Involvement of pyruvate on oxidative stress management in *Giardia lamblia* upon H$_2$O$_2$ exposure
5.1 Introduction:

*Giardia lamblia* (also known as *Giardia intestinalis* or *Giardia duodenalis*) a microaerotolerant, unicellular, gastrointestinal flagellated protozoan causes one of the most common parasitic infections worldwide (Adam, 2001). It lacks conventional mitochondria, peroxisomes and Golgi body (Elias *et al*., 2008) and contributes to an estimated 280 million symptomatic human infections (called giardiasis) every year (Lane & Lloyd, 2002). The symptoms of giardiasis are watery diarrhea, epigastric pain, nausea, vomiting, irritable bowel syndrome and weight loss and it appears 6-15 days after infection (Farthing, 1997). The clinical impact is stronger in case of children with malnutrition and in immunodeficient or undernourished individuals. *Giardia* species can’t invade the gut and secrete no known toxin but recent data suggest that *Giardia* increases intestinal permeability by induction of apoptosis of intestinal epithelial cells (Farthing, 1997; Scott *et al*., 2002; Singer & Nash, 2000).

The anaerobic *Giardia lamblia* rely on fermentative metabolism for ATP production as energy source (Brown *et al*., 1998). Carbohydrates (glucose) are converted to various organic compounds that include alanine, ethanol, acetate and CO₂ (Brown *et al*., 1998; Biagini *et al*., 1998). Pyruvate formed by glycolysis is a key intermediate of energy metabolism and is the precursor of most metabolic end products which are dependent on the ambient values for oxygen tension (Paget *et al*., 1993). Like *Entamoeba histolytica*, *Trichomonas vaginalis*, *Dasytricha ruminantium*, in *Giardia lamblia* the conversion of pyruvate to acetate, an essential metabolic sequence occurs in the cytosol which is the site of all steps of energy metabolism. The energy of thioester bond of acetyl-CoA is always conserved by substrate level phosphorylation. Thus acetate formation plays a crucial role in *Giardia*'s energy metabolism. it has also been postulated that change in redox state of NAD(P)H pools affect the relative rates of end product formation (Mead, 1976). All prokaryotic and eukaryotic cells are known to have a capability to restructure their transcriptomes in order to adapt to the environmental conditions by sensing the endogenous level of various metabolites (Husain *et al*., 2011).

In the environment of high oxygen concentration *Giardia lamblia* consume oxygen up to a threshold level depending on the Species, above which consumption is arrested due to the formation of ROS (Biagini *et al*., 1997; Lloyd *et al*., 2000). There are some resemblances in energy metabolism of *Giardia lamblia* with bacteria. *Giardia* contains the eubacterial like
pyruvate:ferredoxin oxidoreductase (Townson et al., 1996) and pyro-phosphate dependent glycolytic enzymes (Mertens, 1990; Phillips et al., 1997). In *Giardia* cysteine replaces glutathione as the major intracellular pools (Brown et al., 1993) and it has the arginine dihydrolase pathway as an additional energy source (Schofield et al., 1990; Dimopoulos, 2000). The antioxidant defense strategies are totally different from eukaryote. Superoxide dismutase, catalase and non-specific peroxidase activities are imperceptible in *Giardia lamblia* (Brown et al., 1995) but it possesses a thioredoxin reductase like disulphide reductase which has the ability to reduce cysteine (Brown et al., 1998). A recent study has shown that peroxiredoxins are suggested to play an important role in the antioxidant defense of *Giardia* (Mastronicola et al., 2014).

Indeed, pyruvate and α-ketoacids, abundantly present in mammalian cells, can react nonenzymatically with H$_2$O$_2$ (Holleman, 1904) and through this reaction CO$_2$ is liberated and α-ketoacids is converted into the corresponding carboxylic acid:

\[
R\text{-COCOOH} + H_2O_2 \rightarrow R\text{-COOH} + CO_2 + H_2O
\]

The α-keto carbonyl in pyruvate makes it a potent scavenger of reactive oxygen species, particularly H$_2$O$_2$ (Bunton, 1949; Fink, 2001). Pyruvate enters into the cells with the help of monocarboxylate transporter (Kim et al., 2005; Lin et al., 1998). In the present study, two conditions have been preferred to generate oxidative stress in the trophozoites in vitro. Firstly, hydrogen peroxide is a very well-known reactive oxygen species generator. Second is a modified medium that is deprived of cysteine and ascorbic acid. However, the effect of pyruvate in *Giardia* is poorly documented during oxidative stress. In this study, we uncouple the antioxidant potential and metabolic importance of pyruvate in *Giardia lamblia* to identify the molecular components and their mode of action.

### 5.2 Materials and Methods:

**Chemicals**

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

**5.2.1 *Giardia lamblia* Culture**

*Giardia lamblia* Portland1 strain trophozoites were maintained in TYIS-33 medium, supplemented with Penicillin (100 U/ml), Streptomycin (100 mg/ml) and 10% adult bovine serum. All experiments were conducted with trophozoites that had been harvested during the
mid-exponential phase of growth by centrifugation at 2000 rpm for 10 min and resuspended in PBS buffer (PH-7.2) containing 150 mM NaCl, 5 mM K$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$. Pyruvate (sodium salt), menadione, H$_2$O$_2$, acetate, malate, α-ketoglutarate, mannitol, oxaloacetate etc. solutions were prepared freshly on the day of experiment. For experimentation, same set of cells were taken for individual experiment.

5.2.2 Incubation of Cells

Dose and time kinetics of the two different oxidative stress generating conditions have been standardized following the IC$_{50}$ values as reported previously (Raj et al., 2014). Finally, from the standardized data, 0.1 µM H$_2$O$_2$ have been administered to all the long term experiments mentioned below. However, for the short term experiments and for the experimental purposes trophozoites were exposed to 10 µM H$_2$O$_2$ concentration during the study.

5.2.3 Imaging of ROS burst in *Giardia* trophozoites using laser scanning confocal microscopy

Intracellular generation of reactive oxygen intermediates were evaluated by using dichlorodihydrofluoresceine diacetate (H$_2$DCFDA) fluorescent probe according to Schuessel et al., (2006) with some modifications. For monitoring intracellular ROS production, treated and untreated cells were (2x10$^6$ cells/ml) incubated with H$_2$DCFDA (1.5 µM) for 15 min at 37 °C. These cells were previously incubated with different metabolites (pyruvate, acetate, ascorbate etc.) at same concentrations (2 mM). H$_2$DCFDA is able to permeate biological membranes and therefore we have not used any mild detergent for permeabilization and the cells were fixed with 2% paraformaldehyde after washing them with PBS twice. Subsequently, observations were made with a confocal microscope (LSM510, Meta; Carl Zeiss, Thornwood, NY, USA). It should be noted that at least 100 cells/group with identical morphology and with same gain were observed for each condition.

