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RESEARCH ARTICLE

Involvement of pyruvate on oxidative stress management in the microaerophilic protozoan parasite *Giardia lamblia*

Dibyendu Raj[1], Prasanta Saini[1], Tomoyoshi Nozaki[2], and Sandipan Ganguly[1]*

1. Division of Parasitology, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India

2. Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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Corresponding Author

Sandipan Ganguly

Abstract

Background: *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.

Methods: 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 concentrations at different time points were measured during oxidative stress condition in *Giardia*.

Results: Our results provide evidence that exogenously added pyruvate was also able to inhibit lipid peroxidation of stress *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.

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

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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 and 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 secretes 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 and 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 CO_2 (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_2O_2 (Holleman, 1904) and through this reaction CO_2 is liberated and α-ketoacids is converted into the corresponding carboxylic acid:

$$R\text{-}COCOOH + H_2O_2 \longrightarrow R\text{-}COOH + CO_2 + H_2O$$

The α-keto carbonyl in pyruvate makes it a potent scavenger of reactive oxygen species, particularly H_2O_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, hydrogen peroxide (very well-known reactive oxygen species generator) has been preferred to generate oxidative stress in the trophozoites *in vitro*. 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.

METHODS:

Chemicals

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

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_2HPO_4 and 1.8 mM KH_2PO_4. Pyruvate (sodium salt), menadione, H_2O_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.

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_2O_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_2O_2 concentration during the study.

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_2DCFDA) fluorescent probe according to Schuessel et al., (2006) with some modifications. For monitoring intracellular ROS production, treated and untreated cells ($2x10^6$ cells/ml) were incubated with H_2DCFDA (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_2DCFDA 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.

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_2DCFDA 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_2O_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_2DCFDA, fluorescence emission was measured continuously at 530 nm after excitation at 488 nm.

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.

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_2O_2 and menadione (2-methyl-1, 4-napthoquinone) for the indicated time. After the incubation period, trophozoites were washed with PBS and added H_2DCFDA (1.5 µM at final concentration). The H_2DCFDA 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.

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 tochopherol 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.

Estimation of lipid peroxidation induced by H_2O_2 in cultured *Giardia* trophozoites

Sample preparation

The treated and untreated cells (approx. $4x10^7$ 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 K_2HPO_4 and 1.8 mM KH_2PO_4. Washed cells were homogenized in ice-cold PBS in a proportion of $5x10^6$ 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.

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 per mg protein using a molar extinction coefficient of 1.56 $x10^5$ $M^{-1}cm^{-1}$.

Extraction and quantification of intracellular pyruvate concentration at different time points under H_2O_2 stress

Intracellular pyruvate concentration was quantified under oxidative stress condition by using Pyruvate Assay Kit (ab65342). Trophozoites ($4x10^6$ cells/ml) were incubated in TYIS-33 medium and exposed to H_2O_2 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.

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.

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.

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 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 $2^{-\Delta\Delta C_T}$ 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.

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.

RESULTS:

Effects of pyruvate on ROS burst in *Giardia* trophozoites

The H_2DCFDA, 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 (Figure 1).

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_2O_2 than H_2DCFDA-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 (Figure 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 protects *Giardia* trophozoites from the toxicity induced by endogenous H_2O_2

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 H_2O_2 (10 µM). H_2O_2 caused reduction of trophozoites viability to 40.79%, which was significantly lower than the untreated trophozoites (94.66%, $P < 0.001$) (Figure 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 H_2O_2 toxicity. When exposed to 10 µM H_2O_2 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 H_2O_2 (39%, $P < 0.001$).

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 $O_2^{\cdot-}$. The H_2DCFDA assay has been adapted to measure oxidant production in *Giardia lamblia* trophozoites (Biagini et al., 2001) during oxidative stress condition. A progressive increase in the production of reactive oxygen species (as indicated by the augment in fluorescence intensity) with the addition of H_2O_2 (10 µM) increased the rate of reactive oxygen species generation (Figure 4). Prior incubation of H_2DCFDA 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.

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_2O_2 toxicity by improving energy metabolism. For this purpose and to get better effect, *Giardia* trophozoites were exposed for 1 h to H_2O_2 (10 µM) in the presence of α-ketoglutarate and oxaloacetate, which are known to act both as H_2O_2 scavengers and energy substrate metabolites; mannitol, which only possesses H_2O_2 scavenger properties; malate, which is only an energy substrate metabolite or finally β-ketoglutarate, which is neither an H_2O_2 scavenger nor an energy substrate metabolite. These compounds were all added at a concentration of 2 mM.

