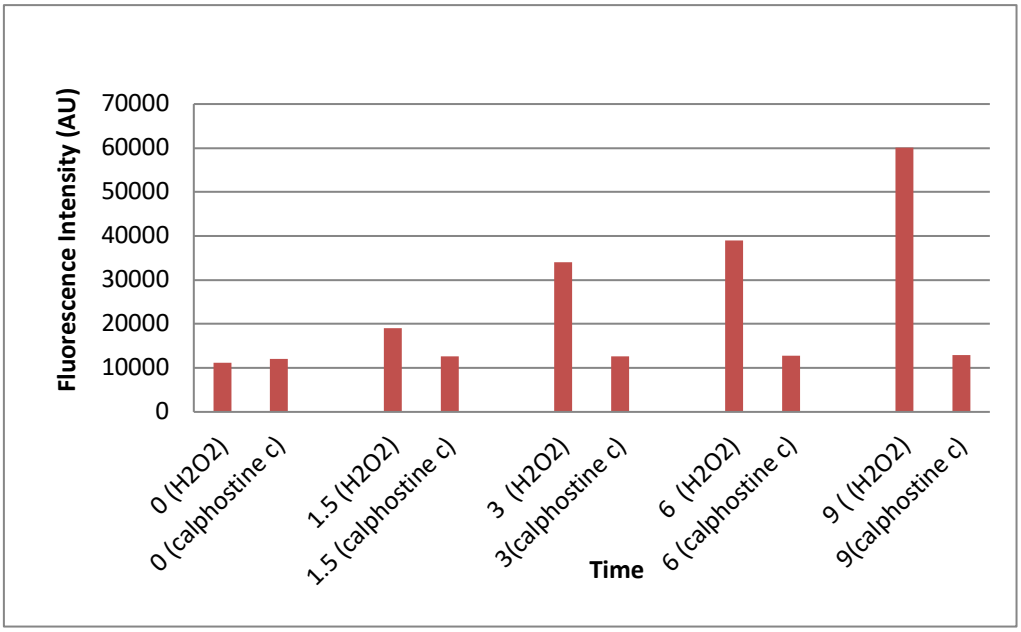
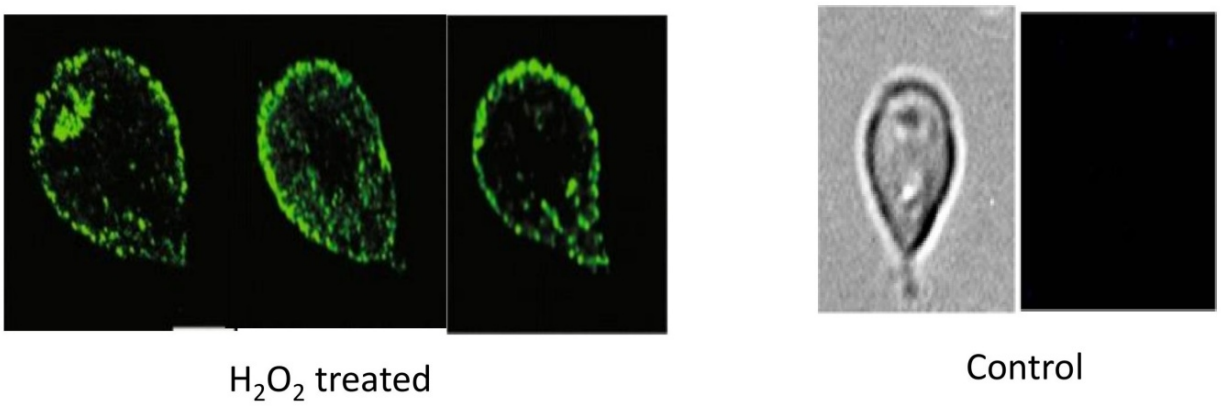


Fig.5.12. The graph represented the data showing PKC played important role in AA production. The free AA increased intracellularly over the time under oxidative stress condition. But after the inhibition of PKC by calphostinCthe AA production decreased. Thus it plays an important role in AA production by the activation of PLB.

5.3.8.gPLB localized to the periphery of the *Giardia* trophozoites

The green flurescence of the pictures are from the secondary antibody tagged with green fluorescently labelled probe. Initially the primary antibody (anti gPLB antibody) made attachment with the intracellular gPLB. Next the secondary antibody was attached with the previous complex. So, the fluorescence emmited from the place actually demonstrated the location of the gPLB. The data showed that after 3 hours of H2O2 and MTZ incubation the gPLB localized to the periphery of the *Giardia* trophozoite.



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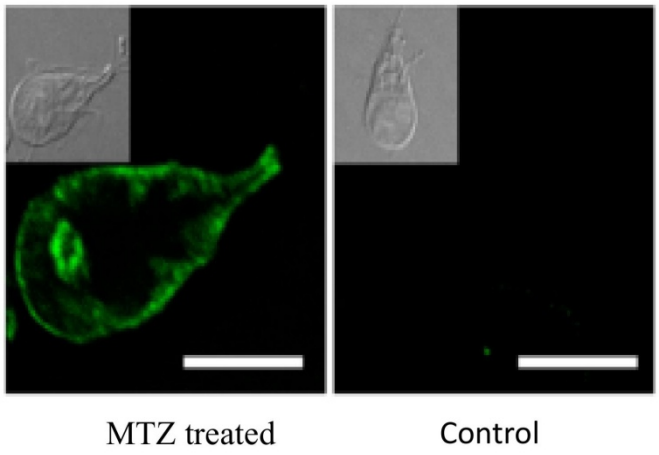


B

Fig.5.13. Localisation of intracellular gPLB under oxidative stress. A. The picture showed that the gPLB was localised to the periphery of the trophozoite after 3 hours H2O2 induction but control trophozoites did not showed any such localisation. B. MTZ induced cells also showed peripheral localisation of gPLB with compared to its control counterpart.

5.4. Discussion

Previous studies have reported a positive correlation between membrane phospholipid peroxidation and enhanced PLA2 activity in several organisms [12]. While we did not identify either PLA1 or PLA2 in *Giardia* trophozoites, we did find evidence of lipid peroxidation and arachidonic acid production *(Das et al., 2001)*. The release of free arachidonic acid from phospholipids is dependent on intracellular PLA2 activities. Phospholipase A enzymes are capable of cleaving the acyl ester bond at either the sn-1 (which is catalyzed by Phospholipase A1) or the sn-2 (catalyzedby Phospholipase A2) position.The Phospholipase B enzymes have three unique activities: a hydrolase that cleaves both the sn-1 and sn-2 fatty acid esters, a lysophospholipase, and a transacylase activity*(Balboa et al., 2006)*.We attempted to investigate whether PLB was responsible for the generation of arachidonic acid within *Giardia* trophozoites as it can hydrolyse the sn-2 position of phospholipid like PLA2 activity.

We observed a six-fold increase in the expression of PLB and lipid peroxidation levels in *Giardia* trophozoites following induction with H2O2.This increase in gPLB expression activated the prostaglandin E2 (PGE2) pathway and resulted in the formation of PGE2. The production of arachidonic acid also increased with the increased PLB expression level. According to the study by Soares et al. (2010),it was determined that alexidine dihydrochloride effectively inhibits PLB *(Soares et al., 2010)*. We utilized a blocker that resulted in a noteworthy decrease in the production of both arachidonic acid and PGE2 in our study.This indicates that when *Giardia* trophozoites are subjected to oxidative stress, they increase their production of phospholipase B (PLB), which can then lead to the production of arachidonic acid.

The PLB enzyme was successfully cloned and purified, with a molecular mass of approximately 56kDa. Bioinformatical analysis showed that the protein had four potential N-glycosylation sites, making it a glycoprotein. The PLBs characterized by other microbes also

have high molecular weight and are highly glycosylated *(Ichimasa et al., 1984; Kuwabara et al., 1988; Oishi et al., 1988)*. In the case of yeast, most PLBs are of high molecular weight and highly glycosylated to protect them against proteolytic degradation *(Maruyama et al., 1990;Fujino et al., 2006)*. Like yeast PLB, this specific protein also is glycosylated. It can be assumed that the modification is the safeguard from proteolytic cleavage. Further glycosylation studies may be needed to fully understand the role of glycosylation in the stability of the protein. The 'NCBI Conserved Protein Domain Search' tool analysis revealed that the protein sequence of gPLB has the capability to break down both acyl ester bonds in glycerophospholipids, indicating its potential role in lipid metabolism. The gPLB enzyme family is similar to the ones found in mammals, flies, and nematodes, but differs from the PLBs observed in yeast. Despite this difference, it is noteworthy that the glycosylation process of gPLB is similar to that of PLB present in yeast.In *Drosophila*, a similar protein known as LAMA was discovered and expressed in neuronal and glial precursors surrounding the lamina. The name "LAMA" was derived from the term "laminin ancestor." Unlike PLB found in bacteria, gPLB acts as a dimer (according to the signal peptide analysis of HHpred) *(Zimmermann et al., 2018)*. It is distinct from PLB gene families found in bacteria and fungi. The deduced amino acid sequence from the 1.6 Kbp nucleotide sequence suggests that the first 15 sequences are signal peptides, indicating that the protein is destined for secretion*(Waterhouse et al., 2018)*. Additionally, the protein contains potential protein kinase phosphorylation sites, suggesting that its activation is regulated by intracellular phosphorylation machinery. In *Giardia* PLB, four specific N-myristoylation sites (at residues 147-152: GLveAY, 328-333: GTynNQ, 414-419: GLmfAA, and 445-450: GIseGQ) were identified. Myristoylation enhances localization and protein-protein interaction in subcellular locations, allowing it to interact with its signalling partner *(Udenwobele et al., 2017)*. The protein has a robust lipase activity optimum at pH 7.5, which is distinct from fungal PLB,

which functions well in acidic pH (2.5-6) and at a temperature of 35°C, which is lower than that of *Streptomyces* sp. PLB (functioning at 50°C) *(England et al., 2005)*. These unique features of *Giardia* PLB make it active at a wide range of pH and temperature, making it a key molecule in initiating the prostaglandin pathway under oxidative stress conditions. The *Giardia* PLB protein is distinct from other reported bacterial and fungal PLBs due to its ability to maintain activity across a wide range of pH and temperature conditions. This unique PLB is upregulated when *Giardia* trophozoites experience oxidative stress and are unable to remove ROS. The upregulation of this protein suggests that it may play a crucial role in initiating the prostaglandin pathway during oxidative stress which is started by free intracellular AA production. Our data also suggested that protein kinaseC played role in AA production by phospholipase activation. Injuries to erythrocytes such as osmotic shock, oxidative stress, or energy depletion can induce the formation of prostaglandin E2, which then activates the Ca2+ permeable cation channel. The *Giardia* trophozoites bear similar consequence or not are subjected to further investigation.

CHAPTER 6

ARACHIDONIC ACID PRODUCTION AND ITS REGULATION UNDER OXIDATIVE STRESS CONDITION

6.1. Introduction

Due to the activity of acyltransferase and phospholipase A2 enzymes, the event of fatty acid incorporation and release occurs in membranes. The incorporation of fatty acid at the hydroxyl group onsn1 position of the glycerol usually has a fully saturated chain and the fatty acid attached atsn2 positionis mono- or polyunsaturated. The fatty acid e.g. arachidonic acid which is released from the membrane deacylationserves as a precursor of prostaglandin biosynthesis *(Das et al., 2001)*. This cyclical process is termed as Lands cycle. Esterified AA on the inner surface of the cell membrane is hydrolyzed to its free form by phospholipase A2 (PLA2)*(Wang et al., 2021)*. To controlthe accumulationtoxic LPLs and fatty acids it is necessary to properly regulate this cycle. This cycle is also considered as the major route for incorporation of AA as most PC and PE in specific tissues are diacyl species *(Sugiura et al., 1995)*, which contain large amounts of this FA at the Sn2 position *(Nakagawaet al., 1985;Yamashita et al., 1997)*. The cycle is also important for the release of free arachidonic acid (AA, a C20:4 unsaturated fatty acid) into cellular phospholipids which is dependent on intracellular PLA2 activities.

Usually, the reacylation pathway is dominant over the phospholipolytic step in un-induced cell keeping AA levels very low. However, when AA deacylation is dominant over AA reacylation in cells the accumulation of free AA occur (*Chilton et al., 1996*). Acyl coenzyme A (CoA) synthetase (ACS) enzymes catalyze the activation of free fatty acids (FAs) which participates lipid biosyntesis and reacylation process. Under stressed condition, Acyl CoA Synthetase downregulated and the reacylation process halts which increases Arachidonic acid accumulates intracellulerly. The free AA is an important bioactive molecule as it can be metabolized by three distinctenzyme systems – cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450(CYP) enzymes and generates a diverse spectrum of biologically active fatty acid mediators. The first reported enzymes for the metabolisation of

AA was COXs which can generate prostaglandins (PGs)and thromboxane A2 (TXA2). This requires the release of the lipid from theplasma membrane by phospholipases and subsequent metabolismby the COX enzymes.

6.2. Materials methods

6.2.1. Intracellular arachidonic acid measurement

The trophozoites were incubated with H2O2 (0.1 μM) and MTZ separately for time course analysis (0-9 hrs). Next, *Giardia* trophozoites ($4x10^7$ cells/ml) were harvested, washed, and homogenized in ice-cold PBS in a proportion of $4x10^6$ cells/ml. Homogenates were centrifuged at 10000g for 15mins, and the collected supernatant was mixed with 125μl of 20% trichloroacetic acid. The mixture was then centrifuged at 15000g for 10mins. The supernatant was collected for Fluorescence measurement using ex.340nm em.390nm. Arachidonic acid (10μl) dissolved in 1 ml methanol was used as the reference standard.

6.2.2. Isolation and PCR of genomic DNA of *Giardia lamblia*

Genomic DNA from *Giardia lamblia* Portland1 strain (ATCCR 30888TM) was isolated usingQIAamp® DNA Mini Kit (QIAGEN, USA). RNase digestion was performed for 30 mins at 37°C to remove RNA contamination. The primers were designed to amplify thecycloxygenase (COX) andacyl CoA Synthetase genes from the Portland1 strain of *Giardia lamblia*, using the sequences of thesegenes obtained from GiardiaDB as a reference. PCRamplification was carried out using Expand Long Template PCR system by Roche (1681834), following PCR conditions - Initial denaturation - 94°c 5mins, denaturation - 94°c 30sec., annealing - 55°c 1.5 mins., elongation - 72°c 3mins, final extension - 72°c 7mins. For the purpose of amplification the following primers were used –

Gene	Primers
Cyclooxygenase	Forward - 5'GCAATTGGCGATTCAAGTAGG 3'
	Reverse – 5' TCCAGGAATCTCAGGATCGTC 3'
Acyl CoA Synthetase	Forward - 5' GAACCCATTCGAAGGCATCC 3'
	Reverse – 5' CGCATCCTCAAAGTCGTCTG 3'

6.2.3. Sequencing and Sequence alignment

Amplicon of specific band size was extracted from horizontal agarose gel and processed using manufacturer's protocol (Gel extraction kit Roche). Next sequencing was performed bidirectionally (using Thermo Fisher Scientific - BigDye Terminator v3.1 cycle sequencing kit) in ABI PRISM^{R}3100 Genetic Analyzer(Applied BioSystems, Waltham, Massachusetts, USA) sequencer. The sequence was aligned with ClustalW (NCBI online) method, and similarity was assessed by BLASTN and BLASTP programs.

6.2.4. RNA isolation cDNA formation and Real-time PCR

The trophozoites, treated with H2O2, MTZ and untreated, were collected and their total RNA was extracted using the TRIZOL (Invitrogen) method according to the protocol described by Raj et al. 2013*(Raj et al., 2014)*. To analyse the role ofcycloxygenase (COX) andacyl CoA Synthetase in AA metabolism in *Giardia,* the expression level of both the gene under oxidative stress were checked. PLB is the first critical enzyme responsible for converting membrane lipids into AA. Next this AA is metabolized by different enzymes and participates in Land's cycle for the reacylation/deacylation of membrane and removal of free AA. To identify the regulation of AA under oxidative stress condition we had performed a real-time PCR assay to study the gene expression of both COX andacyl CoA synthetase using forward and reverse primers for both the genes. Here, the beta-actin gene was used as control gene F-

5’ ACATATGAGCTGCCAGATGG3’ and R- 5’ TCGGGGAGGCCTGCAAAC3’. The experiment was conducted three times with triplicate samples, utilizing the Roche RT-PCR system. The RT-PCR was carried out with the following conditions: denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

6.2.5. SDS-PAGE

SDS-PAGE is the most commonly used gel electrophoretic system for analyzing proteins. This method is based on the separation of proteins according to size and can also be used to determine the relative molecular mass of proteins. SDS is an anionic detergent which binds strongly to and denatures proteins to produce linear polypeptide chains. On average one SDS molecule will be present for every two aminoacids. The presence of β-mercaptoethanol assists in protein denaturation by reducing all disulfide bonds. The detergent binds to the hydrophobic region of the denatured protein in a constant ratio of about 1.4g of SDS/gm of protein. The protein-SDS complex carries net negative charge, hence move towards the anode and the separation is based on the size of the proteins. SDS-PAGE gels were cast with a molar ratio of Bisacrylamide:Acrylamide of 1:29 which had been shown empirically to be capable of resolving polypeptides that differ in size by little as 3%. The Polyacrylamide gel was cast as a separating gelbuffered with Tris-cl buffer at pH 6.8 and topped stacking gel with high percentage of acrylamide and was cast in Tris-cl buffer at pH 8.8. Protein resolved in the gel was stained with Coomaassie brilliant blue. Coomassie blue are electrostatically attracted to charged groups on the protein, forming strong dye:protein complexes that are further augmented by vanderwaals forces, hydrogen bonding and hydrophobic bonding. Next the proteins were transferred to the nitrocellulose membrane. The membrane was overlaid with a primary antibody for a specific COX andacyl CoA synthetase and then with a labelled secondary antibody.