5.2.4 Measurement of total ROS generation in the trophozoites of *Giardia* by using spectrofluorometer

The determination of intracellular oxidant production is based on the oxidation of H$_2$DCFDA to the fluorescent dichlorofluoresceine. *Giardia* trophozoites (approx. 4x10$^6$ cells/ml) were incubated in the presence and absence of different concentration of pyruvate (0-5 mM) in TYIS-33 medium under H$_2$O$_2$ treatment. After 1 h of incubation, medium was removed and trophozoites were washed with PBS. After that, ROS levels in treated and non treated samples were examined. Levels of ROS were measured by a spectrofluorometer.
(QuantaMaster30, Photon Technology International). After addition of H$_2$DCFDA, fluorescence emission was measured continuously at 530 nm after excitation at 488 nm.

### 5.2.5 Quantitative assessment of trophozoites viability based on flowcytometry

Treated and non-treated trophozoites previously incubated with or without pyruvate were harvested and aliquot were made up to 2 x 10$^6$ cells/100 µL into micro centrifuge tubes. Trophozoites were washed two times by adding 1 mL of PBS, centrifuged at 2000 rpm for 10 minutes, and then the buffer was decanted to obtain the pelleted trophozoites. Trophozoites were resuspended in 100 µL of Flow Cytometry Staining Buffer. To adjust flow cytometer settings for PI, 5 µL of PI staining solution was added to a control tube of otherwise unstained cells. The tube was shaken gently and incubated for 1 minute in the dark. Determination of PI fluorescence with a Becton-Dickinson FACSARIA-III flowcytometer (BD Biosciences, San Jose, USA) instrument was performed. Trophozoites were acquired immediately after staining and kept on ice in the dark until measurement.

### 5.2.6 Monitoring of intracellular ROS production induced by menadione in *Giardia* trophozoites

Trophozoites were first washed with fresh medium and pre-incubated for indicated times in the presence of different metabolites. Trophozoites were then incubated in the same fresh medium with H$_2$O$_2$ and menadione (2-methyl-1, 4-napthoquinone) for the indicated time. After the incubation period, trophozoites were washed with PBS and added H$_2$DCFDA (1.5 µM at final concentration). The H$_2$DCFDA loaded cells were incubated in the dark at 37 °C for 15 min. Then, the cells were washed twice with PBS (2000 rpm, 5 min) and resuspended in PBS solution for flowcytometric analysis as described in the previous section. Cells were acquired immediately after staining and always kept on ice in the dark until measurement. All data were analyzed by WinMDI 2.9 software.

### 5.2.7 Effect of pyruvate ion on linoleic acid micelle autoxidation

Fatty acid micelles are a good model to study membrane lipid peroxidation. It has been known that propagation of lipid peroxidation involves the peroxyl radical, a lipid radical (Mead, 1976). The scavenging capacity for lipid radical was measured according to a modified method of Groussard *et al.*, (2000). Linoleic acid (9, 12-octadecadienoic acid) (concentration 10 mM) was dispersed with 0.5% Tween 20 (Merck) in 10 mM phosphate buffer (pH 9) under nitrogen atmosphere. Pyruvate was dissolved in phosphate buffer (pH 7.2, 5 mM) at initial concentration of 0.0001 mM, 0.001 mM, 0.01 mM, 0.1 mM, 0.5 mM, 1
mM, 2 mM respectively and stored at +4 °C. Aliquots of each stock solution were adjusted to pH 7.4 and mixed at time zero to achieve a final linoleic acid concentration of 2.5 mM. Samples were placed in a glass tube and kept in the dark under air at 37 °C. We used tocoferol as a positive control and without metabolite as negative control. Linoleic acid autoxidation was determined by conjugated diene measurement. Measures were performed by using a spectrophotometer (JASCO, V-660) with a UV lamp set at 234 nm.

5.2.8 Estimation of lipid peroxidation induced by H₂O₂ in cultured Giardia trophozoites

5.2.8.1 Sample preparation

The treated and untreated cells (approx. 4x10⁷ cells/ml) were harvested by centrifugation at 2000 rpm for 10 min and resuspended in PBS buffer (pH 7.2) containing 150 mM NaCl, 5 mM K₂HPO₄ and 1.8 mM KH₂PO₄. Washed cells were homogenized in ice-cold PBS in a proportion of 5x10⁶ no. of cells in 1ml of PBS. The homogenates were centrifuged for 15 min, 10000g at +4 °C. Supernatant was collected and 125 μl of 20% trichloroacetic acid was added and mixed properly, then centrifuged at 15000g for 10 min at +4 °C. Supernatant was collected and mixed with 200 μl of 0.8% thioburbituric acid (TBA) reagent and then the mixture was incubated at +100 °C for 60 min. The mixture was kept at room temperature and used for spectrophotometric analysis.

5.2.8.2 Measurement of MDA concentration by spectrophotometer

The process of lipid peroxidation results in the formation of malondialdehyde (MDA). This is a later product in the sequence of lipid peroxidation reactions (Evans et al., 1999; Rael et al., 2004). The TBA assay or MDA assay was used to assess the MDA concentration as described by the Bar-Or et al., (2001) with few modifications. The absorbance of the chromophore was measured at 535 nm. The MDA concentration is presented as nmoles of MDA produced/mg protein using a molar extinction coefficient of 1.56 x10⁵ M⁻¹cm⁻¹.

5.2.9 Extraction and quantification of intracellular pyruvate concentration at different time points under H₂O₂ stress

Intracellular pyruvate concentration was quantified under different oxidative stressed conditions by using Pyruvate Assay Kit (ab65342). Trophozoites (4x10⁶ cells/ml) were incubated in TYIS-33 medium and exposed to H₂O₂ for 0 to 8 h. After homogenization, the mixture was centrifuged for 15 min, 10000g at +4 °C. 6% perchloric acid (PA) was added to lyse the cells and inactivate the enzyme. Supernatant was collected and used for pyruvate
assay according to the manufacturer’s protocol. The pyruvate sample concentration was
determined according to a standard curve established between 0 and 0.5 mM pyruvate.