H_2O_2 at a concentration of 10 µM significantly decreased trophozoites viability compared with control ($P < 0.001$) (Figure 5). The ability of these different compounds to prevent H_2O_2 toxicity was related to their capacity to scavenge H_2O_2 and completely independent of their ability to be used as energy substrate. In particular, acetate was ineffective, whereas oxaloacetate strongly prevent H_2O_2 toxicity 74.3%. Trophozoites viability in H_2O_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 Figure 5).

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 (Figure 6). 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 ($P < 0.01$) (Figure 6A). Scavengers (e.g., tochopherol used as control) of these radicals inhibit the autoxidation reaction significantly at 2, 5, 10 mM concentrations ($P<0.001$) (Figure 6B).

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_2O_2 stress, compared to the stressed trophozoites without pyruvate incubation (Figure 7).

***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_2O_2 stress the intracellular pyruvate level raised linearly up to 1.41 µmol/mg proteins after 2 h (Figure 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_2O_2 caused significant loss of pyruvate in *Giardia*. Finally, at the end of 8 h pyruvate levels in H_2O_2-treated trphozoites were significantly lowers (1.1 µmol/mg proteins, $P < 0.05$) than the trophozoites treated with H_2O_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_2O_2 treatment. Moreover, these data showed that *Giardia* trophozoites have a greater need of pyruvate under H_2O_2 stressed condition than unstressed trophozoites.

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 (Figure 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 (data not shown).

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 (Figure 10, A; Panel A). Trophozoites treated with H_2O_2 (0.10 µM) and H_2O_2 with nicotinamide, an amide form of vitamin B_3 (100 µM) showed less number of healthy cell population (Figure 10 B, C; Panel A) because nicotinamide (100 µM) induced cell death

towards apoptosis. Trophozoites of *Giardia* under H_2O_2 treatment with nicotinamide but previously incubated with pyruvate did not undergo apoptosis and showed higher number of healthy cell population (Figure 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 (Figure 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 trophozites of *Giardia lamblia* by adding a nonmetabolizable antioxidant radical scavenger, N-tert-Butyl-$_{\alpha}$-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 (Figure 10 D, E, F; Panel C).

Recovery rate of H_2O_2 stressed *Giardia* trophozoites by incubation with pyruvate at different time points

We have also quantified the rate of recovery of *Giardia* trophozoites from oxidative stress (Figure 11). Survival rate was estimated 24 h later. We have observed that with time the rate of recovery has increased by pyruvate for hydrogen peroxide stress. Pyruvate thus plays a role in DNA protection and repair in *Giardia* trophozoites during oxidative stress.

Modulation of different metabolic genes under oxidative stress

To better understand the effect of different oxidative stress in transcriptional regulation of gene expression in *Giardia lamblia*, we performed time course analysis of gene expression of pyruvate metabolism pathway upon H_2O_2 (0.10 μM) stress using a quantitative RT-PCR. We have chosen six genes, related to the pyruvate metabolism of *Giardia lamblia* modulated by at least 2 fold at one or more time points in response to H_2O_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 (Figure 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 (Figure 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_2O_2 stress (Figure 12). Other genes that were slightly modulated by H_2O_2 stress included pyruvate kinase, which was induced at early (4-6 h) time points under H_2O_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_2O_2 stress but it was getting down-regulated after (6-8 h) time points upon H_2O_2 stress (Figure 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*.