6.2.6. 3D Homology modelling using predicted amino acid sequence

SWISS Model online modelling servers were used to generate the homology model of the prostaglandin synthase protein. Verif3D software programs were used to validate the generated model; the fittest model was selected from the analysis of this software. The secondary structure was analyzed by the Phyre2 tool of the 3DLigandsite website. Further, the motifs on the protein were predicted by the Prosite tool of Expasy and molecular weight was deduced using ProtParam.

6.2.7. ProstaglandinE2 measurement by Enzyme-Linked Immunosorbent Assay

Prostaglandin concentration was measured using ab 133021- Prostaglandin E2 ELISA kit according to the manufacturer's protocol. Treated and untreated cells ($4x10^6$ cells/ml) were harvested and washed with ice-cold PBS and centrifuged for 5 mins at 2000rpm at 4°c. Supernatants were collected and added to the 96-well plate for prostaglandin measurement. Standards and samples were added to the respective well. AP-conjugate and PGE2 antibodies were added to the appropriate wells and incubated at room temperature on a plate shaker for 2 hours at 500 rpm. The content of the plate was discarded and washed with wash buffer twice. Then, we added pNpp substrate to each well and incubated it at room temperature for 45 minutes without shaking. To stop the reaction, the stop solution was added, and the plate was read immediately at 570nm. Initially, a standard curve was drawn using standard prostaglandin supplied with the kit. Then keeping the standard curve reference PGE2 in stressed condition was measured.

6.2.8. Statistical Analysis

All the experiments were performed thrice in triplicates and the results were expressed as mean±standard error of the mean (SEM). The analysis was evaluated by t-test or 1-way ANOVA (wherever applicable). <0.05 P value was considered statistically significant.

6.3. Results

6.3.1. Oxidative stress increased the concentration of intracellular free arachidonic acid

The fluorescence intensity resulting from the production of arachidonic acid in *Giardia*, when exposed to H2O2and MTZ, has been measuredspectroflourometrically. After three hours of incubation, the fluorescence intensity of the sample extracted from *Giardia* trophozoites under stress increased significantly compared to the control (Fig.6.1.). Increased Fluorescence intensity suggests increasing production of arachidonic acid in the trophozoites.

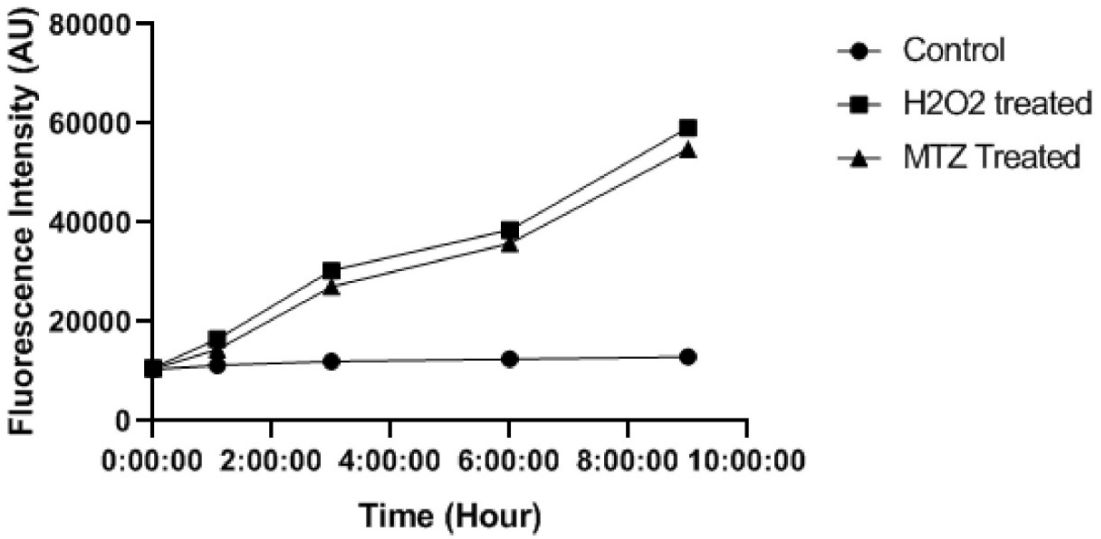


Fig.6.1.Intracellular free Arachidonic acid (AA) production under oxidative stress condition. The concentration of arachidonic acid was measured by a spectrofluorometer at excitation wavelength 340nm and emission wavelength 390nm. The fluorescence intensity is proportional to the amount of free AA in *Giardia* trophozoites. The graph shows that the fluorescence intensity increases with the duration of H2O2and MTZ stress, indicating an increase in AA production.

6.3.2.Presence of Acyl CoA Synthetase in *Giardia* trophozoites

Acyl coenzyme A (CoA) synthetase (ACS) enzymes catalyze the activation of free fatty acids (FAs) which participates lipid biosyntesis and reacylation process. Under stressed condition Acyl CoA Synthetase downregulated and the reacylation process halts which increases intracellular Arachidonic acid accumulation. Hence, this enzyme is important for proper removal of toxic free AA. To investigate the presence of this gene in *Giardia* Portland1 gene it was PCR amplified using the above mentioned primers constructed from the reference stain GL50803_0016667 (Fig.6.2.). The primers amplified nearly 2500 bp fragment which showed 95% sequence similarity.

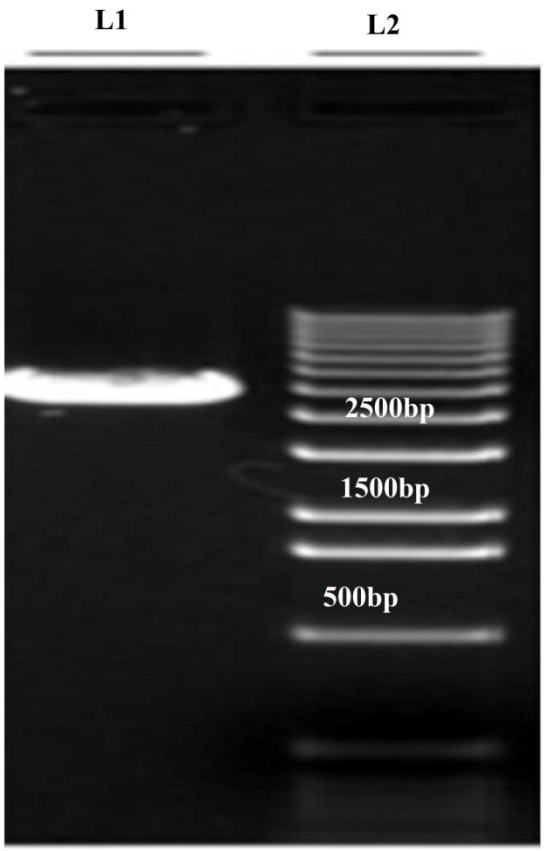


Fig.6.2.PCR amplified *Giardia* DNA using Acyl COA synthetase specific forward and reverse primer. Lane no 1 (L1) was the specific amplified product of Acyl COA synthetase gene and lane 2 was the marker. The primers amplified nearly 2500bp fragment which showed 95% similarity with the specific *Giardia* gene.

6.3.3. Oxidative stress decreases the expression of Acyl COA synthetase

Acyl coenzyme A (CoA) synthetase (ACS) enzymes normally catalyze the activation of free fatty acids (FAs) to CoA esters by a two-step thioesterification reaction and these activated FAs participate in different anabolic and catabolic metabolic pathways, like *de novo* complex lipid biosynthesis, FA β-oxidation, or lipid membrane remodeling. In controlled uninduced cells the ACS expression is high which triggers the free fatty acids to reacylate in cell membrane. In case of *Giardia*trophozoites in normal uninduced cells showed similar positive level of ACS expression over the time (Fig.6.3.A.). In normal cells the free AA levels were also low (Fig.6.3.B.). This indicated the ACS had specific roles in prevention of the free FAs like AA deposition in trophozoites. Our data showed that the oxidative stress (H2O2 and MTZ induction) triggered the down regulation of ACS expression (Fig.6.4.A & 6.4.B) which leaded to the accumulation of free AA in the trophozoites.

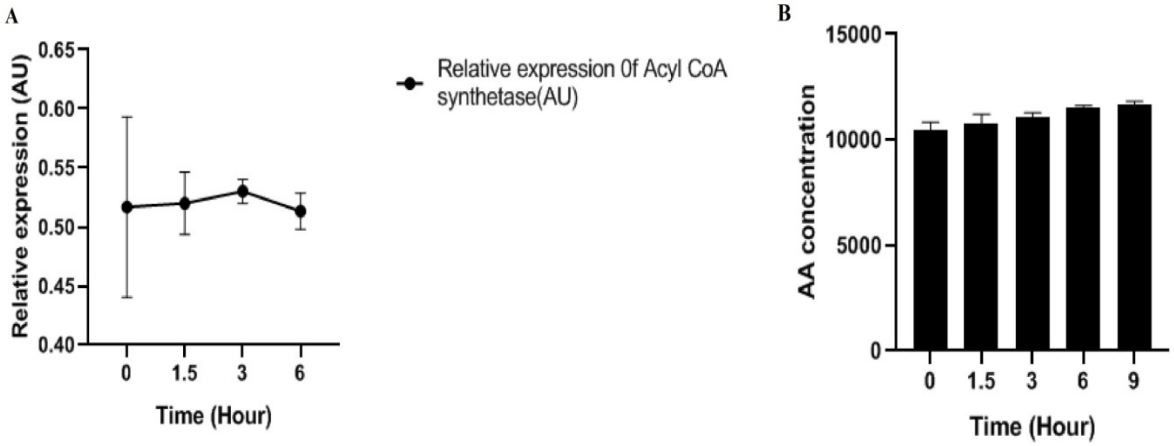


Fig.6.3. A. The relative expressions of ACS were similarly high in the un induced controlled cells over the time. B. The graph showed the AA concentration is low over the time in controlled cells. Both the graph correlate the fact that high expression level of ACS prevents the free AA accumulation in trophozoites.

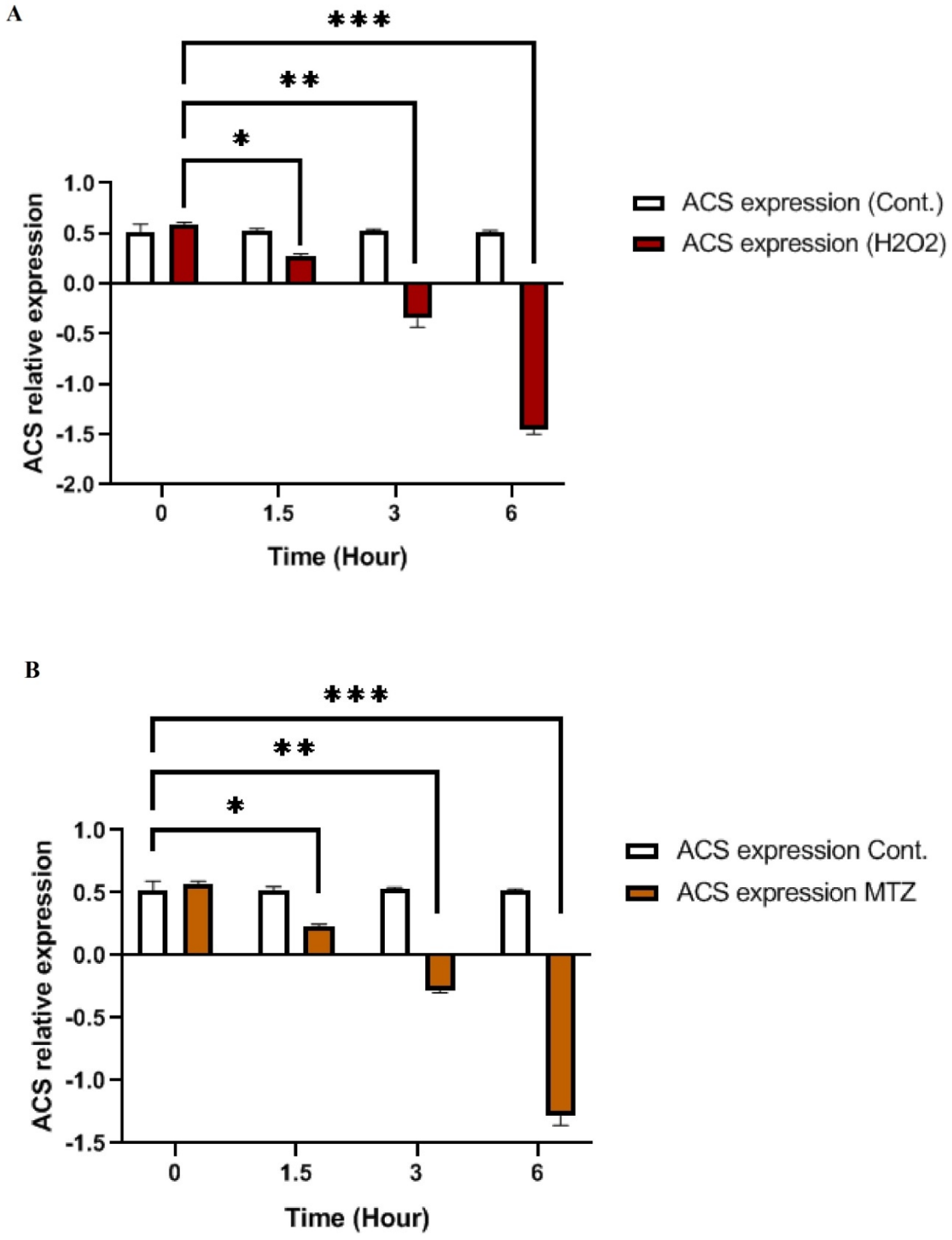


Fig.6.4.A.ACS expression under oxidative stress condition (H2O2 and MTZ). A. The graph represented the ACS expression level under H2O2induction. At initial phase at 0 hours of H2O2administration the ACS expression was similar to the ACS expression of controlled un induced cells. From 1.5 hours the ACS expression level started to down regulate in H2O2treated trophozoites with respect to the untreated one. After 6 hours of induction the down regulation of ACS reached at its lowest level. The down regulation of the enzyme is

significant with the controlled cells (*P=0.000361, **P=0.000063, ***P<0.000001). B. The administration of MTZ showed significant decrease in ACS expression compared to its untreated counterpart (*P=0.000084, **P<0.000001, ***P=0.000002).

6.3.4. Oxidative stressaffects Acyl COA synthetasepreotein expression

In controlled un induced cells the produced free fatty acids are activated and reacylated to the membrane by ACS enzyme. For this reason the enzyme expression is relatively high in these cells. In case of oxidative stress induced cells, the expression of the protein is down regulated. Thus the activation of free fatty acid halts and ceases the re acylation process of membrane. In our study we observed that in oxidative stress induced cells the ACS protein expression decreased with progression of time. The protein expression of the trophozoites after 9 hours of H2O2 induction was lower than the trophozoites after 1.5 hours after H2O2 induction (Fig.6.5.A). The MTZ treatment showed similar kind of result e,g; the higher expression at lower incubation time and lower expression at higher incubation time (Fig.6.5.B). The result significantly stated that oxidative stress has a role in the down regulation ACS protein expression which intern ceased the membrane reacylation and directed to the prostaglandine formation.