5.2.10 DNA fragmentation assay of untreated and stress-induced *Giardia* trophozoites
Pellets of *Giardia* trophozoites (5x10^6/ml) from untreated, H_2O_2-treated were taken. These
were previously incubated with or without pyruvate for 8 h at 35.5 °C. Trophozoites were
harvested and resuspended in digestion buffer (10 mM EDTA, 50 mM Tris, 0.5% SDS
Sarcosine, pH=8.0) containing 0.5 mg/ml proteinase K. Samples were incubated at 37 °C for
1 h after adding DNase-free RNase (0.1 mg/ml). After phenol–chloroform treatment, salt
precipitation and 70% ethanol wash, the pellet was air dried and resuspended in autoclaved
triple distilled water and checked in a 1.5% agarose gel stained with ethidium bromide. DNA
fragmentation assay was also performed with 8 h stress-induced trophozoites reseeded in
fresh TYI-S-33 medium (H_2O_2 free) after 24 h.

5.2.11 Quantifications of cell death with different substrates during stress recovery
Manifestations of cell death were sought using Annexin-V FITC assay kit (IM3546). As
H_2O_2 have been reported to induce DNA damage (Ghosh *et al*., 2009), we have determined
the effects of pyruvate during oxidative stress on DNA damage in *Giardia lamblia*. Trophozoites of *Giardia lamblia* were incubated for 8 h under H_2O_2 with different substrates
at same concentration (2 mM). Then cell samples were washed with ice-cold PBS and
centrifuged for 5 min at 2000 rpm at 4 °C. After that the assay was performed according to
the manufacturer’s protocol and analyzed by flowcytometry.

5.2.12 Gene expression studies
To quantify gene expression by real-time PCR (qRT–PCR), trophozoites were grown until
near confluence was reached. Cells were harvested as described (Raj *et al*., 2014), and RNA
was extracted using the TRIZOL (Invitrogen) method, including a DNase I digestion (to
remove residual genomic DNA) according to the instructions provided by the manufacturer.
RNA was eluted with 20 µL of nuclease-free water and stored at -80 °C.

First-strand cDNA was synthesized using the M-MuLV RT kit (New England Biolabs) as described by the manufacturer with oligo-dT primer for subsequent real-time
PCR (for primer sequences, see Table 5.1). Quantitative PCR was performed with 10 µL of
1:100 diluted cDNA using the FastStart Universal SYBR Green Master (ROX) Kit (Roche)
in a 50 µL standard reaction containing a 0.5 µM concentration of forward and reverse primers (Sigma, USA).

Furthermore, a control PCR included RNA equivalents from samples that had not been reverse transcribed into cDNA (data not shown) to confirm that no DNA was amplified from any residual genomic DNA that might have resisted DNase I digestion (see above). PCR was started by initiating the Taq polymerase reaction at 95 °C (15 min). Subsequent DNA amplification was performed in 40 cycles including denaturation (94 °C for 15 s); annealing (60 °C for 30 s); and extension (72 °C for 30 s). Fluorescence was measured at 72 °C during the temperature shift after each annealing phase. For statistical analysis, three independent experiments were performed. Livak $^{\Delta \Delta C_T}$ method has been adopted to analyze the real time data. Expression levels of the genes were given as values in arbitrary units relative to the amount of constitutively expressed ‘house keeping’ gene actin.

5.2.13 Statistical analysis of results

Each experiment was performed at least thrice in triplicates and the results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was evaluated by t-test or one way ANOVA followed by Kruskal-Wallis test (wherever applicable), using Graph Pad Prism software, version 4 (GraphPad Software Inc, San Diego, CA); P<0.05 was considered as statistically significant.

5.3 Results:

5.3.1 Effects of pyruvate on ROS burst in Giardia trophozoites

The H$_2$DCFDA, a non-fluorescent molecule, is able to enter the cells. After getting entry into the cytosol of the cells esterase activity renders the indicator non-permeate by forming fluorescent product dichlorofluoresceine and the fluorescence intensity of the dye is proportional to the rate of oxidation by reactive oxygen species. To observe cellular fluorescence in the trophozoites, they were examined by using confocal microscopy under different stress conditions. Our results demonstrated that exogenously added physiological concentrations of pyruvate can alter the effective lifetime of reactive oxygen species by scavenging them. The confocal study revealed that pyruvate scavenged ROS production in the stressed Giardia trophozoites (Fig.5.1).
Figure 5.1: H$_2$DCFDA-loaded cells under confocal microscope after H$_2$O$_2$ stress. Increases in fluorescence are representative of increase in the rate of oxidative species generated. Fluorescence was monitored from the suspension of live cells after the addition of H$_2$O$_2$ (10 µM) in the absence and presence of pyruvate (2 mM) and acetate (2 mM). We used mannitol (2 mM) and ascorbate (2 mM) as a positive control and without metabolite as a negative control.

5.3.2 Pyruvate decreases total ROS production in *Giardia* trophozoites during hydrogen peroxide exposure

Antioxidant properties have been widely ascribed to pyruvate. We therefore have investigated whether pyruvate might have those antioxidant capacities in our experimental conditions. Trophozoites were previously incubated with increasing concentrations of pyruvate from 0.0001 to 5 mM and then exposed to hydrogen peroxide (10 µM). The level of ROS was measured in *Giardia* trophozoites with or without pyruvate. The ROS level was increased significantly ($P < 0.01$) by the addition of H$_2$O$_2$ than H$_2$DCFDA-loaded untreated trophozoites. The fluorescence intensity was lowered significantly ($P < 0.05$) in the presence of pyruvate for the range of concentrations from 2 to 5 mM (Fig.5.2). In our experimental conditions, pyruvate could thus decrease the generation of oxidative stress induced by hydrogen peroxide in *Giardia* trophozoites and may also have a direct antioxidant role in *Giardia lamblia* trophozoites.
Pyruvate decreases the level of ROS in the medium of Giardia lamblia trophozoites exposed to H₂O₂. Giardia trophozoites were incubated in TYIS-33 medium under 10 µM H₂O₂ exposure and exposed to increasing doses of pyruvate (from 0-2mM). Levels of ROS were estimated by spectrofluorometry using 2’, 7’-dichlorodihydrofluoresceine diacetate. The data are from three representative experiments.