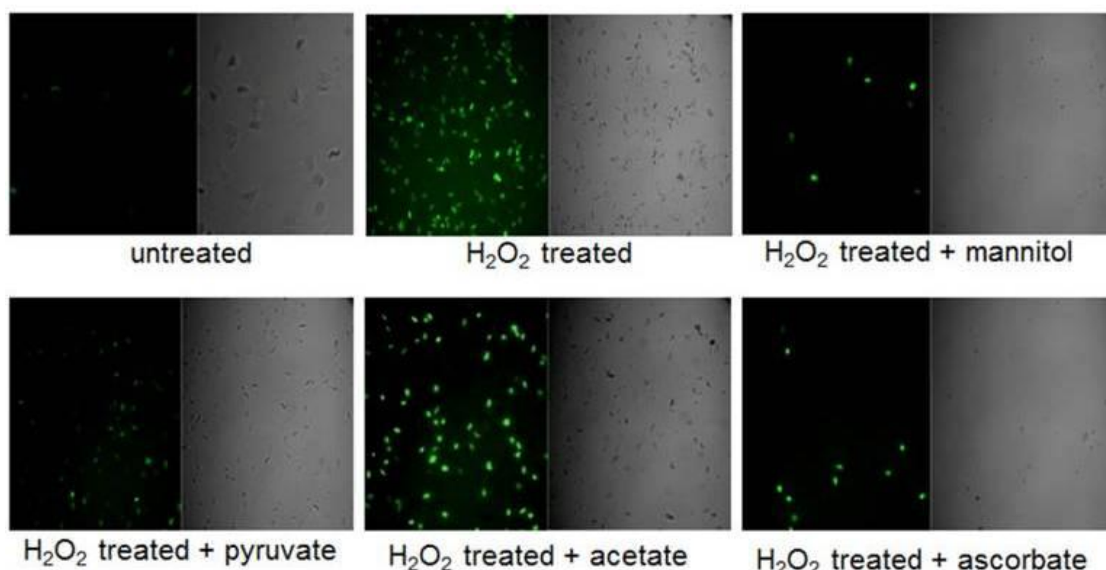

Figure 1: (A) H_2DCFDA-loaded cells under confocal microscope after H_2O_2 stress. Increases in fluorescence are representative of increase in the rate of oxidative species generated. Fluorescence was monitored from the suspension of live cells after the addition of H_2O_2 (10 µM) in the absence and 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. Increases in fluorescence are representative of increase in the rate of oxidative species generated.

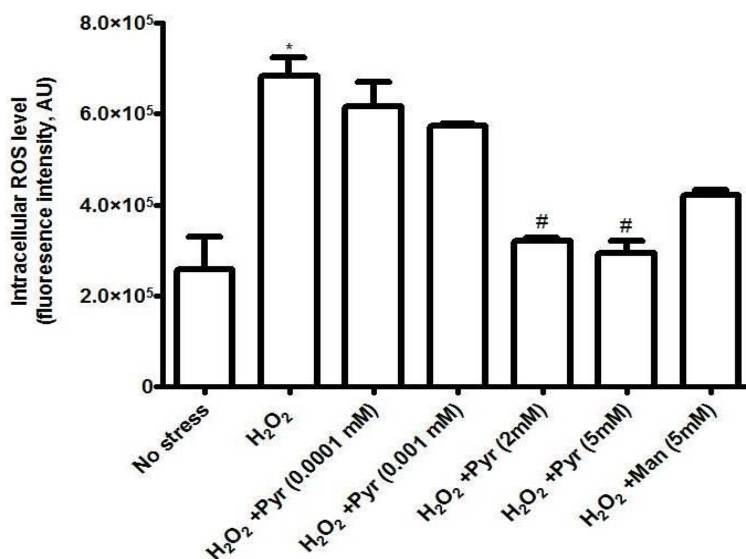

Figure 2: Pyruvate decreases the level of ROS in the medium of *Giardia lamblia* trophozoites exposed to H_2O_2. *Giardia* trophozoites were incubated in TYIS-33 medium under 10 µM H_2O_2 exposure and exposed to increasing doses of pyruvate (from 0-2mM). Levels of ROS were estimated by spectrofluorometry using 2', 7'-dichlorodihydrofluoresceine diacetate. The data are from three representative experiments.