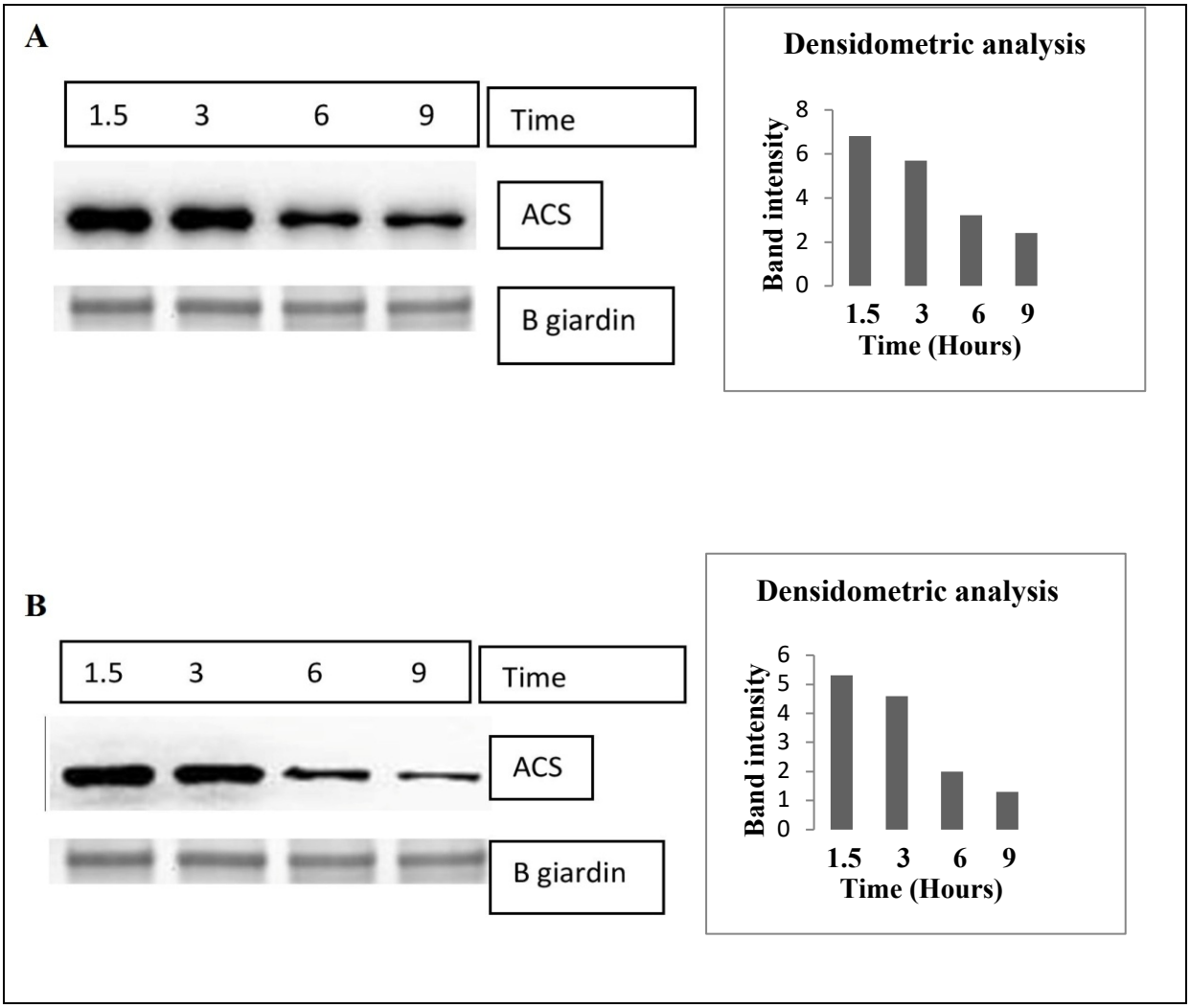


Fig.6.5. ACS protein expression under oxidative stress. A. The expression level of ACS protein under H2O2stress condition. The expression was highest after 1.5 hours of the H2O2induction and lowest after 9 hours of induction. B. The figure showed the expression level of ACS under MTZ induction. The expression was highest after 1.5 hours of induction and gradually decreased over time. The expression was lowest after 9 hours of induction.

6.3.5. Presence of prostaglandin specific synthase in *Giardia* trophozoites

Phospholipases (PLAs) like PLB in *Giardia* releases a 20-carbon unsaturated fatty acid, arachidonic acid (AA). Then it metabolized by the sequential actions of prostaglandin G/H synthase, or cyclooxygenase (COX). It is a bifunctional enzyme containing both peroxidise

and cyclooxygenase activity which exist as distinct isoforms. As oxidative stress increased free AA in *Giardia* trophozoites by decreasing its reacylation process, it was directed to metabolize prostanoid. For the production of prostanoid it is necessary to have specific synthase. To investigate the presence of this specific synthase in *Giardia* Portland1 it was PCR amplified using the above mentioned primers constructed from the reference stain GSB_14521. In gel electrophoresis the fragment were visible near 410 bp. Lane M showed the DNA marker and 1 and 2 showed the amplicon amplified from *Giardia* genome (Fig.6.6).

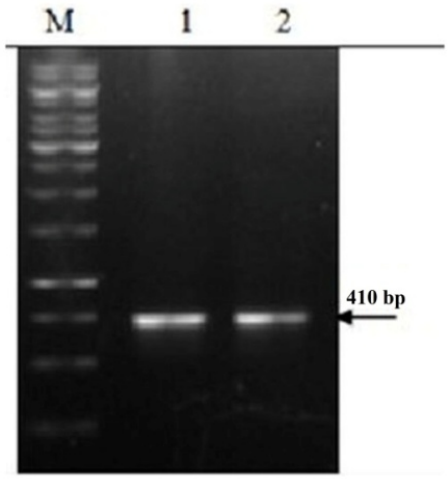


Fig.6.6. PCR amplified *Giardia* DNA using prostaglandinespecific synthase forward and reverse primer. Lane no 1 and 2 were the specific amplified product of synthetase gene and lane M was the marker. The primers amplified nearly 410bp from *Giardia* DNA template.

DNA sequence of prostaglandin synthase

ATGGAAAAATGCAGAAAGCCAGATATAAATTGGGCACAGGGGCATACGCATTTATTCCTTTCTTACGTTTTACCAAGTCTTG
AAAAGGAAACAGCAAATATTCAGGTAACAAGCACTTCTATCCACTTTACTGCAATTGGCGATTCAAGTAGGGATAGATTTGA
TTTTGTTTTGGATTTATACGACGAAATTGATTCAGATAATTATAAAATGAATGTTACAGCACGTGGAGTTTCTTTGTTTATAAT
GAAAGAAGAAAAATGGTGGCCAAGATTGAATAAAGAAGGAAAATTAAATAATATTAAAATTGATTGGGATAAATGGCGTGA
AGAAGACGATCCTGAGATTCCTGGAGTTGATTTTCCATTTGAAGTTCCTGACCCGGAGGATCCTGAAAGTGATGATAATTGA

6.3.6. Prostaglandin specific synthase protein structure of *Giardia*

Using the derived nucleotide sequence of the specific protein the amino acid sequence was predicted for prostaglandin specific synthase of *Giardia* (Fig.6.7.) in SWISS-MODEL tool of ExPasy. The 3D homology model of *Giardia*prostaglandin specific synthase protein was constructed (Fig.6.8.) using the SWISS-MODEL tool of Expasy. After its construction, the protein structure model was subjected to validation using the Verif3D tool. This tool employs a structural protein database to predict various stereochemical parameters of the protein structure, such as loops, sheets, alpha-helices, and the Ramachandran plot. The Ramachandran plot is used to predict the structural stereochemical properties of a protein. The results showed that our model had a Ramachandran favoured percentage of 83.9%, indicating that 83.9% of the model's residues were in the most favoured region of the Ramachandran plot.

Predicted protein sequence

atg gaa aaa tgc aga aag cca gat ata aat tgg gca cag ggg cat acg cat tta ttc ctt tct tac gtt tta cca agt ctt gaa
M E K C R K P D I N W A Q G H T H L F L S Y V L P S L E
aag gaa aca gca aat att cag gta aca agc act tct atc cac ttt act gca att ggc gat tca agt agg gat aga ttt gat
K E T A N I Q V T S T S I H F T A I G D S S R D R F D
ttt gtt ttg gat tta tac gac gaa att gat tca gat aat tat aaa atg aat gtt aca gca cgt gga gtt tct ttg ttt ata atg aaa
F V L D L Y D E I D S D N Y K M N V T A R G V S L F I L M
gaa gaa aaa tgg tgg cca aga ttg aat aaa gaa gga aaa tta aat aat att aaa att gat tgg gat aaa tgg cgt gaa gaa
K E E K W W P R N K E G K L N N I K I D W D K W R E E
gac gat cct gag att cct gga gtt gat ttt cca ttt gaa gtt cct gac ccg gag gat cct gaa agt gat gat aat tga
D D P E I P G V D F P F E V P D P E D P E S D D N -

Fig.6.7. The figure depicted the predicted amino acid sequence which were aligned according to the nucleotide sequence.

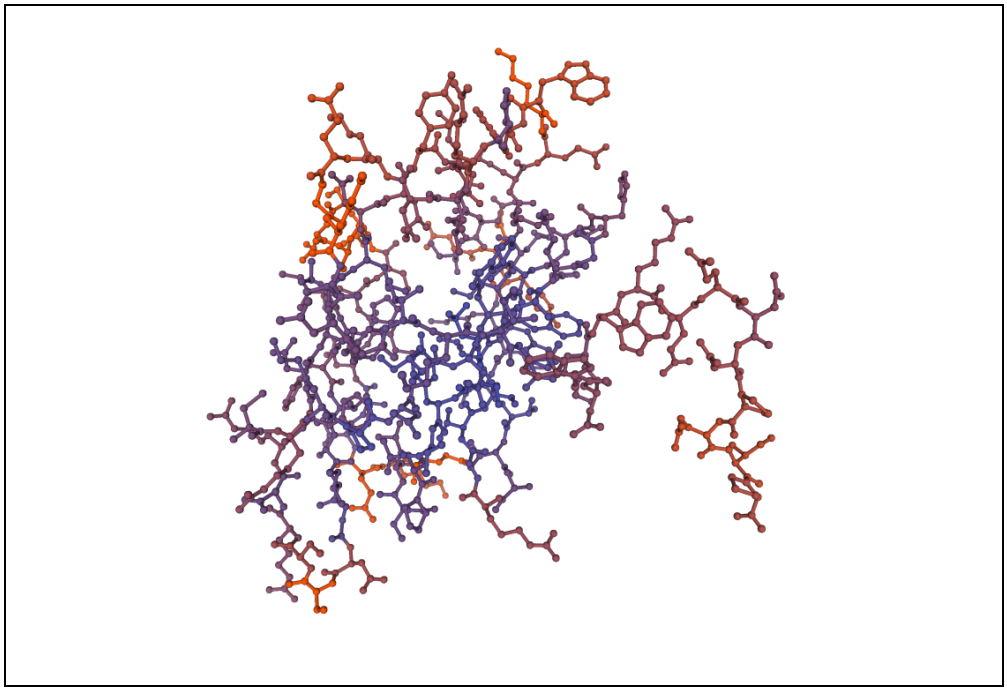


Fig.6.8.Predicted homology model of *Giardia lamblia* prostaglandin specific synthase protein. The model was 83.9% Ramachandran favoured.

6.3.7. Increased AA triggers the up regulation of prostaglandine specific synthase

Prostaglandinsynthase, is responsible for producing prostanoids, such as thromboxaneand prostaglandins, from arachidonic acid. It belongs to a family of isozymes which can convert arachidonic acid to prostaglandin through a short-living prostaglandin G2 intermediate. In our study we discovered with increasing time of oxidative stress the free AA level increased in the trophozoites and with time the mRNA expression level for prostaglandinsynthase was also increased (Fig.6.9.A. &6.9.B.). Extracellularly added AA also increased the expression level of this enzyme (Fig.6.10.). We gradually added increased amount of free AA gradually from 100 µM to 400 µM in *Giardia* trophozoites and measured the expression level of prostaglandin synthase after 3 hours of incubation. The result showed the gradual increase of expression with the increased AA.

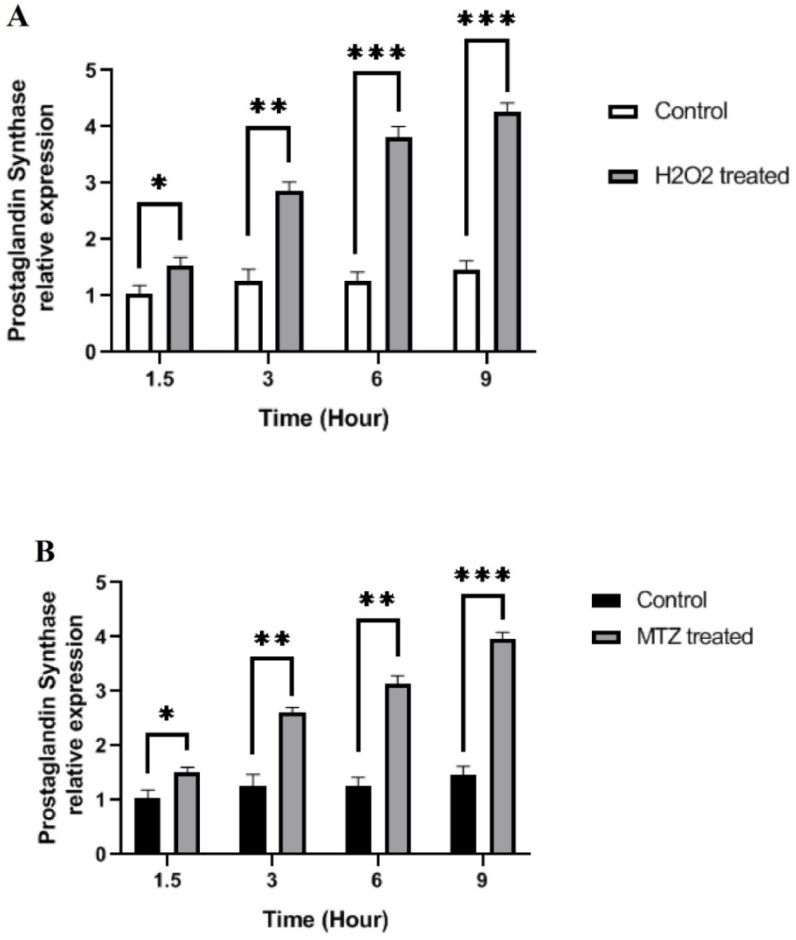


Fig.6.9. Prostaglandin synthase expression under oxidative stress condition (H2O2 and MTZ). A. The graph represented the prostaglandine synthase expression level under H2O2induction. From 1.5 hours the enzyme expression level started to up regulate in H2O2treated trophozoites with respect to the untreated one. After 3 hours of incubation when intracellular AA level is high the expression level of this enzyme also increased significantly. The up regulation of the enzyme is significant with the controlled cells (*P=0.0160, **P=0.00043, ***P=0.000023). B. The graph represented the prostaglandine synthase expression level under MTZ induction. The upregulation of the specific enzyme is significant with time dependant manner under MTZ induce oxidative stress condition. The up regulation of the enzyme is significant with the controlled cells (*P=0.01145, **P=0.00056, ***P=0.000023).

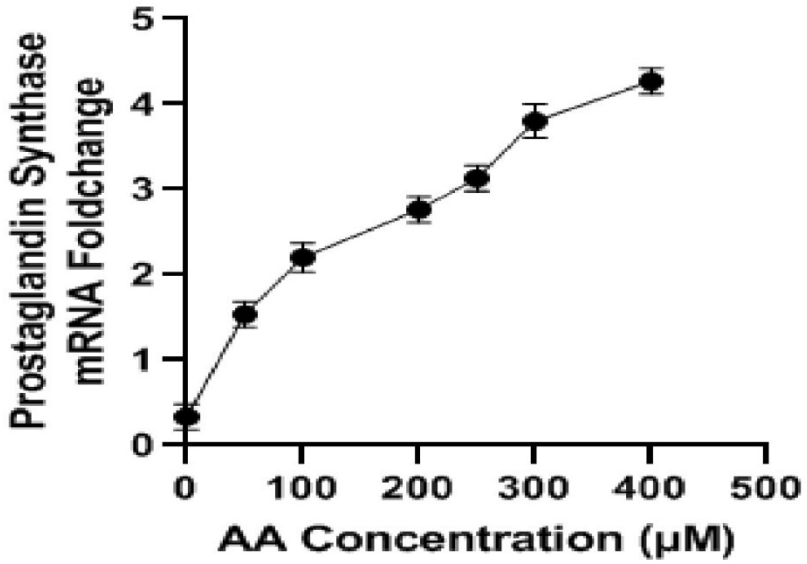


Fig.6.10. The expression level of prostaglandin synthase in presence of AA. The extracellularly added AA increased the prostaglandin synthase expression level. The gradual increase in AA level also increased the enzyme expression level gradually

6.3.8. The level of protein expression is increased in presence of AA

The illustration of prostaglandin specific synthase protein expressions were presented in Fig.6.11. The similar pattern of the specific protein expressions were observed in consecutive three experiments. The protein expression under H2O2 induction showed increased pattern in time dependent manner. The expression was highest after 9 hours of incubation (Fig.6.11.A.). In case of MTZ induction the highest expression was seen after 6 hours of incubation (Fig.6.11.B). We were also able to detect the increased protein expression under extracellular AA induction. The increased AA concentration (50,100,200,300,400 μM) increases the prostaglandin synthase protein expression (Fig.6.11.C). Our data suggested that increased AA concentration leaded to the more protein expression.