5.3.3 Pyruvate protects Giardia trophozoites from the toxicity induced by endogenous H₂O₂

Flow cytometry was used to confirm the antioxidant activity of pyruvate in Giardia trophozoites. Cultured trophozoites were incubated for 2.5 h with increasing concentration of sodium pyruvate in the presence of H₂O₂ (10 µM). H₂O₂ caused reduction of trophozoites viability to 40.79%, which was significantly lower than the untreated trophozoites (94.66%, P < 0.001) (Fig.5.3). Viability of the trophozoites previously incubated with Pyruvate (2 mM) and (5 mM) protects trophozoites significantly at 69.7% and 73.2% (P < 0.05) from H₂O₂ toxicity. When exposed to 10 µM H₂O₂ trophozoites were progressively protected by increasing concentrations of pyruvate. Sodium acetate (2 mM), the decarboxylation product of sodium pyruvate, did not modify the viability of Giardia either in control conditions or in the presence of 10 µM H₂O₂ (39%, P < 0.001).
Figure 5.3: Pyruvate protects *Giardia* trophozoites from H$_2$O$_2$ induced toxicity. Cultured *Giardia* trophozoites were incubated at 35.5 °C under H$_2$O$_2$ (10 µM) for 2.5 h with increasing concentration of pyruvate. Pyruvate and H$_2$O$_2$ were simultaneously applied to the trophozoites. Stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (H$_2$O$_2$ free) and their viability was evaluated after 24 h by using flow cytometry. Acetate was shown not to decrease the rate of ROS generation in *Giardia*. Results are expressed as the percentage of surviving trophozoites compared with control culture. Data are the mean ± SEM of three independent experiments, each performed in triplicate. *P<0.05, compared with control; #P<0.001, compared with stressed sample.

5.3.4 Pyruvate protects trophozoites from the toxicity induced by menadione

The capacity of pyruvate to protect trophozoites against oxidative stress was investigated with the use of menadione. Like other quinones, menadione enters flavoprotein catalyzed redox cycles with molecular oxygen and these results in the formation of large amounts of O$_2^-$. The H$_2$DCFDA assay has been adapted to measure oxidant production in *Giardia lamblia* trophozoites (Biagini et al., 2001) during different oxidative stress conditions. A progressive increase in the production of reactive oxygen species (as indicated by the augment in fluorescence intensity) with the addition of H$_2$O$_2$ (10 µM) increased the rate of reactive oxygen species generation (Fig. 5.4). Prior incubation of H$_2$DCFDA loaded cells with increasing concentration of pyruvate (0.01 mM, 0.5 mM, and 2 mM) for 1 h attenuated the stressed induced fluorescence. Exposure of *Giardia* trophozoites in increasing concentration...
of menadione for 1 h induced ROS generation that was significantly reduced by 2 mM sodium pyruvate.

![Figure 4.](image)

**Figure 5.4: Pyruvate protects trophozoites from menadione-induced toxicity.**
Trophozoites were incubated at 35.5 °C under H$_2$O$_2$ (10 µM) for 2.5 h with increasing concentration of menadione in the absence and presence of pyruvate. Trophozoites were then washed and further incubated for 1 h with or without pyruvate and replaced into the initial culture medium supplemented with the corresponding concentrations of pyruvate. Viability was estimated 24 h later. The various cell populations reflect different treatments: (a) fluorescence arising from H$_2$DCFDA loaded without H$_2$O$_2$ treated cells, (b) H$_2$DCFDA-loaded cells treated with H$_2$O$_2$ (10 µM) previously incubated with pyruvate (2 mM), (c) H$_2$DCFDA-loaded cells treated with H$_2$O$_2$ (10 µM) previously incubated with pyruvate (0.5 mM), (d) H$_2$DCFDA-loaded cells under H$_2$O$_2$ (10 µM) stress previously incubated with pyruvate (2 mM) in the presence of menadione (10 µM), (e) H$_2$DCFDA-loaded cells under H$_2$O$_2$ (10 µM) stress previously incubated with pyruvate (2 mM) in the presence of menadione (30 µM), (f) H$_2$DCFDA-loaded cells under H$_2$O$_2$ (10 µM) treatment, (g) H$_2$DCFDA-loaded cells under H$_2$O$_2$ (10 µM) previously incubated with menadione (10 µM), (h) H$_2$DCFDA-loaded cells under H$_2$O$_2$ (10 µM) previously incubated with menadione (30 µM). All data were analyzed by WinMDI 2.9 software.

5.3.5 **Mechanisms involved in the protective effects of pyruvate and other α-ketoacids against oxidative stress**
Complementary experiments were performed to determine whether pyruvate could also protect *Giardia* against H$_2$O$_2$ toxicity by improving energy metabolism. For this purpose and to get better effect, *Giardia* trophozoites were exposed for 1 h to H$_2$O$_2$ (10 µM) in the presence of α-ketoglutarate and oxaloacetate, which are known to act both as H$_2$O$_2$ scavengers and energy substrate metabolites; mannitol, which only possesses H$_2$O$_2$ scavenger properties; malate, which is only an energy substrate metabolite or finally β-ketoglutarate, which is neither an H$_2$O$_2$ scavenger nor an energy substrate metabolite. These compounds were all added at a concentration of 2 mM.
H$_2$O$_2$ at a concentration of 10 µM significantly decreased trophozoites viability compared with control ($P < 0.001$) (Fig.5.5). The ability of these different compounds to prevent H$_2$O$_2$ toxicity was related to their capacity to scavenge H$_2$O$_2$ and completely independent of their ability to be used as energy substrate. In particular, acetate was ineffective, whereas oxaloacetate strongly prevent H$_2$O$_2$ toxicity 74.3%. Trophozoites viability in H$_2$O$_2$-treated samples supplemented with pyruvate and malate was restored to 86% ($P < 0.01$) and 80%, respectively; however, the trophozoites viability was 53% and 47.1% when previously incubated with mannitol and acetate, respectively, which was significantly lower than the control ($P < 0.001$). In addition, the ability of different α-ketoacids (used at same concentration; 2 mM) to scavenge ROS was closely correlated with their capacity to protect trophozoites; pyruvate = malate > oxaloacetate > α-ketoglutarate > mannitol (which is not an energy substrate metabolite of Giardia). As acetate, β-ketoglutarate was ineffective (not shown in the Fig.5.5).