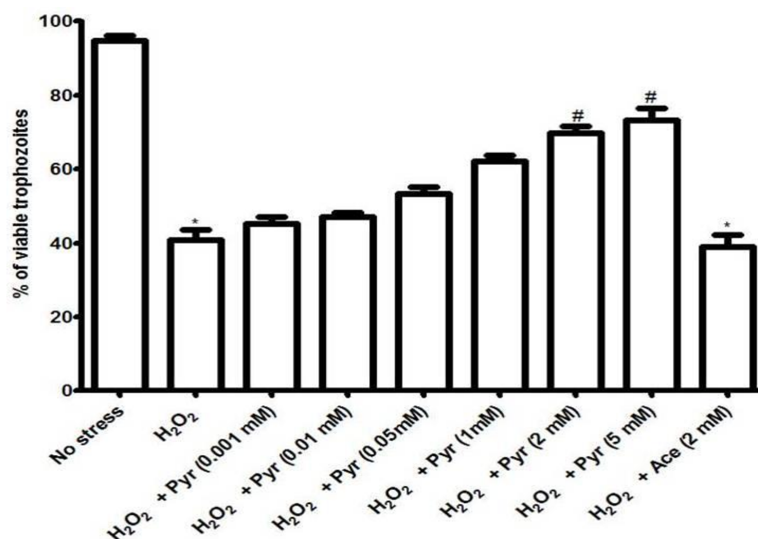

Figure 3: Pyruvate protects *Giardia* trophozoites from H_2O_2 induced toxicity. Cultured *Giardia* trophozoites were incubated at 35.5 °C under H_2O_2 (10 µM) for 2.5 h with increasing concentration of pyruvate. Pyruvate and H_2O_2 were simultaneously applied to the trophozoites. Stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (H_2O_2 free) and their viability was evaluated after 24 h by using flowcytometry. 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.

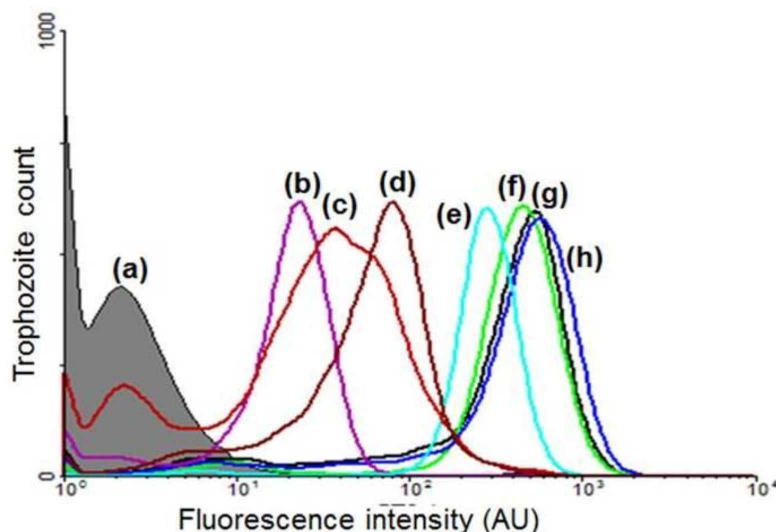

Figure 4: Pyruvate protects trophozoites from menadione-induced toxicity. Trophozoites were incubated at 35.5 °C under H_2O_2 (10 µM) for 2.5 h with increasing concentration of menadione in the absence and presence of pyruvate. Trophozoites were then washed and further incubated for 1 h with or without 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_2DCFDA loaded without H_2O_2 treated cells, (b) H_2DCFDA-loaded cells treated with H_2O_2 (10 µM) previously incubated with pyruvate (2 mM). (c) H_2DCFDA-loaded cells treated with H_2O_2 (10 µM) previously incubated with pyruvate (0.5 mM), (d) H_2DCFDA-loaded cells under H_2O_2 (10 µM) stress previously incubated with pyruvate (2 mM) in the presence of menadione (10 µM), (e) H_2DCFDA-loaded cells under H_2O_2 (10 µM) stress previously incubated with

pyruvate (2 mM) in the presence of menadione (30 µM), (f) H_2DCFDAloaded cells under H_2O_2 (10 µM) treatment, (g) H_2DCFDA-loaded cells under H_2O_2 (10 µM) previously incubated with menadione (10 µM), (h) H_2DCFDA-loaded cells under H_2O_2 (10 µM) previously incubated with menadione (30 µM). All data were analyzed by WinMDI 2.9 software