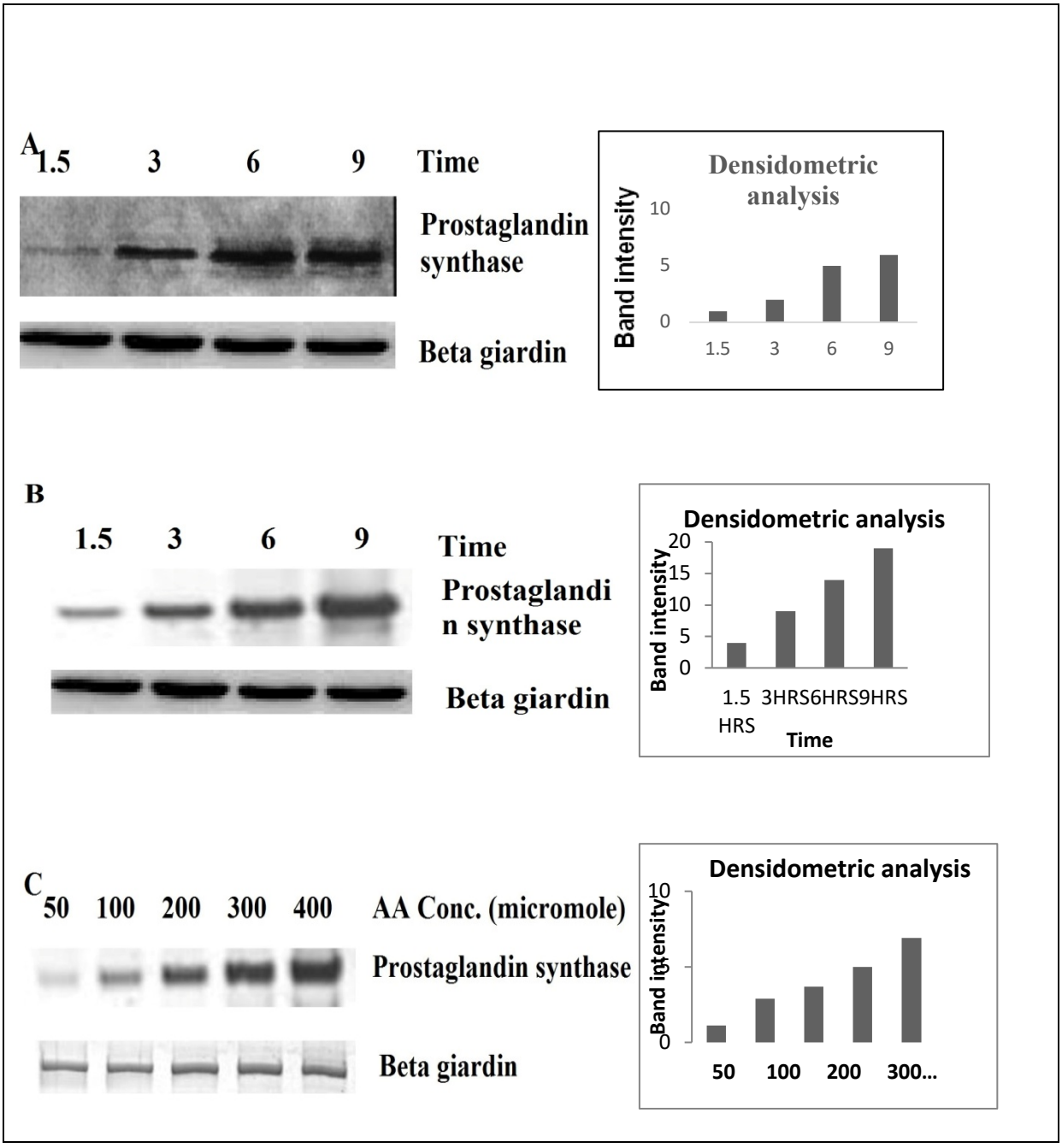


Fig.6.11.The expressions of prostaglandin synthase under oxidative stress condition. A. The specific protein expression was increasing in time dependent manner and highest after 9 hours of incubation with H2O2. B. In case of MTZ induction the highest expression was also seen after 9 hours of incubation. The similar pattern of the specific protein expressions were observed in consecutive three experiments. C. Increased AA concentration gradually increased the prostaglandin specific synhase protein expression. For all the protein expression experiments beta giardin was considered as control.

6.3.8. Increased AA concentration lead to the prostaglandinE_2 production

The production of prostaglandinE2 (PGE2) was monitored post-incubation with H2O2. After 6 hours of incubation the level of intracellular PGE2 was measured and it was increased drastically (Fig.6.12) compared to the controlled one. We observed that the increase in PGE2 concentration (finally 87.33pg/ml inH2O2 treated, 81.7 pg/ml in MTZ treated cells and 24.6 pg/ml in untreated cells)coincides with the increased AA concentration. It infers that the PGE2 pathway is activated via arachidonic acid formation.

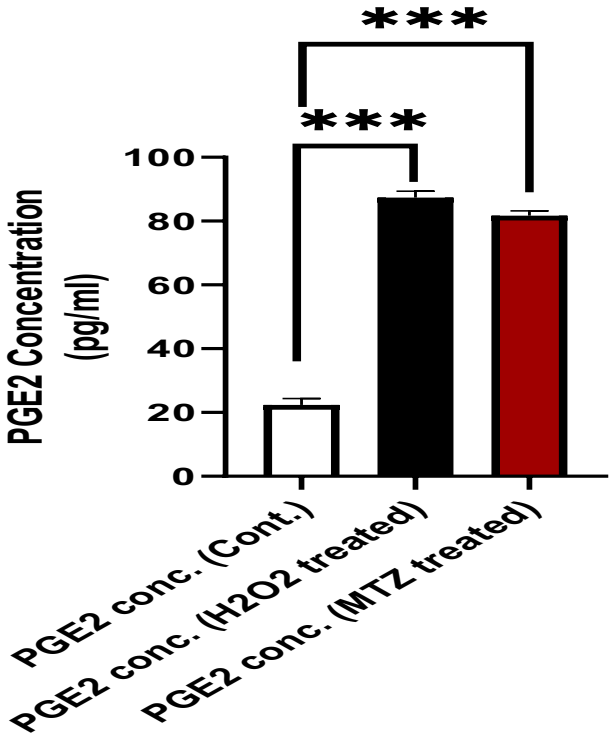


Fig.6.12. The graph shows the levels of Prostaglandin E2 (PGE2) in H2O2 and MTZ-treated cells compared to control cells. The treated cells demonstrated a significant increase in PGE2 production after 6 hours of incubation. After 6 hours of incubation, the PGE2 concentration in H2O2-treated cells reached approximately 87.33pg/ml and MTZ-treated cells 81.7 pg/ml, indicating that oxidative stress induced by H2O2 and MTZ can stimulate PGE2 production. The control cells, on the other hand, maintained a relatively low PGE2 production level throughout the incubation period.

6.4.Discussion

Normal oxidative stress consequentially produces ROS which at low concentration acts as signalling molecule but high levels can be lethal *(Juránek et al., 2005)*. High level of ROS has impact directly or indirectly in the apoptotic or necrotic signalling pathways *(England et al., 2005)*. Most studied effect of ROS is the oxidative modification membrane phospholipid or lipoperoxidation which is a very harmful process. Oxidative stress and lipid peroxidationenhance PLA2 activity which thus gives rise to a number of biologically active mediators such as arachidonic acid (AA). It is a participant ofLands cycle which is a cyclical deacylation/reacylation process. In the mentioned process a fatty acid of membrane phospholipidsis hydrolyzed and another fatty acid is incorporated by theaction of fatty acyl-CoA synthetase. It is considered that the major route for incorporation of AAin the phospholipids of a various types of cells is via Lands cycle *(Chilton et al.,1996)*. under unstimulated cells, the reacylation pathway dominates over the phospholipolytic step which keeps free AA concentration at a basal level. Under stimulation conditions, the rate of AA release exceeds that of reincorporation into phospholipids, hence net accumulation of AA occurs that is followed by its conversion into different oxygenated compounds, collectively called the eicosanoids.

In our present study we had demonstrated that oxidative stress increased the intracellular AA concentration in the *Giardia* trophozoites. In normal uninduced cells the concentration of AA is kept at basal level by the reacylation to the membrane phospholipid with the enzymatic action of acyl CoA synthetase (ACS). As oxidative stress increased net concentration of free AA in trophozoites, we examined the presence and status ACS in this condition. Our data suggested that the ACS was down regulated due to the effect of stress induced by MTZ and H2O2. The down regulation of ACS in induced cells, lead to the prevention of free AA production and hence accumulation of toxic AA. The higher level of free AA induces the

production of prostaglandin by prostaglandin synthase. In *Giardia* trophozoites presence of prostaglandin synthase and its up regulation played an important role in prostaglandin E2 production. Our data showed the enzyme is significantly up regulated after the 6 and 9 hours of oxidative stress induction. In this study we also evaluated the production of prostaglandins. According to the data *Giardia* trophozoites do produce prostaglandinE2 and the concentration of it significantly high in stress induced cells compared to the un induced controlled trophozoites. The fate of the prostaglandin and its effect on trophzoite was determined by the further investigation.

CHAPTER 7

PRODUCTION OF PROSTAGLANDIN2 (PGE2) AND ITS EFFECT ON GIARDIA TROPHOZOITES UNDER STRESSED CONDITION

7.1.Introduction

Prostaglandins, the lipid autacoids, are derived from arachidonic acid (AA). The 20 carbon unsaturated fatty acid, released from the plasma membrane by the action of phospholipases (PLAs), is metabolized by the sequential actions of prostaglandin synthase to form prostanoids. There are four types of bioactive prostaglandins which are ubiquitously produced in vivo depending on the presence of the synthase enzyme in the particular cell type. Each cell usually produces one or two dominating type which performs as autacrine and paracrine lipid mediators for the maintenance of local homeostasis. The prostaglandin production is solely dependent on the bifunctional enzymescolloquially known as COXs*(Smith et al., 2000)*. The enzymes contribute to the generation of autoregulatory and homeostatic prostanoids, and both can contribute to prostanoid release during need. After the formation, PGE2is actively transported through the membrane or diffuses across the plasma-membrane to act at or nearby its site of secretion*(Park et al., 2006)*by binding its four cognate receptors *(Trebino et al.,2003).*Literature survey revealed that Prostaglandine E_2 (PGE_2), can activates the erythrocyte cation channels and thus lead to erythrocyte shrinkage and phosphatidylserin exposure. Injuries to erythrocytes such as osmotic shock, oxidative stress, or energy depletion can induce the formation of prostaglandin E2 and it further activates the K+ channel, leading to hyperpolarization, cell shrinkage, and the exposure of phosphatidylserine, which is a marker for apoptosis (*lang et al.,2005*).

Apoptosis is the process of programmed cell death (PCD) which is used to rid the body of cells that have been damaged beyond repair. It is the most common type of PCD known which involves series of events including activation of caspase, cell shrinkage, DNA fragmentation, phosphatidylserine exposure, formation of apoptotic bodies, loss of mitochondrial membrane potential with cytochrome c release (*Menna-Burreto et al. 2009*).PCD is well regulated cellular process that has been extensively characterized in

multicellular organisms but inmany unicellular eukaryotes PCD is also observed in which *Giardia* is the newly added amitochondrial eukaryote.*Giardia* possesses two forms of PCD – apoptosis and autophagy (*Correa et al (2009)*.Both intrinsic and extrinsic signalling of apoptotic pathways requires different type of caspase machinies. In case of *Giardia*, under oxidative stress condition though phosphatidylserine exposure occurs but no caspase activity is detected (*Bagchi et al.,2012*). Recently it is reported that many unicellular eukaryotes possess caspase independent apoptotic pathway via mitochondrial programmed cell death. Evidential reports also highlighted the involvement of metacaspase in an apoptotic-like PCD in yeast (*Mazzoni et al.,2008*). Due to lack of typical mitochondria, caspase independent apoptosis is unlikely to happen in *Giadia.* AA production and ultimately apoptosis induced by AA and its metabolites is a characteristic feature of ROS generation*(Chen K C., et al. 2009)*.

In our study we examined that under oxidative stress condition when *Giardia* produced excess amount of ROS, the free AA concentration also increased intracellularly. In this study, we tried to measure the prostaglandin concentration under different oxidative condition we would also detect the PGE2 concentration under the influence of increased AA concentration. In our present study, we would examine the cell death type and its relation with AA and its prostanoid.

7.2.Materials Methods

7.2.1. ProstaglandinE2 measurement by Enzyme-Linked Immunosorbent Assay

Prostaglandin concentration was measured using ab 133021- Prostaglandin E2 ELISA kit according to the manufacturer's protocol. Treated and untreated cells ($4x10^6$ cells/ml) were harvested and washed with ice-cold PBS and centrifuged for 5 mins at 2000rpm at 4°c. Supernatants were collected and added to the 96-well plate for prostaglandin measurement.

Standards and samples were added to the respective well. AP-conjugate and PGE2 antibodies were added to the appropriate wells and incubated at room temperature on a plate shaker for 2 hours at 500 rpm. The content of the plate was discarded and washed with wash buffer twice. Then, we added pNpp substrate to each well and incubated it at room temperature for 45 minutes without shaking. To stop the reaction, the stop solution was added, and the plate was read immediately at 570nm. Initially, a standard curve was drawn using standard prostaglandin supplied with the kit. Then keeping the standard curve reference PGE2 in stressed condition was measured.

7.2.2. Intracellular potassium measurement in *Giardia* trophozoites

K+ concentrations may decrease in cells undergoing apoptosis and both physical and fluorescence techniques document an intracellular K+ concentration of 35 mM in apoptotic cells. This loss of K+ also accounts for changes in cell volume (cell shrinkage) that are universally associated with apoptosis. Thus the intracellular potassium concentration was measured by fluorescent dye benzofuran isophthalate (PBFI). The trophozoites were treated with H2O2 and MTZ and incubated for 0 to 9 hours. For another study trophozoite were treated with increased PGE2 concentrations (10,25,50,75 and 100 pg/ml).The treated and untreared controlled cells were harvested by 10 mins cold shock followed by centrifugation at 2000 rpm for 10 mins. The cells were washed with cold PBS and re suspended in it. Next, 5µM working conc. of dye was added to the cells and incubated for 90 mins at 37℃. Finally, the luminescences were measured at a constant emission at 500 nm and excitation ratios at 340 and 380 nm.

7.2.3. Cell viability assay by flow cytometry

The majority of the phosphatidylserine (PS) of mammalian cellmembranenormally resides on the inner leaflet of the bilayer *(Vance, 2008)*.This PS becomes externalized on the outside of

cells during the early phase of apoptosis*(Balasubramanian et al., 2007)*.The surface exposure of PS is one of the recognition signals by which apoptotic cells are removed. Annexin V staining is used to detect the translocation of phosphatidylserine to the outer leaflet of the plasma membrane, a hallmark of apoptosis. Annexin binds to PS in the outer layer of the membrane and is coupled with a fluorescent dye (FITC), which can be measured by flow cytometry. Viability staining is an essential component of any flow cytometry experiment. Dead cells can compromise the integrity of the data by non-specifically binding antibodies; therefore it is essential that dead cells be excluded from analysis. Propidium iodide can be used to stain dead cells so that they may be excluded from analysis in standard live cell surface staining protocols. $1x10^5$ cells were harvested from each of the treated and un treated cells and was resuspend in 500 µL of 1X Annexin V binding buffer of thermofischer scientific. 5 µL of annexin V-FITC and 5 µL of propidium iodide were added in each cell suspension. Finally, cells were incubated at room temperature for 5 min in the dark. The cells were analyzed for annexin V-FITC binding via flow cytometry (Ex = 488 nm; Em = 350 nm) using FITC signal detector.

7.2.3. Cell shrinkage assay

Giardia trophozoites were treated with H2O2 and MTZ and incubated for 0 to 9 hours. Then the cells were harvested and washed twice with ice cold PBS. Next the slides were prepared and mounted with DPX. Then the slides were analysed under confocal microscope toobtain differential interference contrast (DIC) images.

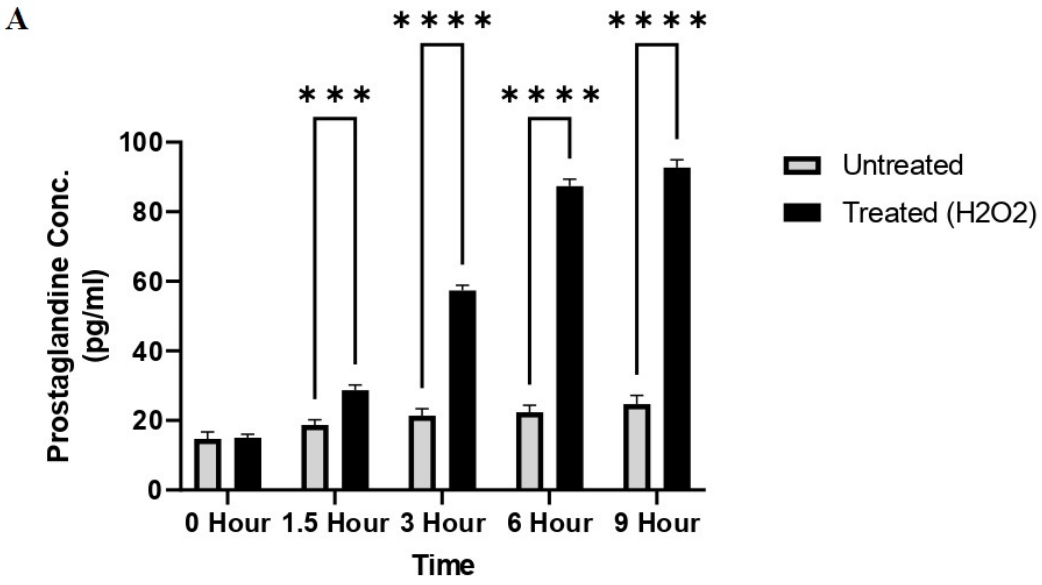
7.2.4. Statistical Analysis

All the experiments were performed thrice in triplicates and the results were expressed as mean±standard error of the mean (SEM). The analysis was evaluated by t-test or 1-way ANOVA (wherever applicable). <0.05 P value was considered statistically significant.