![Figure 5.5: ROS scavenging capacities and antioxidant properties of various α-ketoacids.](image)

Cultured Giardia trophozoites were preincubated for 30 min with a 2 mM concentration of each compound (oxaloacetate, malate, pyruvate, α-ketoglutarate, mannitol, acetate) and further incubated for 2.5 h under H$_2$O$_2$ (10 µM) in their presence or absence. Viability was estimated 24 h later. Data are the mean ± SEM of three independent experiments, each performed in triplicate. *$P<0.05$, compared with control; #$P<0.001$, compared with stressed sample.
5.3.6 Inefficiency of pyruvate to inhibit linoleic acid autoxidation in micelles

Conjugated dienes resulting from the spontaneous autoxidation of linoleic acid (at pH 7.4 and temperature 37 °C) were measured by their absorbance at 234 nm. The conjugated dienes increased rapidly to reach a maximum after few hours and then remained constant. On the basis of linoleic acid autoxidation in micelles, it has been shown that pyruvate did not inhibit lipid peroxidation (not shown) rather acetate showed a greater extent of inhibitory effect on linoleic acid autoxidation at 2, 5 and 10 mM concentrations ($P < 0.01$) (Fig. 5.6.A). Scavengers (e.g., tochopherol used as control) of these radicals inhibit the autoxidation reaction significantly at 2, 5, 10 mM concentrations ($P < 0.001$) (Fig. 5.6.B).

![Graph A and B]

Figure 5.6: Effect of pyruvate, acetate and tochopherol on linoleic acid autoxidation. Micelles of linoleic acid were autoxidized in the dark at 37 °C. Linoleic acid autoxidation was estimated by the absorbance of conjugated dienes at 234 nm. We calculated percentage of diene production and (A) effect of acetate at different concentrations (0-10 mM). We used tochopherol (0-10 mM) as a known positive control (B). Values are mean ± SEM of three independent experiments, each performed in triplicate. *$P < 0.05$, compared with control.

5.3.7 Antioxidant effect of pyruvate on lipid peroxidation

The degree of lipid peroxidation has been measured on the basis of MDA formation, which has been usually used as an index of lipid peroxidation. We have obtained the lipid peroxidation status in Giardia homogenate under oxidative stresses without or with supplementation of puruvate. Lipid peroxidation was found to be increased significantly by 37.5% ($P < 0.001$) in trophozoites under hydrogen peroxide compared to the untreated trophozoites. Administration of pyruvate (5mM) significantly decreased the lipid
peroxidation to 54% ($P < 0.05$) in the trophozoites under H$_2$O$_2$ stress, compared to the stressed trophozoites without pyruvate incubation (Fig.5.7).

Figure 5.7: Effect of pyruvate on level of lipid peroxidation in cultured Giardia trophozoites upon H$_2$O$_2$ stress: (A) MDA concentration in H$_2$O$_2$ stressed Giardia trophozoites after 8 h incubation. Values are mean ± SEM of three independent experiments, each performed in triplicate. *$P < 0.05$, compared with control; **$P < 0.001$, compared with stressed sample.

5.3.8 Giardia trophozoites have greater pyruvate requirements under hydrogen peroxide exposure

The intracellular pyruvate concentration in Giardia lamblia trophozoites were measured during oxidative stressed condition. It was then examined whether Giardia lamblia have the ability to regulate intracellular level of pyruvate in response to oxidative stress. Under H$_2$O$_2$ stress the intracellular pyruvate level raised linearly up to 1.41 µmol/mg proteins after 2 h (Fig.5.8). It was further increased significantly after 4 h up to 2.5 µmol/mg proteins ($P < 0.001$) than the control and maintained at the end of 6 h time points to 1.9 µmol/mg proteins ($P < 0.01$). H$_2$O$_2$ caused significant loss of pyruvate in Giardia. Finally, at the end of 8 h pyruvate levels in H$_2$O$_2$-treated trophozoites were significantly lowers (1.1 µmol/mg proteins, $P < 0.05$) than the trophozoites treated with H$_2$O$_2$ for 4 h. However, the trophozoites seemed to adjust between production and consumption of pyruvate in order to maintain the intracellular pyruvate level at near about 0.5 µmol/mg proteins (the normal pyruvate
concentration in the trophozoites) under H$_2$O$_2$ treatment. Moreover, these data showed that *Giardia* trophozoites have a greater need of pyruvate under H$_2$O$_2$ stressed condition.

**Figure 5.8: Intracellular pyruvate concentration in *Giardia* trophozoites during oxidative stress.** Intracellular pyruvate content was measured in *Giardia lamblia* under H$_2$O$_2$ treatment. The level of intracellular pyruvate was quantified every two hours interval. Values are means ± SEM of three independent experiments, each performed in triplicate. *P*<0.05, compared with control; \(_P<0.001\), compared with 4$^{th}$ h stressed sample, \(^P<0.001\), compared with 2$^{nd}$ h stressed sample.

### 5.3.9 Exogenous pyruvate protects DNA under oxidative stress

It can be stated that increase pyruvate uptake might be related to certain protective actions against the consequences of oxidative stress. In mammalian cells the hallmark of apoptosis is the degradation of genomic DNA. Therefore, we investigated the DNA fragmentation pattern for untreated and stressed-induced trophozoites and also stressed-induced trophozoites previously incubated with pyruvate. The stressed-induced trophozoites showed a DNA fragmentation pattern after 8 h exposure to oxidative stress. The ladder pattern was not clear as a metazoan DNA ladder and showed some degree of smearing with fragmented DNA in low molecular weight region, identified by electrophoresis on a 1.5% agarose gel (Fig.5.9). The untreated and stress-induced trophozoites previously incubated with pyruvate did not
show any characteristics of DNA fragmentation. The 8 h stress-induced trophozoites reseeded in fresh TYIS-33 medium showed DNA fragmentation after 24 h.

Figure 5.9: Effect of pyruvate on DNA fragmentation. Electrophoretic analysis of DNA fragmentation on a 1.5% agarose for *Giardia* trophozoites treated with H$_2$O$_2$ in the absence and presence of pyruvate.

