Chapter 11

Summary
11. Summary

*Giardia lamblia* is a unicellular, early branching eukaryote causing giardiasis, one of the most common human enteric diseases. *Giardia*, a microaerophilic protozoan parasite has to build up mechanisms to protect themselves against oxidative stress within human gut (oxygen concentration 60µM) to establish its pathogenesis. *Giardia lamblia* is devoid of conventional mechanisms of oxidative stress management system, including superoxide dismutase, catalase, peroxidase, and glutathione cycling, which are present in most eukaryotes. NADH oxidase is a major component of the electron transport chain of *Giardia lamblia*, which in concurrence with disulphide reductase, protects oxygen-labile proteins such as pyruvate: ferredoxin oxidoreductase against oxidative stress by sustaining a reduced intracellular environment. It also contains the arginine dihydrolase pathway, which occurs in number of anaerobic prokaryotes, includes substrate level phosphorylation and adequately active to make a major contribution to ATP production.

To study differential gene expression under three types of oxidative stress, a *Giardia* genomic DNA array was constructed and hybridized with labeled c-DNA of without stressed and stressed cells. The transcriptomic data has been analyzed and further validated using Real Time PCR. We identified that out of 9,216 genes represented on the array, more than 200 genes encoding proteins with functions in metabolism, oxidative stress management, signaling, reproduction and cell division, programmed cell death and cytoskeleton. We recognized genes modulated by at least ≥2 fold at a significant time point in response to oxidative stress.

The study has highlighted the genes that are differentially expressed during the three experimental conditions regulates the stress management pathway differently to achieve redox homeostasis. Identification of some unique genes in oxidative stress regulation may help in new drug designing for this common enteric parasite prone to drug resistance. Additionally, these data suggest the major role of this early divergent ancient eukaryote in anaerobic to aerobic organism evolution.

*Giardia lamblia*, a microaerophilic protozoon produces energy by fermentative metabolism. It is devoid of conventional mechanisms of oxidative stress management, including superoxide dismutase, catalase, peroxidase, and glutathione cycling, which are present in most eukaryotes. To protect and establish its pathogenesis within human gut it has to develop antioxidant defense strategies to grapple with elevated oxygen tensions, which are harmful for its survival.
Intracellular reactive oxygen species (ROS) generation by *Giardia* suspension was monitored with the help of a dichlorodihydrofluoresceine diacetate based assay. Linoleic acid micelles were employed to investigate the lipid radical scavenging activity of pyruvate. In this study, we examined the effects of pyruvate addition during oxidative stress on DNA damage in *Giardia*. The pyruvate concentration at different time points were measured during different oxidative stress conditions in *Giardia*.

Our results provide evidence that exogenously added pyruvate was also able to inhibit lipid peroxidation of stressed *Giardia* and effects of pyruvate were concentration dependent but no inhibition of lipid peroxidation by pyruvate was observed in the micelle model. We have demonstrated trophozoites have the ability to regulate intracellular level of pyruvate during oxidative stress. Pyruvate recovers *Giardia* trophozoites from oxidative stress by decreasing the number of DNA breaks and might favor DNA repair.

Our results clearly denote that pyruvate acts as a protector and a key regulatory factor of stressed *Giardia lamblia*.

We previously reported that cysteine-ascorbate deprivation induces apoptotic like cell death in *Giardia lamblia*. It was further investigated upon the hypothesis that metabolic oxidative stress was the causative process behind the cytotoxicity. In the present study, the effect of pyruvate as a physiological antioxidant on oxidative stress in *Giardia* by cysteine-ascorbate deprivation has been explored. In this study, we have examined the effects of pyruvate addition, during cysteine-ascorbate associated deprivation stress on DNA damage in *Giardia*. The pyruvate concentration at different time points were measured during cysteine-ascorbate deprivation stress conditions in *Giardia*. The exogenous addition of a physiologically relevant concentration of pyruvate to *Giardia* suspensions 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 favour DNA repair.

Metronidazole has been used for the treatment of infections for giardiasis. Anaerobic parasitic infections caused by different protozoan parasite respond favorably to metronidazole therapy. The trophozoites must fight against oxidative stress generated by metronidazole. Metronidazole reduction was driven by pyruvate, but progressive damage to the radical generating system was observed. There are different enzymes involved in response to
metronidazole stress in *Giardia* such as pyruvate ferredoxin oxidoreductase, NADH oxidase and peroredoxin etc.

The present study aims to establish the effects of pyruvate in *Giardia* exposed to metronidazole treatment. Intracellular reactive oxygen species (ROS) generation by *Giardia* suspension was monitored in the presence and absence of pyruvate with the help of a dichlorodihydrofluoresceine diacetate (H$_2$DCFDA) based assay. In this study, we examined the effects of pyruvate addition during metronidazole stress on DNA damage in *Giardia*. We have investigated the expression levels of some genes to show their relevance to metronidazole stress.

Exogenously addition of physiologically relevant concentration of pyruvate was shown to induce the rate of ROS generation in *Giardia* suspension treated with metronidazole. Our results provide evidence that exogenously added pyruvate was also induce lipid peroxidation of stressed *Giardia*. pyruvate can reduce metronidazole and form different types of nitroso radical derivative which can damage DNA. We have shown that expression levels of different metabolic genes which are significantly up or down regulated during metronidazole treatment. This suggests that these genes are involved in combating against metronidazole.