7.3. Results

7.3.1. Induced production of PGE2 by Giardia lamblia under oxidative stress

The production of PGE2 was monitored post-incubation with H2O2and MTZ. The level increased drastically at 3 and 6 hours and 9 hours of incubation (Fig no: 5). Our data showed a significant amount of increased PGE2 production over the time in treated cells comparison with the untreated controlled one (finally 92.66 pg/ml inH2O2 treated, 82.33pg/ml in MTZ treated and 24.6 pg/ml in untreated cells) (Fig.7.1.A and 7.1.B.). We observed that the PLB up-regulation and increased in PGE2 coincides at 3 hours and 6 hours post H2O2 induction. We also observed that PLB inhibiter, alexidine dihydrochloride (0.25 µM), reduced AA (Fig.5.11.A and 5.11.B) and PGE2 production (Fig.7.1.C.). It infers that the PGE2 pathway is activated via arachidonic acid formation by PLB.



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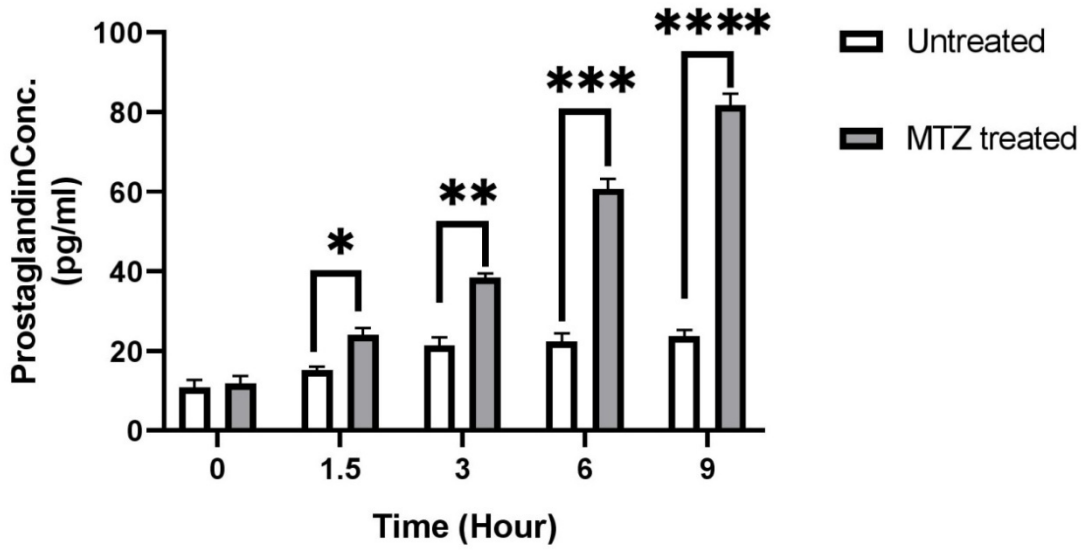
ProstaglandinConc. (pg/ml)
100
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6
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Untreated
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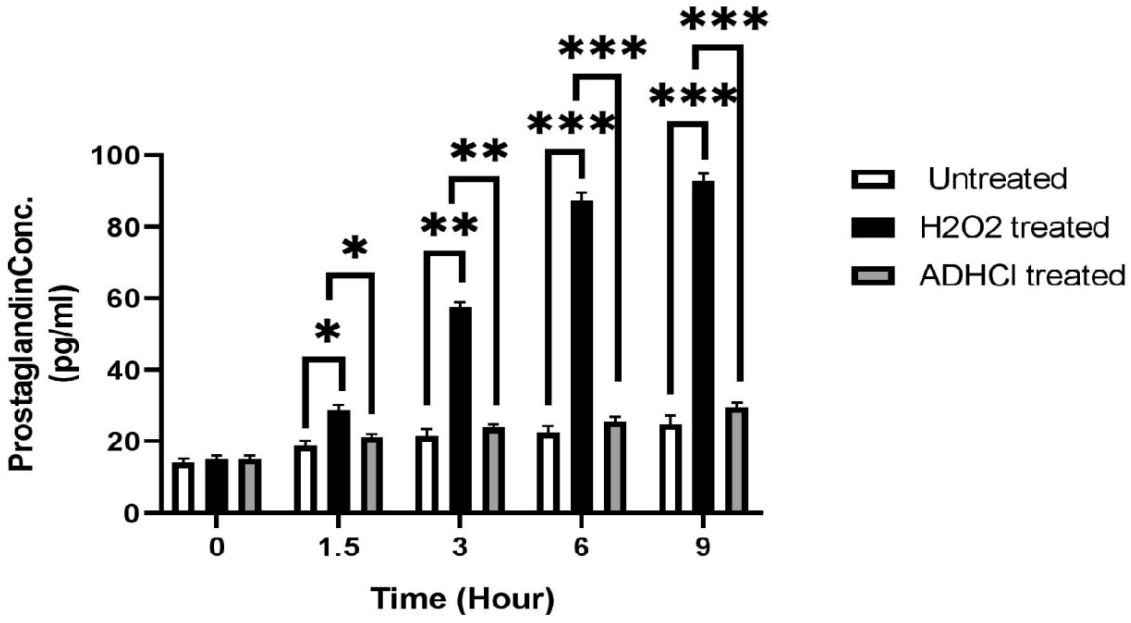
C


ProstaglandinConc. (pg/ml)
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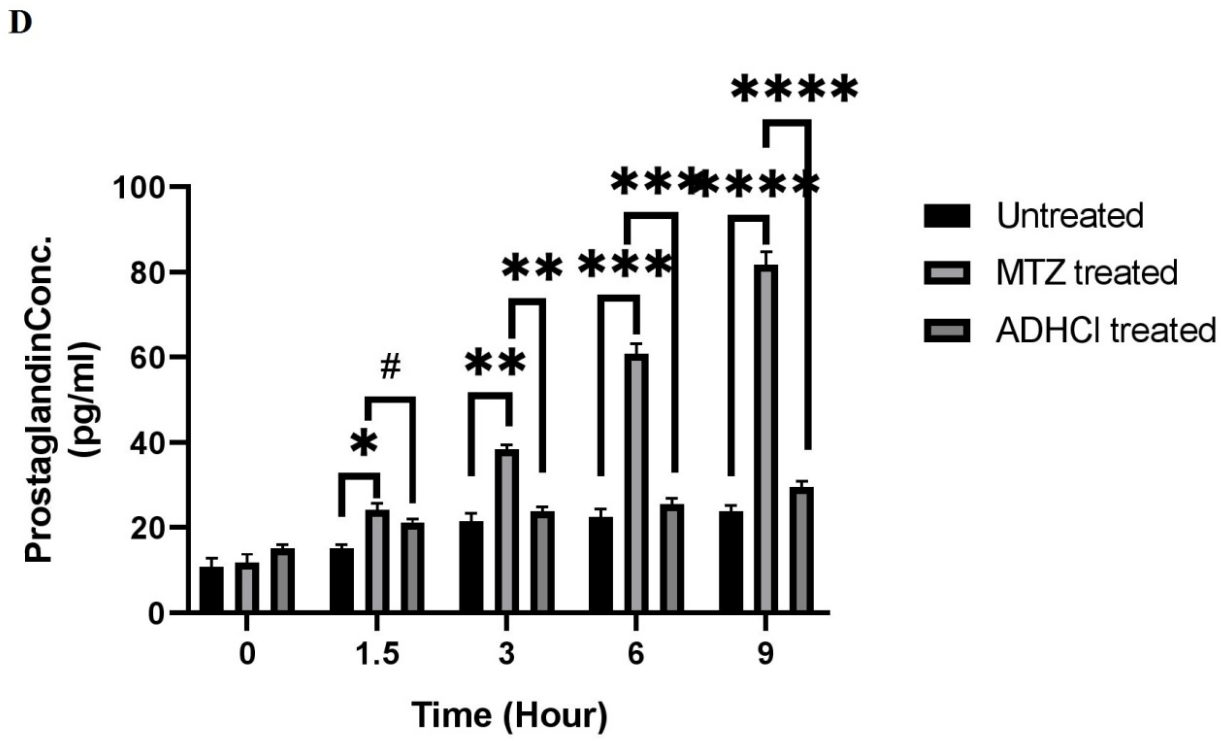
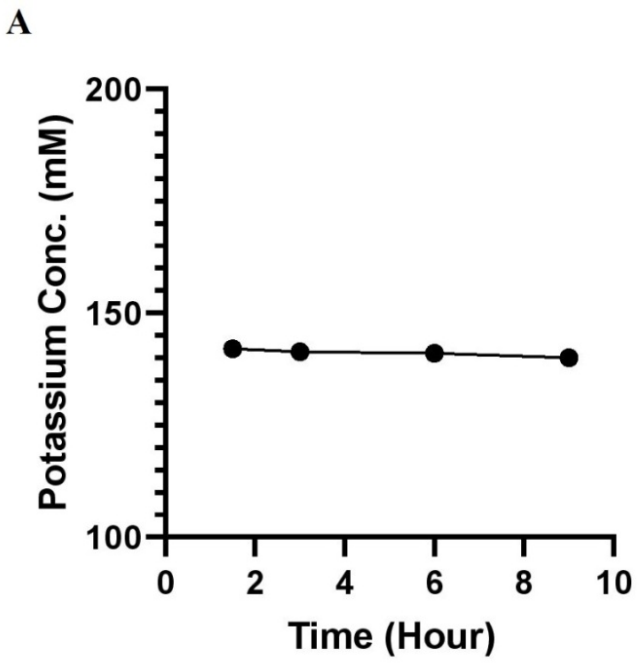


Fig.7.1. The graphs represent the prostaglandin production over the time under oxidative stress. A. The graph showed the levels of Prostaglandin E2 (PGE2) in H2O2-treated cells compared to control cells. The treated cells demonstrated a significant increase in PGE2 production after 3 hours of incubation, which continued to rise over time. After 9 hours of incubation, the PGE2 concentration in treated cells reached approximately 90 pg/ml, indicating that oxidative stress induced by H2O2 can stimulate PGE2 production. The control cells, on the other hand, maintained a relatively stable PGE2 production level throughout the incubation period. B. The graph represented the PGE2 production in MTZ treated cells. Significant increase in PGE2 production after 3 hours of incubation was seen which continued to rise over time (*P=0.0015,**P=0.00025, ***P=0.00003,****P=000008).C. The image showed the significant reduction in PGE2 formation with the treatment of PLB inhibitor ADHCl in H2O2 treated cells (*P=0.0013,**P=000017,***P=000003). D. According to the graph PGE2 formation was significantly reduced by ADHCl in MTZtreated cells (*P=0.0015, **P=0.000246.***P=0.000035,****P=0.000008).

7.3.2.Protaglandin reduced intracellular potassium concentration in trophozoites

Many cellular processes like enzyme activity, cell volume regulation, cell growth and determination of membrane potential is dependent on the high intracellular potassium concentration. Here K+ channels play an important role by permitting the dynamic equilibrium of this ion such that net influx and efflux of K+ are equalunder steady-state.

Thus, intracellular K+ level stay well above than that of the extracellular concentration *(Scott et al., 2018)*. The K+ sensitive fluorescent dye, K+-binding benzofuran isophthalate (PBFI), has provided a noninvasive technique for determining K+ concentration. Initially the normal K+ concentration was measured in untreated trophozoites over the time (Fig.7.2.A.). The graph represented that the trophozoites maintained a steady K+ concentration (around 140 mM ±5) over the time. The oxidative stress (both H2O2 and MTZ stress) decreased the intracellular K+ concentration over the time (Fig.7.2.B & 7.2.B). In both the cases the PGE2 concentration was high in respective time frame (Fig.7.1.A & 7.1.B) which indirectly correlate the reduced intracellular K+ concentration. To directly analyse the role PGE2 in reduced K+ concentration we also treated trophozoites with increased PGE2 concentrations. The data of this analysis showed that the increasing PGE2 concentration reduced the intracellular K+ concentration. Thus it can be concluded that increased PGE2 production under the influence of oxidative stress decreased the intracellular K+ concentration of the *Giardia* trophozoites.



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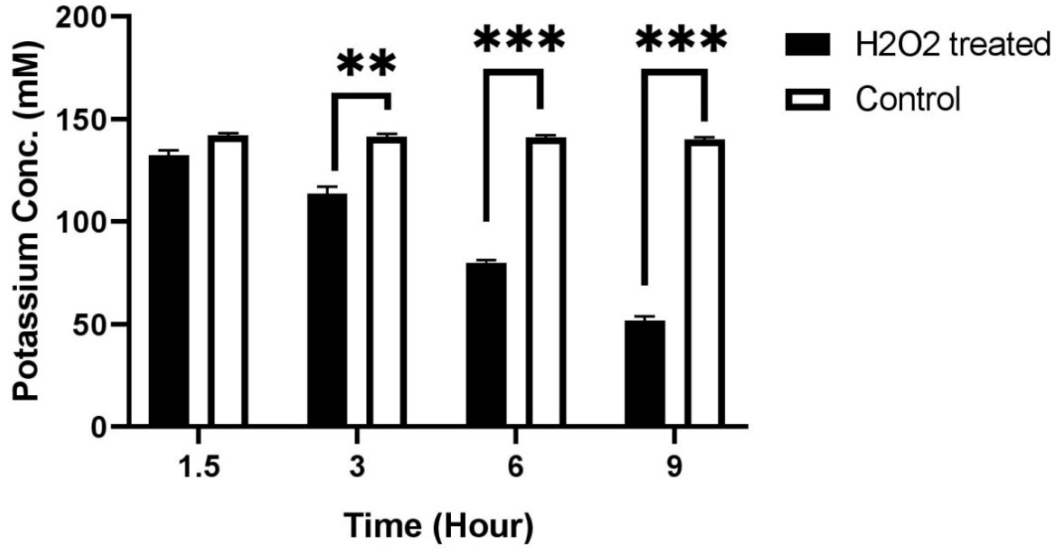
200
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50
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Potassium Conc. (mM)
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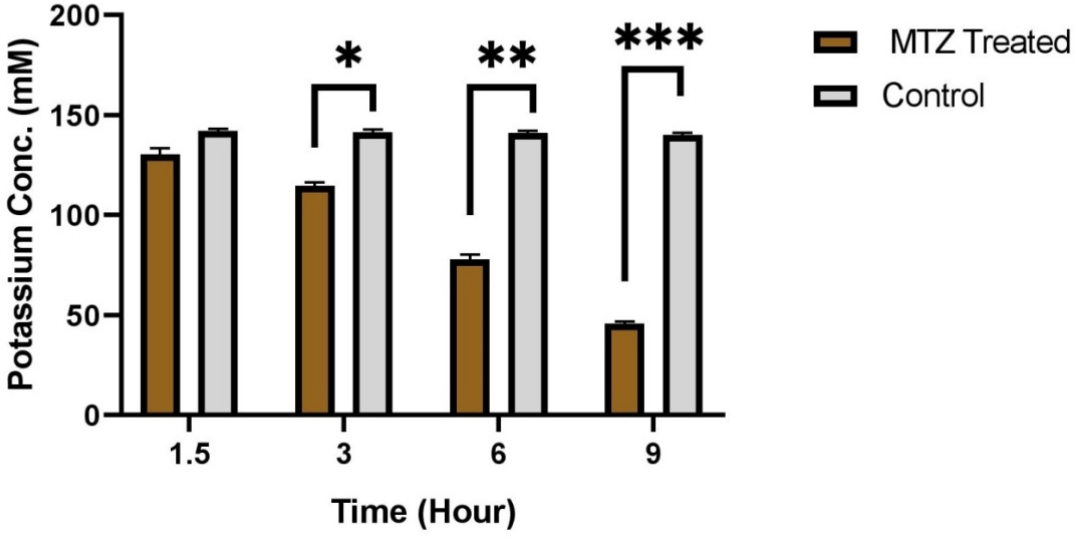
9
Time (Hour)
H2O2 treated
Control

C


200
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Potassium Conc. (mM)
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Time (Hour)
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Control

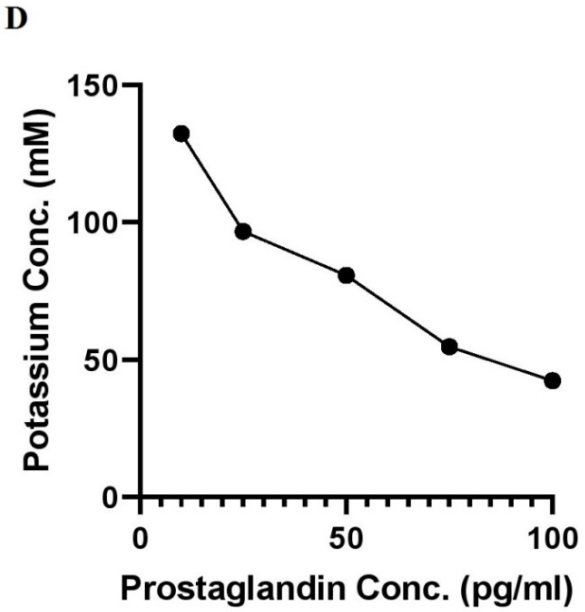


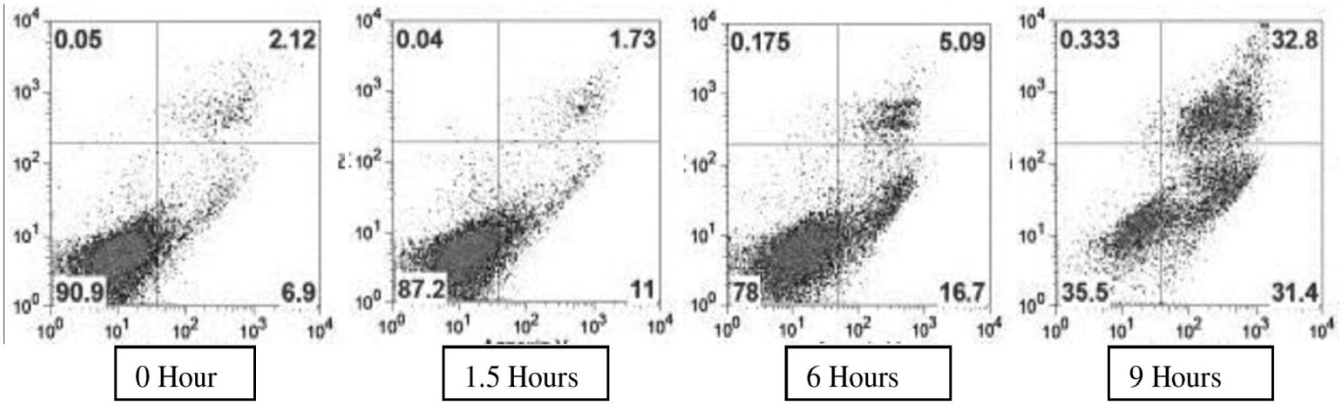
Fig.7.2. Status of intracellular potassium concentration in *Giardia* trophozoites. A. The graph represented the intracellular potassium (K^+) concentration in *Giardia* trophozoite which showed that trophozoites maintained significantly high intracellular K^+ concentration (approximately 140 mM). B. Represented the significant K^+ concentration depletion over the time under the influence of H2O2 induction (**P=00022,***P<0.00001). C. MTZ treatment reduced intracellular K^+ significantly from approx. 140-50 mM (*P= 0.0033,**P=00005,***P=000002). D. The data showed increased PGE2 concentration (10-100 pg/ml) decreased K^+ concentration (p value <0.0001).