5.3.10 Externalization of phosphatidyl serine upon exposure to hydrogen peroxide

Externalization of phosphatidyl serine (PS) is one of the indicators of apoptosis in the eukaryotic cells. Fluorescein isothiocyanate (FITC)-tagged annexin can bind with the PS when it flips outside the membrane and the fluorescence signal can be measured in a fluorescent microscope. A characteristic green ring formation can be observed in the membrane of an ideal apoptotic cell. In our study we have used this assay to check the mode of death in stress-induced cells. Normal live cells do not show any ring formation whereas in case of all the experimental sets treated with different oxidative stress producing agents reveal a clear ring formation in their outer membrane part (Figures not shown) confirming the mode of cell death to be apoptotic. Cells stained with FITC and PI can be targeted for observing
their viability and mode of death in a flow cytometer. The gated region in the flow cytometer denotes the type of cells according to their location in the four coordinates. Live cells do not take the stains and lie in the left hand bottom panel (Q3) whereas early apoptotic cell population taking FITC stain lies in right hand bottom panel (Q4) and late apoptotic cell population taking FITC and PI stained because of their increased membrane permeability lies in the right hand upper panel (Q2). All the necrotic cells taking the PI stain lie in the left hand upper panel (Q1). In our experiment, in case of un-stressed Giardia most of the live cells have not taken any type of stains and consequently lie in the left hand bottom panel (Fig.10. A; Panel A). Trophozoites treated with H$_2$O$_2$ (0.10 µM) and H$_2$O$_2$ with nicotinamide, an amide form of vitamin B$_3$ (100 µM) showed less number of healthy cell population (Fig.10. B, C; Panel A) because nicotinamide (100 µM) induced cell death towards apoptosis. Trophozoites of Giardia under H$_2$O$_2$ treatment with nicotinamide but previously incubated with pyruvate did not undergo apoptosis and showed higher number of healthy cell population (Fig.10. F; Panel A) than when treated with other metabolites (e.g., acetate, ascorbate). We thought that pyruvate might rescue trophozoites by scavenging ROS but it was exciting when we added pyruvate exogenously after 6 h stress and incubated for another 2 h more and analyzed further. We observed that the rate of recovery of the stressed Giardia trophozoites increased with the increase in pyruvate concentration (Fig. 10.B, C; Panel C). In these rescue studies the major goal for administering pyruvate was to provide the substrate distal to glycolysis, thereby boosting ATP generation and acetate production. We tried to rescue trophozoites of Giardia lamblia by adding a nonmetabolizable antioxidant radical scavenger, N-tert-Butyl-α-phenylnitrone (PBN), to the external medium. After we found that PBN protection at 1 mM was not as good as that 5 mM pyruvate. We addressed the concern that PBN might do better protection than pyruvate if it were at a higher concentration. We showed that switching the 1 mM PBN concentration to 5 mM made no difference in cell recovery (Fig.10.D, E, F; Panel C).
Figure 5.10: Cell death assay: Assay of cell death after stress induction for 8 h and effect of pyruvate on stress induced *Giardia lamblia* during recovery by flowcytometry using an annexin-V-FITC staining kit. Determination of healthy cell population in: Panel A: A. without stress, B. H$_2$O$_2$ stress, C. H$_2$O$_2$ stress preincubated with nicotinamide (100 µM), D. H$_2$O$_2$ stress previously incubated with ascorbate (2 mM), E. H$_2$O$_2$ stress previously incubated with acetate (2 mM) in the presence of nicotinamide, F. H$_2$O$_2$ stress previously incubated with pyruvate (2 mM) in the presence of nicotinamide; Panel B: A. without stress, B. After H$_2$O$_2$ stress (for 6 h) cells were incubated with exogenously added pyruvate (2 mM) for another 2 h, C. After cysteine-ascorbate deprived medium stress (for 6 h after 2 h initial incubation) cells were incubated with exogenously added pyruvate (2 mM) for another 2 h, D. H$_2$O$_2$ stress (8 h) pre-incubated with PBN (1 mM), E. H$_2$O$_2$ stress (8 h) preincubated with PBN (5 mM), F. cysteine-ascorbate deprived medium stress (8 h) pre-incubated with PBN (5 mM). The data are from one representative experiment.
5.3.11 Recovery rate of H\textsubscript{2}O\textsubscript{2} stressed \textit{Giardia} trophozoites by incubation with pyruvate at different time points
We have also quantified the rate of recovery of \textit{Giardia} trophozoites from oxidative stress (Fig.5.11). Survival rate was estimated 24 h later. We have observed that with time the rate of recovery has increased by pyruvate for both stresses. Pyruvate thus plays a role in DNA protection and repair in \textit{Giardia} trophozoites during oxidative stress.

![Graph showing recovery rate of H\textsubscript{2}O\textsubscript{2} stressed \textit{Giardia} trophozoites by incubation with pyruvate at different time points.](image)

**Figure 5.11: Quantification of viable cells.** Counts of viable cells are given as percentages of the total number of cells. \textit{Giardia} trophozoites were incubated under H\textsubscript{2}O\textsubscript{2} for 2.5 h after that pyruvate was added and then further incubated for 30-120 min. Stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (H\textsubscript{2}O\textsubscript{2} free) supplemented with the corresponding concentration of pyruvate and their survival rate was evaluated after 24 h by using flow cytometry. Results are expressed as the percentage of surviving trophozoites compared with control culture. Data are the mean ± SEM of three independent experiments, each performed in triplicate. *P<0.05, compared with control; #P<0.001, compared with stressed sample. Results are from three independent experiments, each performed thrice. Pyr = pyruvate.