In this study, we demonstrate that metronidazole radical anions are generated in the cytoplasm of *Giardia lamblia* under metronidazole exposure previously incubated with pyruvate as a source of reducing power, and that these free radicals can arrive at the organelle membrane and produce lipid radicals by lipid peroxidation and undergoes apoptotic death.

*Giardia lamblia*, anaerobic, unicellular protozoan, causative agent of diarrhoeal disease, resides in the small intestinal lumen in close apposition to epithelial cells. Since the concrete pathway of disease mechanisms of giardiasis are poorly understood, exposing the parasite under different oxidative stress conditions to mimic the environment like the intestinal tract of the host to provide clues to understand the pathogenesis. Here we examined the hypothesis that with the exposure of the different oxidative stress with *Giardia lamblia* trophozoites might lead to release of specific proteins. The proteome of *Giardia lamblia* at its untreated conditions were compared with the trophozoites treated with hydrogen peroxide, cysteine-ascorbate deprived medium and metronidazole by using two-dimensional SDS-PAGE gel electrophoresis. Protein spots that increased in the extracts of treated trophozoites compared to untreated trophozoites were identified by MALDI-TOF mass spectrometry.
Expression patterns of five of the identified proteins were examined during exposure of the hydrogen peroxide, cysteine-ascorbate deprived medium and metronidazole by real-time PCR. The secreted *Giardia lamblia* proteins were localized to the cytoplasm and the inside of the plasma membrane of trophozoites.

In trophozoites of *Giardia*, hydroxyl and superoxide radicals are generated during oxidative stress (Fig. 11.1). These radicals then initiate membrane lipid peroxidation. Thus, pyruvate, by eliminating hydroxyl and superoxide radical, prevented lipid peroxidation. It was exciting to observe that acetate showed inhibition of lipid peroxidation but the key mechanism still remains unknown. In our present study, it was shown that intracellular pyruvate concentration was found to be increasing and it decreased after several hours. It can be explained by the inactivation of pyruvate: ferredoxin oxidoreductase, an enzyme which is sensitive to oxidative stress. However, it might also result from a metabolic need. Pyruvate concentration can also be increased by the activities of pyruvate dikinase and malate dehydrogenase that *Giardia* can produce more pyruvate. This is indicating an enhancement of glycolysis activity in the presence of pyruvate. Increased glycolysis and arginine metabolism might thus maintain the ATP supply during cysteine-ascorbate deprived medium stress. Such an increase in the intracellular pyruvate in response to elevated O$_2$ concentration has been reported to have protective effects against oxidative stress (Herbener, 1976).

![Figure 11.1: Schematic representation of the oxidative stress management in *Giardia*](image)

(A) Healthy cell, (B) Stressed cells accumulates intracellular ROS, (C) Concentration of intracellular ROS generation augmented, (D) Intracellular pyruvate concentration increases by the up-regulation of malate dehydrogenase and pyruvate phosphate dikinase. (E) As ROS
generation is a cyclic and also a spontaneous process it produces alcoxyl, peroxyl or lipid radicals by lipid peroxidation. (F) Lipid radicals are not scavenged by pyruvate so it produces more lipid radicals. (G) Intracellular acetate concentration is up-regulated as acetyl CoA synthase up-regulated. (H) On further stress, as pyruvate ferredoxin oxidoreductase is down-regulated so acetate production is inhibited and ultimate fate is death.

As because of the down regulation of pyruvate ferredoxin oxidoreductase on further stress acetate production is inhibited and as trophozoites have no option remains for survival strategy commits suicide (Fig.11.2). Calcium up-regulation occurs as the consequence of cellular signals and prostaglandin pathway activates. Arachidonic acid released due to the activity of phospholipase A2 on sn-2 position of phospholipids.

![Cells after committed suicide](image)

**Figure 11.2: Flow chart of probable mechanism of apoptotic like death in *Giardia lamblia*.**

The calcium regulation revealed that intracellular free calcium is required to activate a pathway which is called prostaglandin pathway. Intracellular unesterified arachidonic acid signals apoptosis. To confirm the prostaglandin production we have measured concentration of prostaglandin in both normal and stressed *Giardia*. This is the first study that we have
identified the presence of prostaglandin pathway in *Giardia lamblia*. So, due to oxidative stress arachidonic acid induced calcium dependent prostaglandin mediated apoptotic like death occurs in *Giardia lamblia*.

This study has unveiled the importance of learning the total effects of alterations in the level of intermediary metabolites in causing stress and coping with it by transducing signals to genes to reach a stable state of equilibrium. This study has revealed the important clinical and mechanistic associations between oxidative stresses and specific metabolites or metabolite groups. We have generated information from our study that provides a compendium of associations between pathological situations with oxidative stress and metabolites. This study will be helpful to learn key mechanistic aspects and the information generated from the experiments can be used to develop methods that serve as a tool for chemotherapeutic interventions and new drug designing.