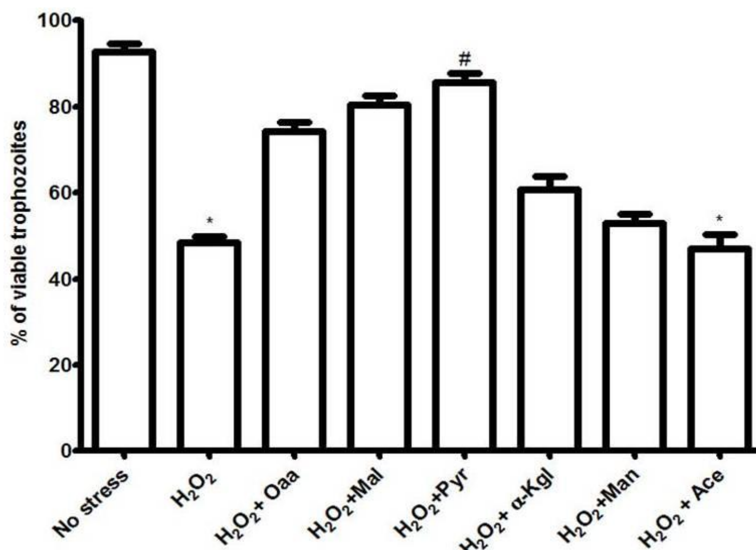

Figure 5: ROS scavenging capacities and antioxidant properties of various α-ketoacids. Cultured *Giardia* trophozoites were preincubated for 30 min with a 2 mM concentration of each compound (oxaloacetate, malate, pyruvate, α-ketoglutarate, mannitol, acetate) and further incubated for 2.5 h under H_2O_2 (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.

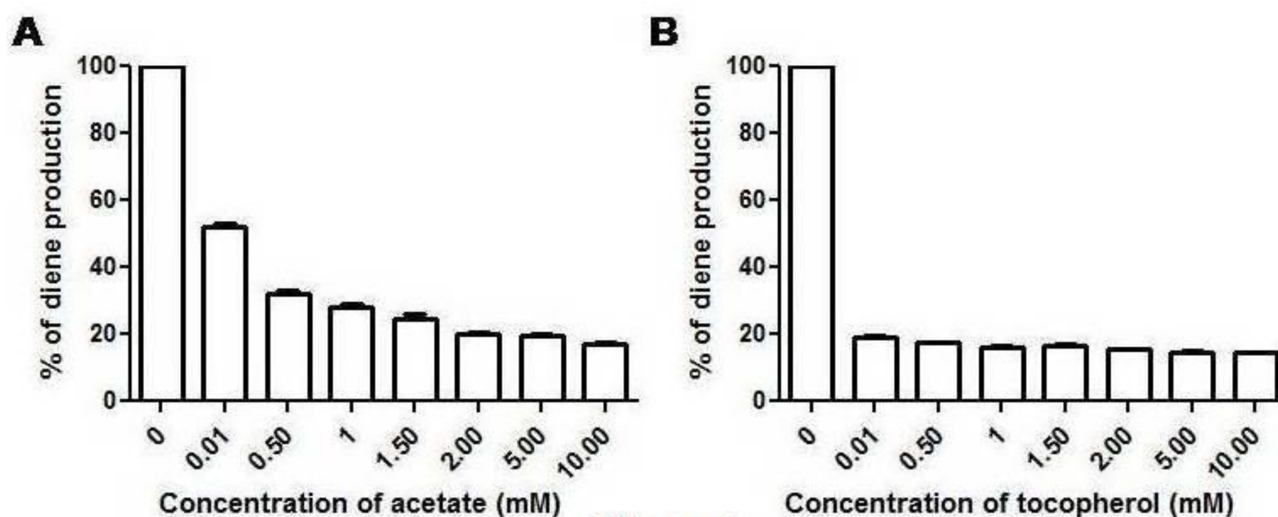

Figure 6: Effect of acetate and tochopherol on linoleic acid autoxidation. Micelles of linoleic acid were autoxidized in the dark at 37 °C. Linoleic acid autoxidation was estimated by the absorbance of conjugated dienes at 234 nm. We calculated percentage of diene production and **(A)** effect of acetate 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.