5.3.12 Modulation of different metabolic genes under oxidative stress
To better understand the effect of different oxidative stress in transcriptional regulation of gene expression in \textit{Giardia lamblia}, we performed time course analysis of gene expression of pyruvate metabolism pathway upon H\textsubscript{2}O\textsubscript{2} (0.10 µM) stress using a quantitative RT-PCR. We have chosen six genes, related to the pyruvate metabolism of \textit{Giardia lamblia} modulated by at
least 2 fold at one or more time points in response to H$_2$O$_2$. The metabolism of *Giardia* species is essentially fermentative, with glucose and amino acids being used as energy sources (Jaroll et al., 1995). Arginine is metabolized by Arginine dihydrolase pathway (Knodler et al., 1995) and aspartate can be metabolized to pyruvate via a variety of enzymes (Mendis et al., 1992). In our study, we have shown that arginine deiminase (ARGD)-encoding gene was down-regulated in *Giardia* trophozoites under hydrogen peroxide stress (Fig.5.12). Glucose is metabolized by a glycolytic pathway that is modified such that ATP is replaced by pyrophosphate at several key points (Mertens, 1993). This makes the pathway reversible and produces an increase in net ATP synthesis. In *Giardia lamblia*, pyruvate can be produced by three different pathways. Phosphoenolpyruvate carboxyphosphotransferase together with malate dehydrogenase and malate dehydrogenase (decarboxylating), serves as a pathway to convert phosphoenol pyruvate (PEP) into pyruvate (Lindmark, 1980). In response to hydrogen peroxide stress, the Malate dehydrogenase (MDH) gene was down-regulated after (4-8 h) time points (Fig.5.12). However, pyruvate phosphate dikinase (PDK) (Bruderer et al., 1996; Park and Sinskey, 1997) and pyruvate kinase (PK) (Ellis et al., 1993) can also convert phosphoenolpyruvate into pyruvate in *Giardia*. Pyruvate phosphate dikinase, an enzyme that catalyzes the irreversible conversion of phosphoenolpyruvate (PEP) to pyruvate, the energy generating step of pyruvate biosynthetic pathway, was up-regulated till 8 h under the H$_2$O$_2$ stress (Fig.5.12). Other genes that were slightly modulated by H$_2$O$_2$ stress included pyruvate kinase, which was induced at early (4-6 h) time points under H$_2$O$_2$ stress (Figure 12) but it was getting down-regulated after (8 h) time points. This has suggested that the three-enzyme pathway may have an alternative function, such as transferring equivalents from NADH to NADPH (Lindmark, 1980; Ellis et al., 1993). Pyruvate ferredoxin oxidoreductase (PFOR) (Townson et al., 1996), an enzyme system involved in the conversion of pyruvate to acetyl-coA and finally from acetyl-CoA to acetate is formed by the enzyme acetyl-CoA synthase (ACS). *Giardia* displays a significant sensitivity to O$_2$ (Lloyd et al. 2000) that are attributed to the expression of O$_2$-labile key metabolic enzymes such as PFOR (Townson et al., 1996). In our study, PFOR-encoding gene was up-regulated during first couple of hours under H$_2$O$_2$ stress but it was getting down-regulated after (6-8 h) time points upon H$_2$O$_2$ stress (Fig.5.12). The enzyme acetyl-CoA synthase transcript was up-regulated during stress. However, the hydrogen peroxide stress has been shown to significantly modulate the metabolic flux across pyruvate metabolism in *Giardia lamblia*. 

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Figure 5.12: Effect of H₂O₂ on the expression of genes involved in pyruvate metabolism. Modulation of transcripts encoding enzymes involved in pyruvate metabolism. A. Gene expression (fold change) under H₂O₂ stress. Data are shown as fold change in relative expression compared with Actin on the basis of Comparative Ct (2^(-ΔΔCt)) method. Values are shown as mean ± SEM of three independent experiments, each performed in triplicate. (Gene abbreviation used: Metabolic enzymes: PFOR: Pyruvate-ferredoxin oxidoreductase, MALDH: Malate dehydrogenase, ARGD: Arginine deiminase, PK: Pyruvate kinase, PDK: Pyruvate dikinase, ACS: Acetyl coA synthase).
Table 5. 1. List of primers used in Real-Time PCR.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Size (bp)</th>
<th>Code</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate ferredoxin-oxidoreductase</td>
<td>Gl50803_114609</td>
<td>3762</td>
<td>PFOR</td>
<td>Forward: 5´- ATCCAACGCGACCCAGAAG-3´</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5´- GTTCACTGCTTACTCCGCC-3´</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>Gl50803_3331</td>
<td>996</td>
<td>MALDH</td>
<td>Forward: 5´- GGAGACATGCTGGGTACGA-3´</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5´- CGGCAGGAACTCAAGCATA-3´</td>
</tr>
<tr>
<td>Pyruvate phosphate-dikinase</td>
<td>Gl50803_9909</td>
<td>2655</td>
<td>PDK</td>
<td>Forward: 5´- TGGAACCAACGATCTTACACAG-3´</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5´- GGAGACATAGTTCAGGCAATC-3´</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Gl50803_3206</td>
<td>1965</td>
<td>PK</td>
<td>Forward: 5´- CAGACCAGAAAAAGCACATCAG-3´</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5´- GCGGTCCAATTCTTGAAGCACAACCTAC-3´</td>
</tr>
<tr>
<td>Acetyl co-A synthase</td>
<td>Gl50803_13608</td>
<td>2181</td>
<td>ACS</td>
<td>Forward: 5´- ACTGAGATCCTGGGTACAG-3´</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>Reverse: 5´- ACGATGGACTCAAGGTAAGG-3´</td>
</tr>
<tr>
<td>Arginine deiminase</td>
<td>Gl50803_112103</td>
<td>1743</td>
<td>ARGD</td>
<td>Forward: 5´- GGCGAAGGCAAATGTTGAGT-3´</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Reverse: 5´- CGGACGATCGTGTAACCATT-3´</td>
</tr>
</tbody>
</table>
5.4 Discussion:
The present study demonstrates that extracellular pyruvate protects *Giardia* trophozoites against the cytotoxicity induced by endogenous reactive oxygen species (ROS) generated during the treatments of hydrogen peroxide (H$_2$O$_2$). The antioxidant protective effect of α-ketoacids has already been investigated both in vitro in several cell types (Andrae et al., 1985) and in vivo in whole organs such as heart (Crestanello *et al*., 1995). However, to date, the exact role of pyruvate and the effect of different α-ketoacids in protecting *Giardia* trophozoites from ROS attack are not yet clear.

Biological membranes are readily permeable to pyruvate and it can therefore be assumed that the extracellular addition of pyruvate would raise the levels of intracellular pyruvate (Biagini *et al*., 2001). The H$_2$DCFDA assay was performed to monitor the intracellular generation of reactive oxygen species in *Giardia lamblia*. Likewise, the addition of H$_2$O$_2$ (which is uncharged and therefore freely penetrates the plasma membrane) was shown to increase the rate of intracellular generation of reactive oxygen species (as indicated by the augment in fluorescence intensity). Similarly, the addition of menadione increased the rate of intracellular generation of reactive oxygen species. Our results indicate that menadione induced generation of reactive oxygen species in stressed *Giardia* trophozoites were reduced by the addition of pyruvate. It was also shown to reduce the generation of total fluorescence arising from the oxidation of the H$_2$DCFDA by spectrofluorometer and may also have played a direct antioxidant role in *Giardia*.

Our results indicate that pyruvate and related α-ketoacids improve the survival of cultured trophozoites exposed to H$_2$O$_2$. Several observations suggest that the protective effect of pyruvate results rather from its ability to react with H$_2$O$_2$ to form acetate, H$_2$O and CO$_2$ than from the improvement of energy metabolism of *Giardia*. The protective effect of pyruvate was reproduced by several α-ketoacids, which share with pyruvate the ability to react with hydrogen peroxide; these compounds include mannitol, which is not an energy substrate. The protective effect of the different α-ketoacids against oxidative stress was closely correlated with their ability to scavenge ROS.