7.3.2. Externalization of phosphatidylserin of trophozoite membrane

Externalization of phosphatidyl serin (PS) is one of the indicators of apoptosis in the eukaryotic cells.Flourescein isothiocyanate (FITC) tagged annexin can bind with PS when it flips outside the membrane and the fluorescence can be measured via flowcytometer. In our study we have used this assay to check death percent of *Giardia* trophozoiets and mode of its death. In our study, it was confirmed that the both H2O2 and MTZ induced cellular damage leaded to increased apoptotic cell death in trophozoites over the time (Fig.7.3). The H2O2stress increased the phosphatidyl serin exposure to the outer leaflet of the trophozoite membrane which was attached to FITC tagged annexin V and produced green fluorescence. Considering increased fluorescence, the trophozoites were sorted by flowcytometer to live

early and late apoptotic cell populations. The apoptotic cell populations over the time gradually moved from early to late apoptotic phase (Fig.7.3.A & 7.3.B). The MTZ induction also induced increased apoptosis in *Giardia* trophozoites (Fig.7.3.C & 7.3.D).

A



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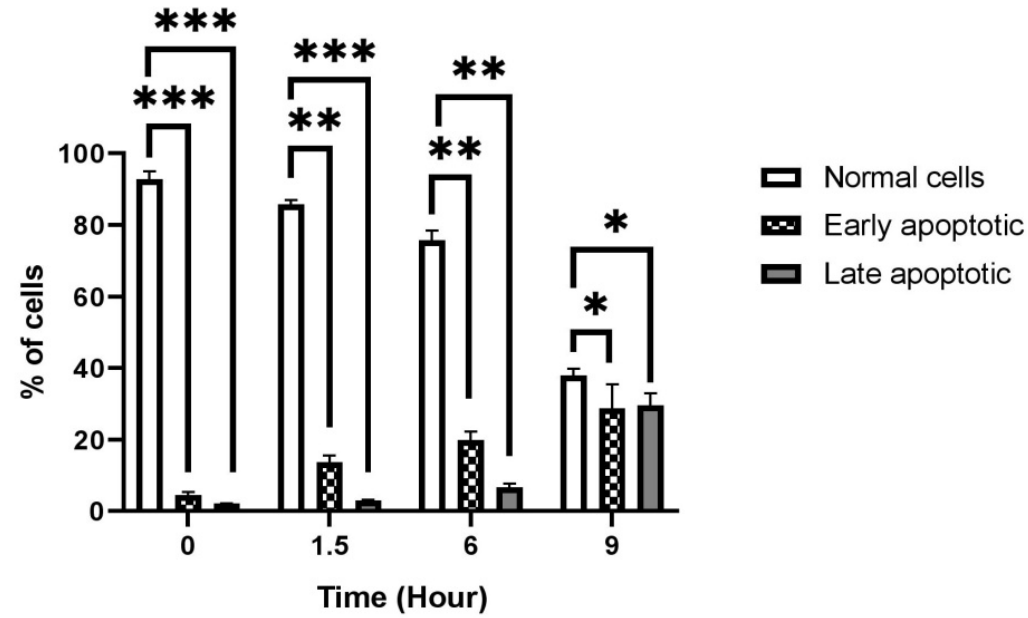

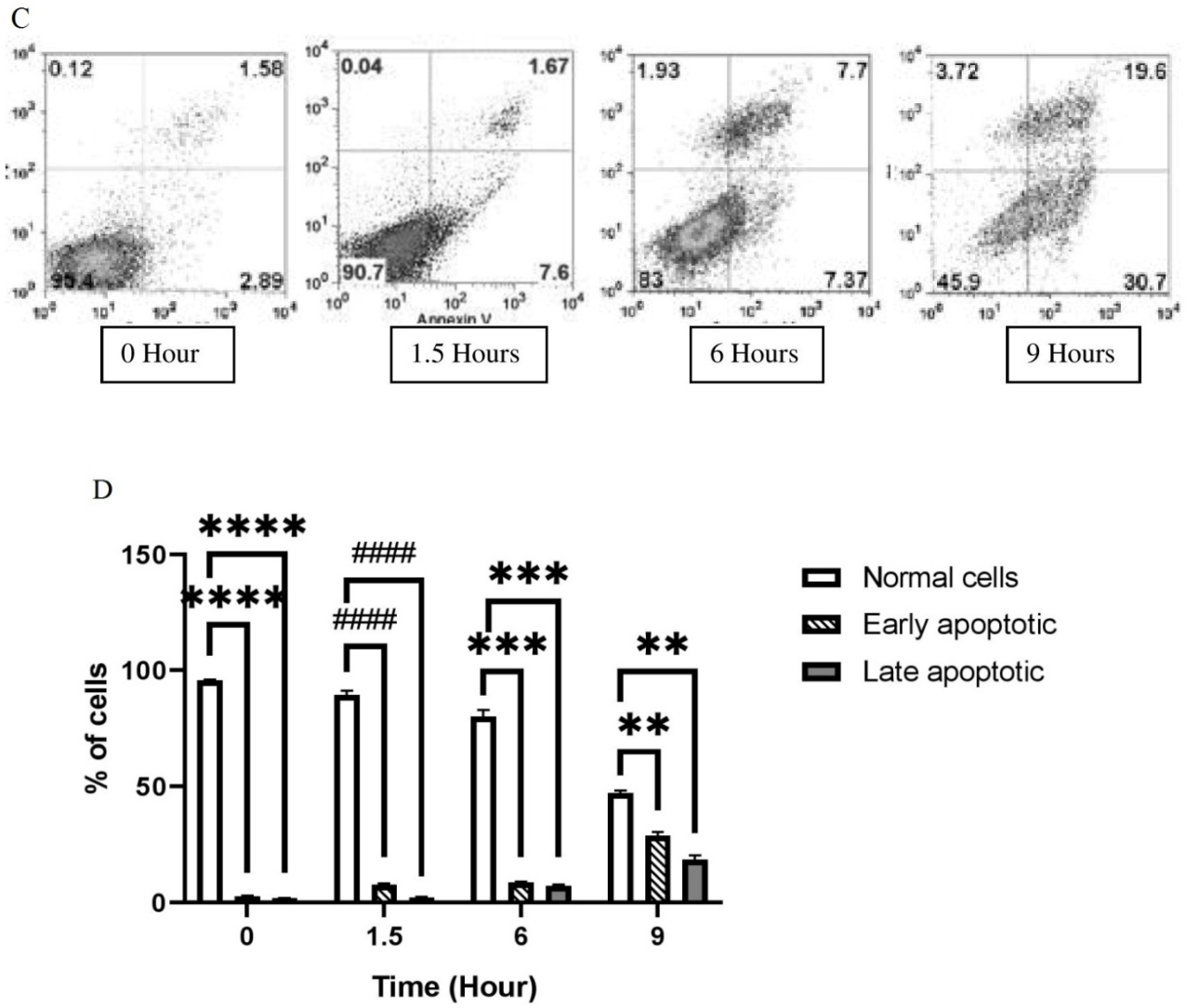

Fig.7.3. Apoptotic stages of *Giardia* trophozoites over the time under oxidative stress condition. A. The panel represented the data obtained from flowcytometer showing that the percent of live cells decreased and percent of apoptotic cells increased over the time under H2O2 stressed condition. B. The graph represented the significant no of trophozoits were live at initial phase but gradually no of live cells were decreased and early and late apoptotic cells increased (***P<0.000001,**P=0.000012,*P=0.0087). C. The panel showed that the percent of live cells decreased and percent of apoptotic cells increased over the time under MTZ stressed condition and the cells moved from early apoptotic to late apoptotic phase. D. The graph represented the percent of live early apoptotic and late apoptotic cells in different time frame. It revealed that the apoptosis increased significantly over the time and the cells gradually entered from early to late apoptosis. (****P<0.000001,***P=0.000022, **P=0.00096,$^{\#\#\#\#}$P=0.000002).

7.3.3. Increased PGE2 and decreased K^+ concentration increased trophozoite apoptosis

To analyse the effect of PGE2 in trophozoite death we extracellularly introduced increasing concentration of PGE2 and noted the cell death percentage. Our data suggested that increasing PGE2 concentration (10, 25, 50, 75, 100 pg/ml) escalated trophozoite apoptosis significantly over time (Fig.7.4.A). *Giardia* trophozoites maintain a constant K+ concentration in un induced controlled condition (approx. 140 mM). But in case of high PGE2 concentration, the intracellular K+ concentration decreased. This decreased K+ concentration enhanced the apoptosis in *Giardia* trophozoites (Fig.7.4.B).

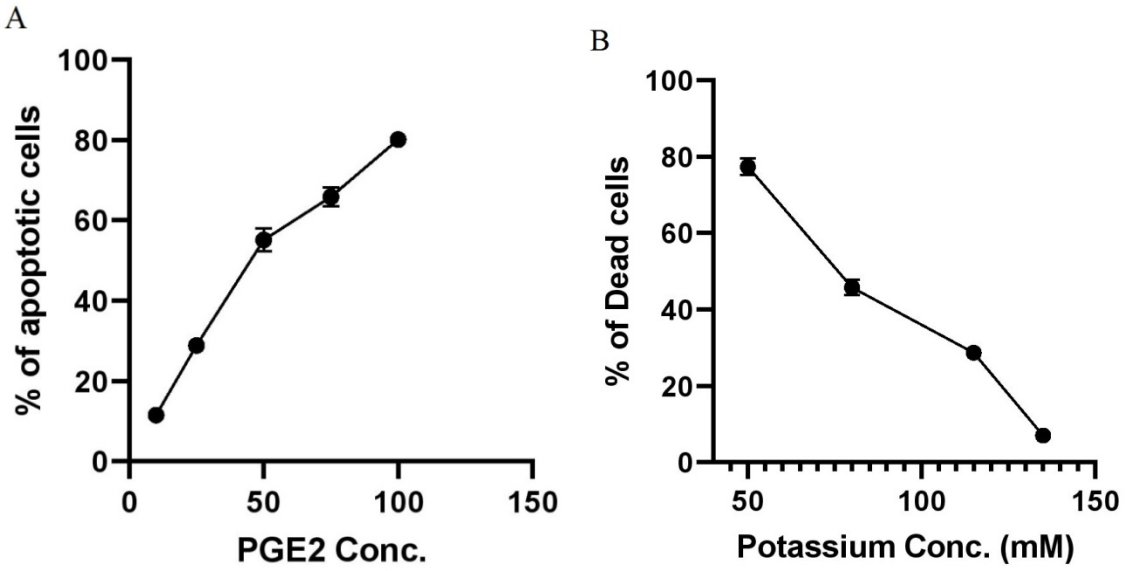

Fig.7.4. Apoptosis in trophozoite under the effect of different stimuli. A.Extracellularly added increased concentration PGE2 increased apoptosis in Giardia trophozoites significantly (p value <0.0001). B. The data showed that while under oxidative stress when K+ concentration decreased the apoptosis in the trophozoites increased (p value < 0.0001).

7.3.4. Oxidative stress enhanced Giardia trophozoite cell shrinkage

Shrinkage in cellular volume is a ubiquitous characteristic of programmed cell death observed mostly all kind of apoptotic cells. This decrease in cellular volume is due to the fluxes of intracellular ions including K+*(Bortner et al.,2003)*.K+ efflux is critical for cell

shrinkage and death by apoptosis. The exactmechanismfor the loss of intracellular potassiumand subsequent cell shrinkage has not properly designated but it is suggested that Na/K-ATPase inhibition play a crucial role in K+ efflux. So we finally examined the cellular morphology under oxidative stressed condition over the time. Our data displayed that the control uninduced trophozoites exhibited normal cellular morphology whereas the H2O2 and MTZ treated displayed shrinked trophozoites over the time (Fig.7.5). This data coincide with the reduced intracellular K+ concentration over the time under H2O2 and MTZ treatment.

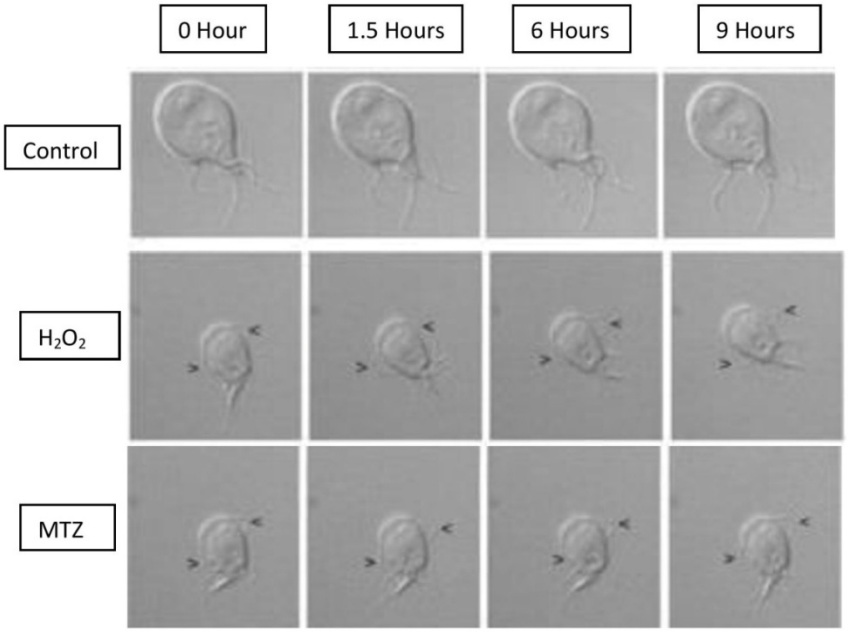

Fig.7.5. The trophozoite shrinkage under oxidative stress condition. In controlled un induced condition the trophozoite morphology is normal but in H2O2 and MTZ treated condition the trophozoites shrinked.

7.4. Discussion

Arachidonic acidmetabolismby cyclooxygenase produces prostaglandin E2 (PGE2) which is an antifibrotic lipid mediator. However, PGE2 is considered a potent antiapoptotic/prosurvival mediator in normal epithelial cells, it can promote apoptosis through activation of multiple apoptotic pathways in fibroblasts *(Huang et al., 2009)*.

Apoptosis is programmed cell death which is a catabolic process that is composed of breakdown of cellular macromolecules by distinct enzymes and later recycled in healthy cells. It also involves many physiological and pathological phenomena like cell shrinkage, DNA fragmentation, phosphatidyl serine exposure etc. A significant loss of intracellular potassium occurs at the time of apoptotic cell shrinkage. Measurements of K+ concentration in shrunken and non-shrunken cells demonstrated that only shrunken cells contain a reduced concentration of intracellular K+*(Bortner et al.,2003)*.

Our data suggested that two oxidative stress inducing condition (H2O2 and MTZ treatment) in *Giardia* trophozoites increased PGE2 production via the metabolism of AA. Our data also deduced that this increased PGE2 condition decreased intracellular K+ concentration of trophozoites. The influx and efflux of K+ are equalunder steady-state keeping the concentration high (approx. 140mM). In apoptoticaly dying cells this concentration decreases. Our experiment suggested that the intracellular K+ concentration gradually deceased in trophozoites over the time when cell is under oxidative stress leading to apoptosis. However, the controlled un-induced cells maintained a steady high level of K+ concentration over the time. We also observed that the oxidative stress leading to reduced K+ concentration induced cell shrinkage in trophozoites and phosphtidyl serine externalization. The phosphatidyl serine exposure is the marker for apoptosis and it confirms that *Giardia* trophozoites undergone apoptosis due to oxidative stress.