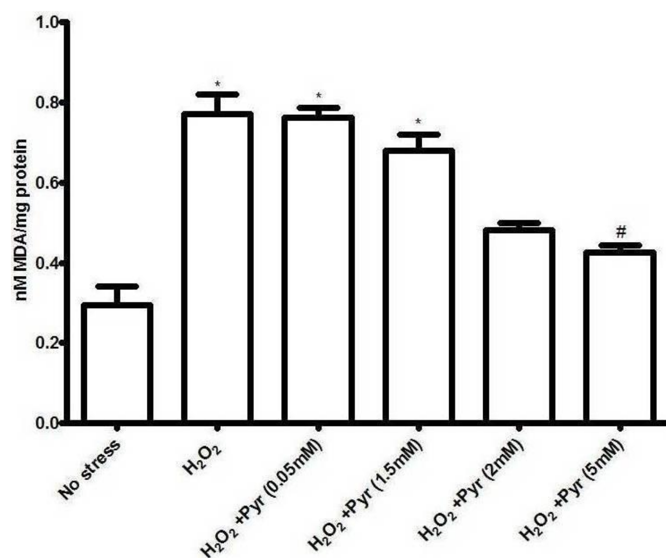

Figure 7: Effect of pyruvate on level of lipid peroxidation in cultured *Giardia* trophozoites upon H_2O_2 stress: (A) MDA concentration in H_2O_2 stressed *Giardia* trophozoites after 8 h incubation. Values are mean ± SEM of three independent experiments, each performed in triplicate. $^{*}P<0.05$, compared with control; $^{\#}P<0.001$, compared with stressed sample.

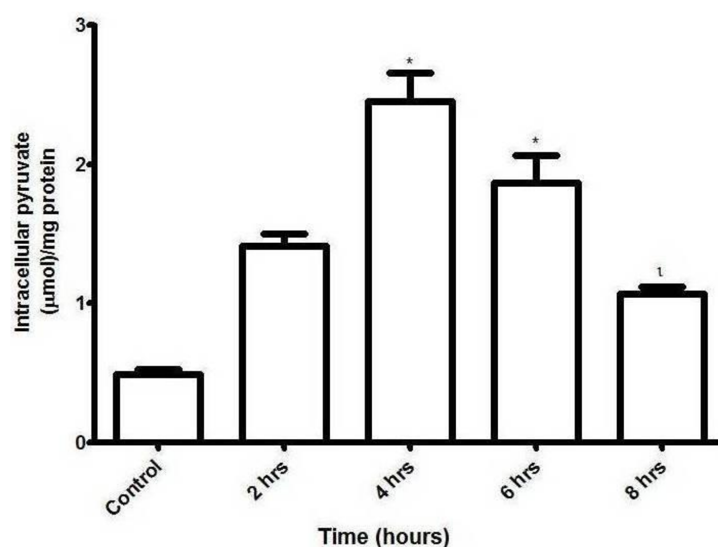

Figure 8: Intracellular pyruvate concentration in *Giardia* trophozoites during oxidative stress. Intracellular pyruvate content was measured in *Giardia lamblia* under H_2O_2 treatment. The level of intracellular pyruvate was quantified every two hours interval. Values are means ± SEM of three independent experiments, each performed in triplicate. $^{*}P<0.05$, compared with control; $^{l}P<0.001$, compared with 4th h stressed sample, $^{\tau}P<0.001$, compared with 2nd h stressed sample.

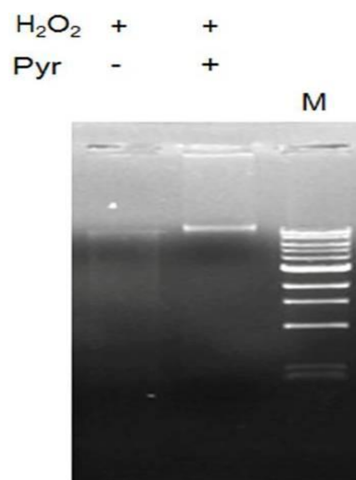

Figure 9: Effect of pyruvate on DNA fragmentation. Electrophoretic analysis of DNA fragmentation on a 1.5% agarose for *Giardia* trophozites treated with H_2O_2 in the absence and presence of pyruvate.