It has been reported that elevated ROS generation is correlated with a decline in the production of ATP as well as protein synthesis and increase in DNA breakage and lipid peroxidation (Herbener, 1976; Vorbeck *et al*., 1982). The decrease of ROS level may be endorsed either to their reduced generation or to the enhancement in antioxidant levels. Based on linoleic acid autoxidation in micelles (Cillard *et al*., 1980), that pyruvate did not inhibit lipid peroxidation. No induction period was observed. Indeed, in a micelle system, the
The autoxidation process involves lipid radicals such as peroxyl radical (ROO•) and alkoxyl radical (RO•). The levels of conjugated dienes increased rapidly to reach a maximum level and then remained constant. The addition of pyruvate at 0-10 mM was without any effect on the formation of conjugated dienes during the indicated time period. Thus it can be presumed that pyruvate did not scavenge lipid radicals.

Lipid peroxidation is used as an index for measuring the damage that occurs in cell membranes as a result of lipid destruction by free radicals (Sandhu and Kaur, 2003). In this system, pyruvate was able to inhibit lipid peroxidation. This incongruity between a cellular antilipoperoxidant effect of pyruvate and its ineffectiveness to prevent diene production in micelles could be explained by the property of pyruvate to scavenge radicals such as H2O2•− radical, O2•−, OH− but not lipid radicals (Floriano-Sánchez et al., 2006). In Giardia trophozoites hydroxyl and superoxide radicals are generated during different oxidative stress. These radicals then initiate membrane lipid peroxidation. Thus pyruvate, by eliminating hydroxyl and superoxide radical, prevented lipid peroxidation. In other words, pyruvate acted at the initiation step of lipid peroxidation but not at the propagation step, which involves lipid radicals. Such findings are not unexpected because pyruvate is not liposoluble. Vitamin-C, well-known antioxidant agent reacts rapidly with O2•− and OH− but not with peroxyl radical (Groussard et al., 2000). In contrast, the familiar liposoluble vitamin-E is a powerful scavenger of lipid radical (Burton and Ingold, 1989). It was surprising that acetate showed inhibition of linoleic acid autoxidation i.e. with increased concentration of acetate diene formation decreased.

In our present study, it was observed that intracellular pyruvate concentration was increased and after several hours it was found to be decreasing. The higher pyruvate concentrations upon oxidative stress may be due to the inactivation of pyruvate:ferredoxin oxidoreductase, an enzyme which is sensitive to oxidative stress. Our present work has provided new insights into the role of pyruvate in Giardia trophozoites during oxidative stress. We observed that trophozoites of Giardia are inclined to maintain intracellular pyruvate at constant level 0.5 µmol/mg proteins (data not shown). Oxidative stress inhibits such regulation, whereas exogenously added pyruvate restores it. As we have already indicated that the effect of pyruvate against H2O2 toxicity may be attributable to its ability to degrade H2O2 through a nonenzymatic oxidative decarboxylation leading to the formation of CO2, H2O and acetate (Holleman, 1904; Bunton, 1949). This reaction may occur in the intracellular medium, leading to the degradation of equal amounts of H2O2 and pyruvate. On the contrary, because of its high membrane permeability H2O2 may depress energy
metabolism through degradation of intracellular pyruvate (and other α-ketoacids). Supporting this hypothesis, intracellular levels of pyruvate were markedly reduced in trophozoites of *Giardia* when exposed to H$_2$O$_2$ (0.10 µM) after few hours.

Induction of DNA damage by oxidative stress is a well-known phenomenon mainly caused by reactive oxygen species. A functioning antioxidant system is necessary to reduce such damage. In *Giardia lamblia* despite the absence of an effect of glutathione, pyruvate has beneficial effects on DNA breaks under oxidative stress. Particularly, the increased uptake of pyruvate might allow *Giardia* trophozoites to maintain their antioxidative capabilities in a way independent of glutathione during oxidative stress. DNA breaks decrease faster in stressed trophozoites of *Giardia* previously incubated with pyruvate, suggesting stimulation of DNA repair. Some studies found that pyruvate has antioxidant mechanism such as the activation of different anti-apoptotic pathways (Husain *et al*., 2001).

Essentially, impairment of the regulation of the intracellular metabolite and the increase of pyruvate uptake induced by oxidative stress illustrated that alteration of pyruvate metabolism has taken place. The greater uptake might be due to an increased requirement to maintain antioxidant potential in *Giardia lamblia*. However, it might also result from a metabolic need. Pyruvate increases the ratio of acetate production to glucose consumption and also increased the activities of pyruvate dikinase, pyruvate kinase and also malate dehydrogenase that *Giardia* can produce more pyruvate. This is indicating enhancement of glycolysis activity in the presence of pyruvate. Increased glycolysis might thus maintain the ATP supply during oxidative 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).

This study has unveiled for the first time the dynamics of the transcriptional and metabolic regulatory networks during oxidative stress. Oxidative stress management was thought to be controlled by NADH oxidase, flavodiiron protein etc (Mastronocola *et al*., 2011). However, the transcriptomic results have shown that the oxidative regulation is not only controlled by some metabolic genes, but also different types of other proteins take a significant role in ROS detoxification (Raj *et al*., 2014). In the two experimental conditions it has been observed that different types of stress generating conditions regulate the pyruvate metabolism pathway differently. Some metabolic genes like pyruvate dikinase, acetyl-coA synthase etc. are always up-regulated during both stresses. However, the exact mechanism underlying the pyruvate effects on oxidative stress management in *Giardia lamblia* has yet to be further examined.
5.5 Conclusion:

We can infer from this study that pyruvate in *Giardia lamblia* confirms the significance and the multilevel and also very complex implications in response to oxidative stress. As *Giardia* does not contain the components of respiratory chain (e.g. cytochromes), by controlling pyruvate level it keeps the harmful consequences of ROS at bay. Modulation of the fate of pyruvate in one direction or the other can be important for homeostatic response of *Giardia* to oxidative stress. This could alter functioning of the antioxidant system and have protective effects against DNA damage induced by oxidative stresses. Alterations of pyruvate metabolism are observed in *Giardia* due to high oxygen environment. This could be advantageous for *Giardia* trophozoites in such stressful conditions.