CHAPTER 8

CONCLUSION

8.1. Conclusion

Giardia lamblia, early-branched amitochondrian protozoa, causesgiardiasis in human and cattles. *Giardia*can tolerate and respire in O_2 till 50 μM but above this limit it ceases O_2consumption. Due to the O_2metabolism, the intracellular ROS is generated in trophozoites.The oxidative stress due to ROS generation in *Giardia*affects plasma membrane permeability, cell volume, externalization of phosphatidyl serine leading to trophozoite death (*Lloyd et al., 2000*; *Paget et al., 2004*). In our studies we have discovered similar phenomenon as phosphatitdylserine exposure under ROS induction confirming it as apoptosis. Though the process of cell death is apoptotic type, the *Giardia*trophozoiets does not follow the conventional apoptotic pathways due to absence of caspase machineries. So we proposed that the apoptosis of *Giardia* was AA and its metabolite induced unique one. The pathway is dependent upon the activation and the synthesis of PGE_2.

To thoroughly understand the role of oxidative stress in *Giardia* trophozoites we created stressed condition in cultured trophozoite using H2O2 and metronidazole (MTZ). In our study we were able to detect significant amount of intracellular ROS generation in suspension of *Giardia* trophozoites using H2DCFDA under both the conditions over the time (from 0-9 hours). With the increased treatment time the ROS generation is also increased, which induced different signalling molecule generation in trophozoites. One of the key molecules that can trigger ROS signalling in cells is arachidonic acid (AA). Increased concentration of free AA in cells plays a crucial role to induce further signalling and increased lipid peroxidation. As our data suggest increased induction of AA in cells increased the MDA concentration in trophozoites. We also observed increased MDA production, significantly by 37.5% in trophozoites treated with H2O2and 28.3% in MTZ treatment compared to control ones. MDA production is the marker for intracellular lipid peroxidation. The data suggested

increased ROS generation triggered AA production which further induced the lipid peroxidation in *Giardia.*

The formation of AA needs a potent cellular phospholipase usually phospholipase A2 (PLA2). Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites *(María et al.,2006; Mark et al., 1989)*. In the case of *Trypanosoma,* PLA2 activity is stimulated by Ca^{2+} and the release of AA. As our study revealed AA formation in trophozoite under oxidative stress condition we further investigated if the parasite shows increased intracellular calcium ion (Ca^{2+}) concentration. Our study showed the concentration of intracellular $Ca^{2+}$was increased till 2 hours of both treatment but then gradually decreased and simultaneously after that from 3hours the AA production increased significantly. This type of pattern indicates that Ca^{2+}act as inducer molecule to activate intracellular enzyme which can further produce AA from phospholipids. The accumulation of $Ca^{2+}$was visible throughout the periphery of the trophozoites which indicate the enzyme is effective on membrane phospholipids.

Previous studies have reported a positive correlation between membrane phospholipid peroxidation and enhanced PLA2 activity in several organisms [12]. While we did not identify either PLA1 or PLA2 in *Giardia* trophozoites, we did find evidence of lipid peroxidation and arachidonic acid production *(Das et al., 2001)*. The release of free arachidonic acid from phospholipids is dependent on intracellular PLA2 activities. Phospholipase A enzymes are capable of cleaving the acyl ester bond at either the sn-1 (which is catalyzed by Phospholipase A1) or the sn-2 (catalyzedby Phospholipase A2) position.The Phospholipase B enzymes have three unique activities: a hydrolase that cleaves both the sn-1 and sn-2 fatty acid esters, a lysophospholipase, and a transacylase activity*(Balboa et al., 2006)*.We attempted to investigate whether PLB was responsible for

the generation of arachidonic acid within *Giardia* trophozoites as it can hydrolyse the sn-2 position of phospholipid like PLA2 activity.

We observed a six-fold increase in the expression of PLB and lipid peroxidation levels in *Giardia* trophozoites following induction with H2O2.This increase in gPLB expression activated the prostaglandin E2 (PGE2) pathway and resulted in the formation of PGE2. The production of arachidonic acid also increased with the increased PLB expression level. According to the study by Soares et al. (2010),it was determined that alexidine dihydrochloride effectively inhibits PLB *(Soares et al., 2010)*. We utilized a blocker that resulted in a noteworthy decrease in the production of both arachidonic acid and PGE2 in our study.This indicates that when *Giardia* trophozoites are subjected to oxidative stress, they increase their production of phospholipase B (PLB), which can then lead to the production of arachidonic acid.

The PLB enzyme was successfully cloned and purified, with a molecular mass of approximately 56kDa. Bioinformatical analysis showed that the protein had four potential N-glycosylation sites, making it a glycoprotein. The PLBs characterized by other microbes also have high molecular weight and are highly glycosylated *(Ichimasa et al., 1984; Kuwabara et al., 1988; Oishi et al., 1988)*. In the case of yeast, most PLBs are of high molecular weight and highly glycosylated to protect themselves against proteolytic degradation *(Maruyama et al., 1990;Fujino et al., 2006).* Like yeast PLB, this specific protein also is glycosylated. It can be assumed that the modification is the safeguard from proteolytic cleavage. Further glycosylation studies may be needed to fully understand the role of glycosylation in the stability of the protein. The 'NCBI Conserved Protein Domain Search' tool analysis revealed that the protein sequence of gPLB has the capability to break down both acyl ester bonds in glycerophospholipids, indicating its potential role in lipid metabolism. The gPLB enzyme family is similar to the ones found in mammals, flies, and nematodes, but differs from the

PLBs observed in yeast. Despite this difference, it is noteworthy that the glycosylation process of gPLB is similar to that of PLB present in yeast.In*Drosophila*, a similar protein known as LAMA was discovered and expressed in neuronal and glial precursors surrounding the lamina. The name "LAMA" was derived from the term "laminin ancestor." Unlike PLB found in bacteria, gPLB acts as a dimer (according to the signal peptide analysis of HHpred) *(Zimmermann et al., 2018)*. It is distinct from PLB gene families found in bacteria and fungi. The deduced amino acid sequence from the 1.6 Kbp nucleotide sequence suggests that the first 15 sequences are signal peptides, indicating that the protein is destined for secretion*(Waterhouse et al., 2018)*. Additionally, the protein contains potential protein kinase phosphorylation sites, suggesting that its activation is regulated by intracellular phosphorylation machinery. In *Giardia* PLB, four specific N-myristoylation sites (at residues 147-152: GLveAY, 328-333: GTynNQ, 414-419: GLmfAA, and 445-450: GIseGQ) were identified. Myristoylation enhances localization and protein-protein interaction in subcellular locations, allowing it to interact with its signalling partner *(Udenwobele et al., 2017).* The protein has a robust lipase activity optimum at pH 7.5, which is distinct from fungal PLB, which functions well in acidic pH (2.5-6) and at a temperature of 35°C, which is lower than that of *Streptomyces* sp. PLB (functioning at 50°C) *(England et al., 2005)*. These unique features of *Giardia* PLB make it active at a wide range of pH and temperature, making it a key molecule in initiating the prostaglandin pathway under oxidative stress conditions. The *Giardia* PLB protein is distinct from other reported bacterial and fungal PLBs due to its ability to maintain activity across a wide range of pH and temperature conditions. This unique PLB is upregulated when *Giardia* trophozoites experience oxidative stress and are unable to remove ROS. The upregulation of this protein suggests that it may play a crucial role in initiating the prostaglandin pathway during oxidative stress which is started by free intracellular AA production. Injuries to erythrocytes such as osmotic shock, oxidative stress,

or energy depletion can induce the formation of prostaglandin E2, which then activates the Ca2+ permeable cation channel. The *Giardia* trophozoites bear similar consequence or not are subjected to further investigation.

Normal oxidative stress consequentially produces ROS which at low concentration acts as signalling molecule but high levels can be lethal *(Juránek et al., 2005)*. High level of ROS has impact directly or indirectly in the apoptotic or necrotic signalling pathways *(England et al., 2005)*. Most studied effect of ROS is the oxidative modification membrane phospholipid or lipoperoxidation which is a very harmful process. Oxidative stress and lipid peroxidationenhance PLA2 activity which thus gives rise to a number of biologically active mediators such as arachidonic acid (AA). It is a participant ofLands cycle which is a cyclical deacylation/reacylation process. In the mentioned process a fatty acid of membrane phospholipidsis hydrolyzed and another fatty acid is incorporated by theaction of fatty acyl-CoA synthetase. It is considered that the major route for incorporation of AAin the phospholipids of a various types of cells is via Lands cycle *(Chilton et al.,1996)*. under unstimulated cells, the reacylation pathway dominates over the phospholipolytic step which keeps free AA concentration at a basal level. Under stimulation conditions, the rate of AA release exceeds that of reincorporation into phospholipids, hence net accumulation of AA occurs that is followed by its conversion into different oxygenated compounds, collectively called the eicosanoids.

In our present study we had demonstrated that oxidative stress increased the intracellular AA concentration in the *Giardia* trophozoites. In normal uninduced cells the concentration of AA is kept at basal level by the reacylation to the membrane phospholipid with the enzymatic action of acyl CoA synthetase (ACS). As oxidative stress increased net concentration of free AA in trophozoites, we examined the presence and status ACS in this condition. Our data suggested that the ACS was down regulated due to the effect of stress induced by MTZ and

H2O2. The down regulation of ACS in induced cells, lead to the prevention of free AA production and hence accumulation of toxic AA. The higher level of free AA induces the production of prostaglandin by prostaglandin synthase. In *Giardia* trophozoites presence of prostaglandin synthase and its up regulation played an important role in prostaglandin E2 production. Our data showed the enzyme is significantly up regulated after the 6 and 9 hours of oxidative stress induction. In this study we also evaluated the production of prostaglandins. According to the data *Giardia* trophozoites do produce prostaglandinE2 and the concentration of it significantly high in stress induced cells compared to the un induced controlled trophozoites. The fate of the prostaglandin and its effect on trophzoite was determined by the further investigation.

Arachidonic acidmetabolismby cyclooxygenase produces prostaglandin E2 (PGE2) which is an antifibrotic lipid mediator. However, PGE2 is considered a potent antiapoptotic/prosurvival mediator in normal epithelial cells, it can promote apoptosis through activation of multiple apoptotic pathways in fibroblasts *(Huang et al., 2009)*. Apoptosis is programmed cell death which is a catabolic process that is composed of breakdown of cellular macromolecules by distinct enzymes and later recycled in healthy cells. It also involves many physiological and pathological phenomena like cell shrinkage, DNA fragmentation, phosphatidyl serine exposure etc. A significant loss of intracellular potassium occurs at the time of apoptotic cell shrinkage. Measurements of K+ concentration in shrunken and non-shrunken cells demonstrated that only shrunken cells contain a reduced concentration of intracellular K+*(Bortner et al.,2003)*.

Our data suggested that two oxidative stress inducing condition (H2O2 and MTZ treatment) in *Giardia* trophozoites increased PGE2 production via the metabolism of AA. Our data also deduced that this increased PGE2 condition decreased intracellular K+ concentration of trophozoites. The influx and efflux of K+ are equalunder steady-state keeping the

concentration high (approx. 140mM). In apoptoticaly dying cells this concentration decreases. Our experiment suggested that the intracellular K+ concentration gradually deceased in trophozoites over the time when cell is under oxidative stress leading to apoptosis. However, the controlled un-induced cells maintained a steady high level of K+ concentration over the time. We also observed that the oxidative stress leading to reduced K+ concentration induced cell shrinkage in trophozoites and phosphtidyl serine externalization. The phosphatidyl serine exposure is the marker for apoptosis and it confirms that *Giardia* trophozoites undergone apoptosis due to oxidative stress.

8.2. Summery

- Oxidative stress increased Protein kinase activation and Ca^{2+} upregulation in *Giardia* trophozoites.
- Intracellular activated PK and increased calcium synergistically increased PLB expression.
- Induced PLB then started to produce intracellular free Arachidonic acid (AA).
- The production of free AA is a highly regulated process that represents a balance between two competing reactions, namely, phospholipid deacylation by phospholipase A2 (PLA2) enzymes and reacylation and transfer into various phospholipid pools by acyltransferases and transacylases. in uninduced cells, reacylation dominates, and hence, the bulk of cellular AA is found in the esterified form. In stimulated cells, the dominant reaction is the PLA2-mediated deacylation, which results in dramatic releases of free AA. In our study it was found that induced trophozoites directed free AA towards membrane deacylation process by suppressing the expression of acyl CoA synthetase.

- The released free AA by PLB was then available for eicosanoid synthesis and by the effect of prostaglandine specific synthase the Prostaglandin E2(PGE2) was produced.
- Prostaglandin E2 (PGE2) increased theefflux of potassium ion (K+). Hence, theintracellular K+ concentration was depleted which leads to cell shrinkage and phosphatidyl serine exposuure. Both are the marker for apoptosis.

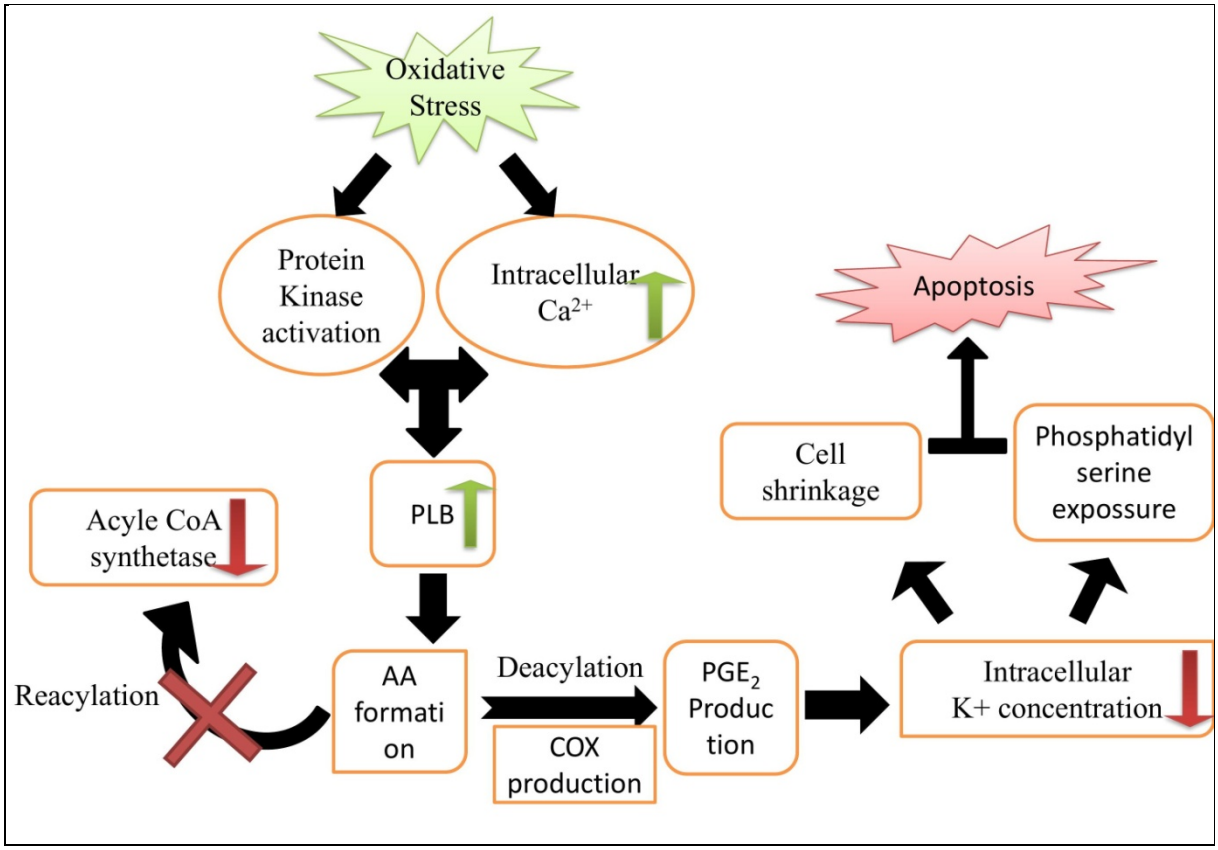

Fig.8.1.Schematic representation of apoptotic cell death in Giardia trophozoites under oxidative stress condition.

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PUBLICATIONS

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2. Raj Dibyendu, Chowdhury Punam, Sarkar Rituparna, Saito-Nakano Yumiko, Okamoto Keinosuke, Dutta Shanta, Nozaki Tomoyoshi, Ganguly Sandipan. (2018). Pyruvate Protects *Giardia* Trophozoites from Cysteine-Ascorbate Deprived Medium Induced Cytotoxicity. The Korean journal of parasitology. 56. 1-9.doi: 10.3347/kjp.2018.56.1.1.

3.Ganguly Sandipan, Barkataki Sharad, SangaPrerna, BoopathiKangusamy, KanagasabaiKaliaperumal, DevikaShanmugasundaram, KarmakarSumallya, ChowdhuryPunam, Sarkar Rituparna, RajDibyendu, James Leo, Dutta Shanta, Campbell Suzy, MurhekarManoj. (2022). Epidemiology of Soil-Transmitted Helminth Infections among Primary School Children in the States of Chhattisgarh, Telangana, and Tripura, India, 2015–2016. The American Journal of Tropical Medicine and Hygiene. 107. 10.4269/ajtmh.21-1185.

4. Ganguly Sandipan, Barkataki Sharad,KarmakarSumallya, SangaPrerna, Boopathi, Kangusamy, KanagasabaiKaliyaperumal, Kamaraj P., ChowdhuryPunam, SarkarRituparna,RajDibyendu, James Leo, Dutta Shanta, SehgalRakesh, Jha Priya,Murhekar Manoj. (2017). High prevalence of soil-transmitted helminth infections among primary school children, Uttar Pradesh, India, 2015. Infectious Diseases of Poverty. 6. 10.1186/s40249-017-0354-7.