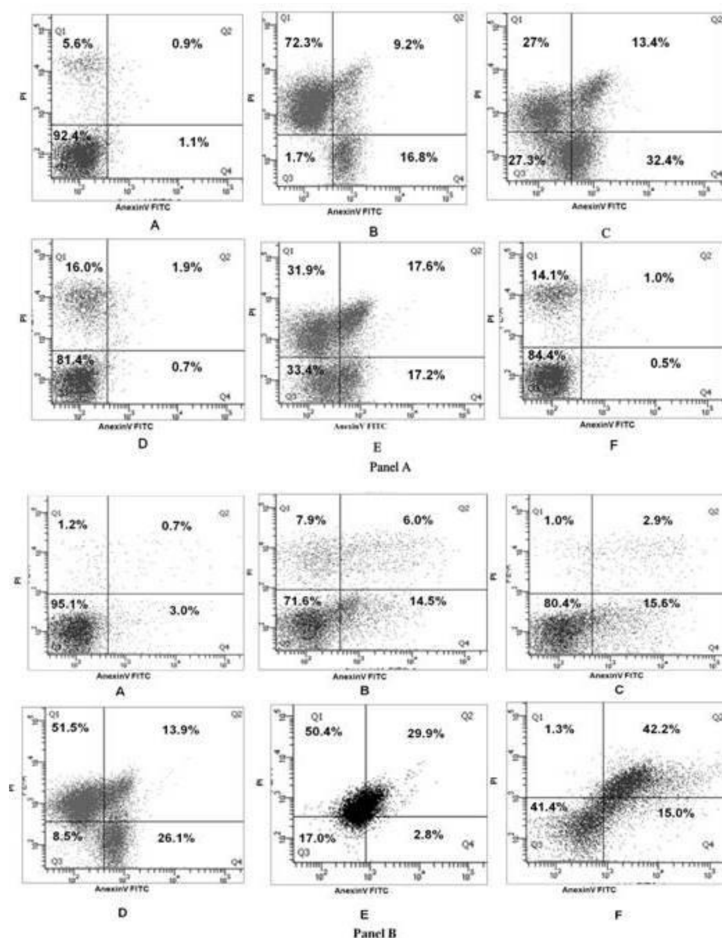

Figure 10: Cell death assay: Assay of cell death after stress induction for 8 h and effect of pyruvate on stress induced *Giardia lamblia* during recovery by flowcytometry using an annexin-V-FITC staining kit. Determination of

healthy cell population in: **Panel A:** A. without stress, B. H_2O_2 stress, C. H_2O_2 stress preincubated with nicotinamide (100 µM), D. H_2O_2 stress previously incubated with ascorbate (2 mM), E. H_2O_2 stress previously incubated with acetate (2 mM) in the presence of nicotinamide, F. H_2O_2 stress previously incubated with pyruvate (2 mM) in the presence of nicotinamide; **Panel B:** A. without stress, B. After H_2O_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_2O_2 stress (8 h) pre-incubated with PBN (1 mM), E. H_2O_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.

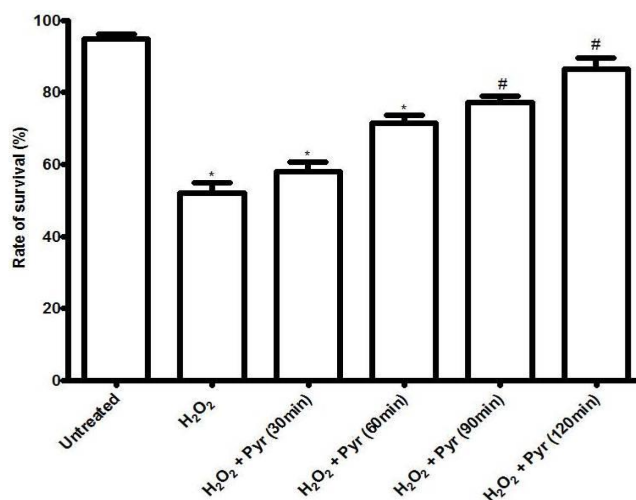

Figure 11: Quantification of viable cells. Counts of viable cells are given as percentages of the total number of cells. *Giardia* trophozoites were incubated under H_2O_2 for 2.5 h after that pyruvate was added and then further incubated for 30-120 min. Stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (H_2O_2 free) supplemented with the corresponding concentration of pyruvate and their survival rate was evaluated after 24 h by using flowcytometry. 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.