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POSTER PRESENTATION

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2. Sarkar R., Ganguly S., ROS induced novel apoptotic pathway in *Giardia,* International Conference on Microbiology in the New Millennium: from Molecules to Community, Bose Institute, Kolkata, India.

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Functional characterization of phospholipase B enzyme from *Giardia lamblia*

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ABSTRACT

The microaerotolarent amitochondriate protozoan *Giardia lamblia* causes Giardiasis and produces a unique enzyme called Phospholipase B (PLB) in contrast to higher eukaryotes. The enzyme is produced upon induction with oxidative (H2O2) stress, thus leading to prostaglandin E2 (PGE2) production. It exists in dimeric form, and its molecular weight is 56 kDa. This PLB was extracellularly cloned in the pET21d vector. The ORF is 1620 bp (Genbank accession no. -OM939681) long and codes for a protein 539 amino acid long, with a 15 amino acid long amino-terminal signal peptide. The highest enzyme activity of PLB was identified at pH 7.5 and 35 °C. This specific enzyme was also active at 50 °C pH 10, but activity was low. We also analyzed the expression of PLB protein in *G. lamblia,* which was significantly induced under *increased* oxidative stress.

1. Introduction

Early-branched amitochondriate protozoa, *Giardia lamblia*, causes diarrheal diseases in humans and animals. Approximately 280 million cases of Giardiasis are reported globally (Lane and Lloyd, 2002).*Giardia* infection can be asymptomatic or symptomatic; symptoms include diarrhoea, abdominal discomfort, vomiting, malabsorption, and weight loss (Kamda and Singer, 2009). The life cycle of this intestinal parasite consists of two morphological forms: trophozoites and cysts. The trophozoite form colonizes in the small intestine, whereas the resistant cysts are responsible for the transmission of the disease through contaminated food and water. Previous epidemiological studies showed that humans, cattle, and other mammals can be the hosts for this parasite and chronic infection has a negative impact on the global economy (Giangaspero et al., 2005). Available data supports the zoonotic transmission of this parasite among different host species through contaminated water (Monis and Thompson, 2003; Smith et al., 2007; Bajer, 2008). After entry to the small intestine, two binucleated trophozoites are emerged from the cyst and localize in the duodenum. Inside the host gut environment, the trophozoites are exposed to complex and ever-changing hydrogen ion concentrations and comparatively high oxygen levels (60 μM) (Davenport, 1977; Atkinson and Zuckerman, 1980).

Reactive oxygen species (ROS) production is an inevitable consequence of normal oxidative metabolism. Low levels of ROS can play a role in cell signalling by acting as direct cellular stimuli or as receptor-directed stimuli (Balboa and Balsinde, 2006). When in high levels ROS exerts many direct and indirect effects on cell signalling pathways, which finally result in the induction of apoptosis or necrosis (England and Cotter, 2005). One of the effects of ROS on cells is lipoperoxidation, i.e., the oxidative modification of membrane phospholipids. Lipid peroxidation predominantly occurs at the sn-2 position of phospholipids, as most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. This position is also targeted by phospholipase A2 (PLA2) for cleavage (Cummings et al., 2000). Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms (Sapirstein and Bonventre, 2000). Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites (Balboa and Balsinde, 2006; Lister

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Epidemiology of Soil-Transmitted Helminth Infections among Primary School Children in the States of Chhattisgarh, Telangana, and Tripura, India, 2015–2016

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Abstract. Soil-transmitted helminth (STH) infections are highly prevalent in many developing countries, affecting the poorest and most deprived communities. We conducted school-based surveys among children studying in first to fifth standard in government schools in the Indian States of Chhattisgarh, Telangana, and Tripura to estimate the prevalence and intensity of STH infections during November 2015 and January 2016. We adopted a two-stage cluster sampling design, with a random selection of districts within each agro-climatic zone in the first stage. In the second stage, government primary schools were selected by probability proportional to size method from the selected districts. We collected information about demographic details, water, sanitation, and hygiene (WASH) characteristics and stool samples from the school children. Stool samples were tested using Kato-Katz method. Stool samples from 3,313 school children (Chhattisgarh: 1,442, Telangana: 1,443, and Tripura: 428) were examined. The overall prevalence of any STH infection was 80.2% (95% confidence interval [CI]: 73.3–85.7) in Chhattisgarh, 60.7% (95% CI: 53.8–67.2) in Telangana, and 59.8% (95% CI: 49.0–69.7) in Tripura. *Ascaris lumbricoides* was the most prevalent STH infection in all three states. Most of the STH infections were of light intensity. Our study findings indicate that STH infections were highly prevalent among the school children in Chhattisgarh, Telangana, and Tripura, indicating the need for strengthening STH control program in these states. The prevalence estimates from the survey would serve as a baseline for documenting the impact of the National Deworming Day programs in these states.

INTRODUCTION

Ascaris lumbricoides, *Trichuris trichiura*, and hookworms *Necator americanus* and *Ancylostoma duodenale* are the commonest soil-transmitted helminths (STHs), accounting for loss of nearly two million disability-adjusted life years.[1] STH are some of the most common infections contributing heavily to intestinal damage, anemia, and impaired physical growth and cognitive performance in children.[2] Periodic anthelminthic treatment reduces the number of individuals with heavy infections; reduces environmental contamination and risk of infection for other people; reduces micronutrient loss (e.g., iron loss through intestinal bleeding in hookworm infection); and improves nutritional status, cognitive function, and learning ability.[3] School-based deworming programs are considered as simple, safe, cost-effective, and scalable interventions to reach high-risk populations.[4,5]

In 2014, the WHO estimated that by number, India has the highest burden of STH infections in the world, with 223 million children aged 1–14 years at risk.[6] Although the published studies indicate heterogenous burden of STH in the country, with prevalence ranging from 0.6% to 91%, with *A. lumbricoides* as the predominant species,[7] large-scale surveys estimating the prevalence at the state level are limited. Such estimates are required to determine the frequency of preventive chemotherapy.[8] Results of two multi-site statewide surveys in Bihar ($N = 1,279$, conducted in 2011) and Uttar Pradesh ($N = 6,421$, conducted in 2015) indicated high STH prevalence, ranging between 68% and 76%.[3,9] Besides these two studies, information about STH prevalence and intensity data were not available from other Indian states.

Ad hoc deworming had been conducted in some states in India, often as part of other initiatives, particularly annual mass drug administration (MDA) using a single dose of 400 mg of albendazole in districts where lymphatic filariasis (LF) was endemic, and provision of deworming tablets within the Weekly Iron and Folic Acid Supplementation (WIFS) Program in some areas.[10] Although the reported coverage of MDA was generally higher (more than 80%),[11] there is no information about validated coverage of LF-MDA, and more importantly compliance. In 2014, the Government of India made concerted efforts to scale up STH control activities to meet the WHO global commitment to overcome the impact of neglected tropical diseases.[12] As a key step toward this, cross-sectional, cluster-sampled, school-based surveys were conducted in several States, including Chhattisgarh and Telangana (late 2015), and Tripura (early 2016)—totalling a geographic area of 314,568 km and population of over 67.5 million people.[13] The objective of these surveys was to estimate the prevalence and intensity of STH infections among school-aged children studying in first to fifth standard in these states. The secondary objectives were to estimate the prevalence according to age group, sex, and by agro-climatic zone and to develop geospatial predictive maps of STH prevalence, encompassing the environmental diversity of each state.

MATERIALS AND METHODS

Ethics, consent, and permission. The Institutional Ethics Committee of the Indian Council of Medical Research—National Institute of Epidemiology, Chennai, approved study protocols for each state. Written informed consent from parents of all students assenting to participate in the study

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PREVALENCE MAPPING OF SOIL TRANSMITTED HELMINTHS IN NORTH EASTERN STATES OF INDIA – FIRST REPORT.

Parasitology

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KEYWORDS

INTRODUCTION

Soil transmitted helminthiases (STH) is a major health problem in tropical and subtropical regions of the world, with 1.5 billion people affected worldwide. [1] There are four intestinal nematode species, namely roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*), and the hookworms (*Necator americanus* and *Ancylostoma duodenale*), that fall under the group of soil transmitted helminths. [2] Sub-Saharan Africa, East Asia, China, India and South America, contribute highest number of STH infections in the world. WHO estimates show that > 1.2 billion people are affected with ascariasis. Trichuriasis and hookworm infection have been estimated to be ~700–800 million each, globally. [3] The warm and moist climate of tropics and subtropics favours hatching and embryonation of the eggs. Soil contaminated with the egg or larvae leads to widespread infection of all the STH. [2] Besides, inadequate sanitation, open defecation, poor hygiene practices and socio-economic status in those countries contribute to high disease burden among population. STH infections causes nutritional deficiency, anaemia, growth retardation and impaired cognitive development which may result in absenteeism and disability adjusted life years (DALYs) loss. [2,4] School age children (SAC) are very much prone to be affected and normally harbour worm burdens greater than adults. According to WHO estimation, over 267 million preschool-age children and over 568 million school-age children are at risk of STH infection worldwide, of which 241 million are from India alone. [1, 4] WHO recommended annual or biannual school-based deworming can be an effective strategy to control infection in SAC. [4] Most of the studies on STH epidemiology in India have been carried out in northern, western and southern parts of this country. Currently there is limited information available on the STH disease burden in the North-eastern states viz. Assam, Manipur, Mizoram, Arunachal Pradesh, Meghalaya, Tripura and Nagaland. This pilot study, was conducted among the children studying in government primary schools in those states in order to generate a preliminary estimate of current prevalence and intensity of STH which would help in formulating deworming frequency.

MATERIALS AND METHODS

2.1. Ethics, and Permission

Permission to the conduct the survey in selected schools had been obtained from Ministry of Health & Family Welfare, Govt. of India as well as of each individual states and also from Ministry of Secondary Education of each state. This cross-sectional study was designed according to WHO recommendations and approved by Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases (NICED), Kolkata. Written consent was obtained from the guardians prior to including the school children in the survey.

2.2. Study setting

The North-east of India originally comprises 7 sister states viz. Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Assam. As per 2011 census the total population of Northeast India is around 45 million.

2.3. Sampling procedure and sample size

For collection of data, WHO recommended Sentinel site Approach was followed. A list of randomly selected primary schools from both rural and urban areas in each state was prepared. Fifty students (age group 5-12years), who were present on the day of survey, were selected at random from each school.

2.4. Data collection

The study was conducted by Division of Parasitology, ICMR-NICED, Kolkata, during December, 2015- February, 2016. The field team demonstrated the aim, objective and procedure of fresh stool sample collection to the Head of the respective school and to the guardians of all the participants. Guardians and participants both were interviewed for relevant information like sanitation system, drinking water system, house condition, family income etc.

2.5. Sample collection

Fresh stool sample, defecated in the morning, was collected in a plastic screw cap bottle. Bottles were put in a zip lock bag and delivered to the temporary laboratory facility in ice box within an hour of collection.

2.6. Stool sample processing

The sample was processed using the Vestergaard Frandsen Kato-Katz kit following the manufacturer's protocol [5]. Two slides were prepared for each sample and were checked for the helminth egg. From the slide count egg per gram was calculated to measure the intensity of the worm infection.

2.7. Quality control

For the purpose of quality control, 10% slides were rechecked. An independent expert also reviewed the slides and survey procedure as well.

2.8. Data analysis

Excel 2010 were used to calculate the prevalence and to prepare graphical representation of the collected data.

RESULTS

The cross-sectional study, included 20 primary schools in 7 Northeast states, majority of which were in rural areas, with a source of drinking water and toilet facilities within the premises. A total of 1116 individuals was enrolled in this survey, out of which 1071 (96%) faecal samples were properly collected and transported for examination, following the sample acceptance criteria. Rest of the samples (45) were rejected as they did not pass the acceptance criteria.

All of the study participants were in the age group of 5-12 years. 531

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ORIGINAL ARTICLE

Pyruvate Protects *Giardia* Trophozoites from Cysteine-Ascorbate Deprived Medium Induced Cytotoxicity

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Abstract: *Giardia lamblia*, an anaerobic, amitochondriate protozoan parasite causes parasitic infection giardiasis in children and young adults. It produces pyruvate, a major metabolic product for its fermentative metabolism. The current study was undertaken to explore the effects of pyruvate as a physiological antioxidant during oxidative stress in *Giardia* by cysteine-ascorbate deprivation and further investigation upon the hypothesis that oxidative stress due to metabolism was the reason behind the cytotoxicity. We have estimated intracellular reactive oxygen species generation due to cysteine-ascorbate deprivation in *Giardia*. In the present study, we have examined the effects of extracellular addition of pyruvate, during oxidative stress generated from cysteine-ascorbate deprivation in culture media on DNA damage in *Giardia*. The intracellular pyruvate concentrations at several time points were measured in the trophozoites during stress. Trophozoites viability under cysteine-ascorbate deprived (CAD) medium in presence and absence of extracellular pyruvate has also been measured. The exogenous addition of a physiologically relevant concentration of pyruvate to trophozoites suspension was shown to attenuate the rate of ROS generation. We have demonstrated that *Giardia* protects itself from destructive consequences of ROS by maintaining the intracellular pyruvate concentration. Pyruvate recovers *Giardia* trophozoites from oxidative stress by decreasing the number of DNA breaks that might favor DNA repair.

Key words: *Giardia lamblia*, oxidative stress, cysteine-ascorbate, antioxidant, pyruvate, reactive oxygen species, cytotoxicity

INTRODUCTION

Giardia lamblia, an enteric parasite causing giardiasis, has a simple life cycle consisting of encystation and excystation [1]. It is responsible for foodborne and waterborne diarrhea worldwide [2]. It imparts to an approximately 280 million symptomatic infections in human yearly [3], and it has been included as part of WHO Neglected Disease Initiative since 2004 [4]. The symptoms of giardiasis appear after 6-15 days of infection, and it is characterized by diarrhea or greasy stool, fatigue, nausea, bloating, abdominal cramps, excessive gas, and headache [5]. The impact of clinical complications is high in case of malnourished children and immunodeficient individuals. Although it cannot cross intestinal lining and does not secrete any toxin, recent data suggest that *Giardia* increases intestinal permeability by induction of apoptosis of intestinal epithelial cells. There are many drugs available, but metronidazole is the most commonly used drug for treatment. It is incapable to tolerate elevated oxygen pressure and in the upper intestine where this organism generally inhabits, the oxygen (O_2) concentration there has been measured at 60 μM [6]. In addition to this, the amitochondriate parasite lacks some of the conventional enzymes that detoxify reactive oxygen species (ROS), like superoxide dismutase (SOD), catalase, peroxidase, and glutathione reductase [7]. Cysteine is not incorporated de novo and is not synthesized from cystine. It appears to be imported into the cell by passive diffusion, although active transport may account for some of the acquisition of cysteine [8]. Trophozoites containing free thiol (-SH) groups on their surface was described previously by the toxicity of thiol-blocking agents that are unable to penetrate intact cells [9]. It suggests that the toxicity is due to the reaction between the agents and surface proteins [10]. The trophozoites

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Infectious Diseases of Poverty

RESEARCH ARTICLE Open Access

High prevalence of soil-transmitted helminth infections among primary school children, Uttar Pradesh, India, 2015

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Abstract

Background: Soil-transmitted helminth (STH) infections often affect the poorest and most deprived communities. In order to generate reliable data for planning a school based deworming program, we conducted a survey among primary school children studying in government schools in the Indian state of Uttar Pradesh. The objectives of our survey were to estimate the prevalence and intensity of STH infections.

Methods: We conducted a cross-sectional survey among children studying in 130 primary schools from 9 agro-climatic zones, during May – August 2015. Information about socio-demographic details, defecation and hand-hygiene practices, and stool samples were collected from the school children. Stool samples were examined using the Kato-Katz method.

Results: Stool samples from 6421 school children were examined. The overall weighted prevalence of any STH in the State was 75.6% (95% *CI*: 71.2–79.5). The prevalence was more than 50% in six of the nine agro-climatic zones. *A. lumbricoides* was the most prevalent STH (prevalence: 69.6%), followed by hookworm (prevalence: 22.6%) and *T. trichura* (4.6%). The majority of the STH infections were of low intensity. The practice of open defecation and not washing hands with soap after defecation and residence in kutcha house were significant risk factors of STH infection.

Conclusions: STH prevalence among primary school children in Uttar Pradesh was high. Given the WHO guidelines on deworming frequency according to STH prevalence, Govt of Uttar Pradesh needs to implement a school-based deworming program with bi-annual frequency. The findings of our survey would also help monitor the performance of school based deworming programme.

Keywords: Soil-transmitted helminths, Uttar Pradesh, India

Multilingual abstracts

Please see Additional file 1 for translations of the abstract into the five official working languages of the United Nations.

Background

Infection with four species of nematodes – the roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*), and the hookworms (*Necator americanus* and *Ancylostoma duodenale*), collectively referred to as soil-transmitted helminths (STH), are among the most common neglected tropical diseases worldwide [1]. STH are widely distributed in tropical and subtropical areas with warm and moist climates, and are more prevalent where sanitation and hygiene of the population is poor. The World Health Organization (WHO) estimates that 880 million children are at risk of STH infection worldwide and require treatment [2], of which 241 million are in India [3]. The greatest numbers of STH infections occur

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