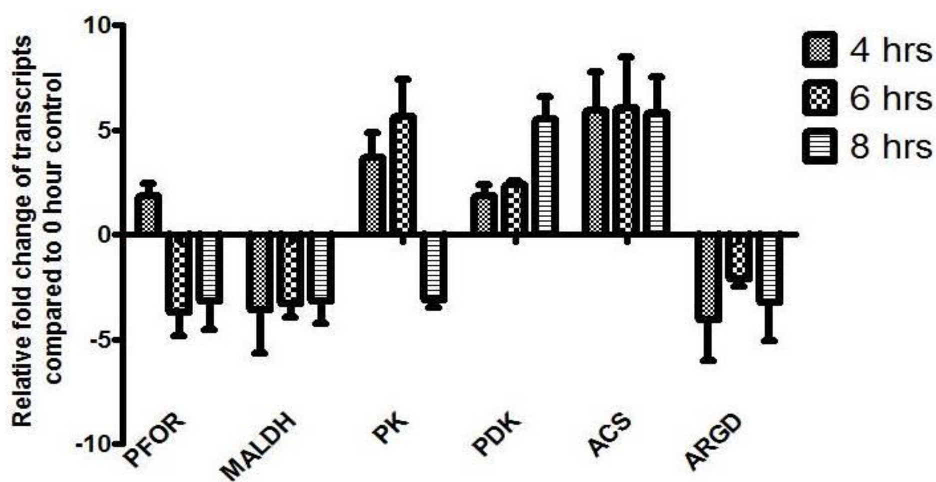

Figure 12: Effect of H_2O_2 on the expression of genes involved in pyruvate metabolism. Modulation of transcripts encoding enzymes involved in pyruvate metabolism. **A.** Gene expression (fold change) under H_2O_2 stress. Data are shown as fold change in relative expression compared with Actin on the basis of Comparative Ct ($2^{-\Delta\Delta Ct}$) method. Values are shown as mean ± SEM of three independent experiments, each performed in triplicate. (Gene abbreviation used: **Metabolic enzymes:** PFOR: Pyruvate-ferredoxin oxidoreductade, MALDH: Malate dehydrogenase, ARGD: Arginine deiminase, PK: Pyruvate kinase, PDK: Pyruvate dikinase, ACS: Acetyl coA synthase).

Table 1. List of primers used in Real-Time PCR.

Gene Name	Gene ID	Size (bp)	Code	Primer Sequence
Pyruvate ferredoxin-oxidoreductase	Gl50803_114609	3762	PFOR	Forward: 5-ATCCAACGCGACCCAGAAG-3
				Reverse: 5- GTTCACTGCTTACTCCGCC-3
Malate dehydrogenase	Gl50803_3331	996	MALDH	Forward: 5- GGAGACATGCTGGGCTACGA-3
				Reverse: 5- CGGCAGGAACCTCAAGCATA-3
Pyruvate phosphate-dikinase	Gl50803_9909	2655	PDK	Forward: 5-TGGAACCAACGATCTTACACAG-3
				Reverse: 5-GGAGACATAGTTCAGGCCAATC-3
Pyruvate kinase	Gl50803_3206	1965	PK	Forward: 5-CAGACCAGAAAAAGCACATCAG-3
				Reverse: 5-GCGGTCCATTCTTGAAAACTAC-3
Acetyl co-A synthase	Gl50803_13608	2181	ACS	Forward: 5-ACTGAGATCCTGGGGTACAAG-3
				Reverse: 5-ACGATGGACTCAAGGTAAAGG-3
Arginine deiminase	Gl50803_112103	1743	ARGD	Forward: 5- GGCGAAGGCAAATGTTGAGT-3
				Reverse: 5- CGGACGATCGTGTAACCATTTT-3

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_2O_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_2DCFDA assay was performed to monitor the intracellular generation of reactive oxygen species in *Giardia lamblia*. Likewise, the addition of H_2O_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_2DCFDA 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_2O_2. Several observations suggest that the protective effect of pyruvate results rather from its ability to react with H_2O_2 to form acetate, H_2O 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 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 $H_2O_2^-$ radical, $O_2^{·-}$, $^·OH$ but not lipid radicals (Floriano-Sánchez et al., 2006). In *Giardia* trophozoites hydroxyl and super oxide 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 $O_2^{·-}$ 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 H_2O_2 toxicity may be attributable to its ability to degrade H_2O_2 through a nonenzymatic oxidative decarboxylation leading to the formation of CO_2, H_2O and acetate (Holleman, 1904; Bunton, 1949). This reaction may occur in the intracellular medium, leading to the degradation of equal amounts of H_2O_2 and pyruvate. On the contrary, because of its high membrane permeability H_2O_2 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_2O_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 (data not shown) 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). However, the exact mechanism underlying the pyruvate effects on oxidative stress management in *Giardia lamblia* has yet to be further examined.

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 stress. Alterations of pyruvate metabolism are observed in *Giardia* due to high oxygen environment. This could be advantageous for *Giardia* trophozoites in such stressful condition.

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REFERENCES:

Adam, R. D. (2001): The biology of *Giardia* spp. Microbial Rev., 55: 706-732.

Andrae, U., Singh, J., and Ziegler-Skylakakis, K. (1985): Pyruvate and related α-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. Toxicol Lett., 28:93–98.

Bar-Or, D., Rael, L. T., Lau, E. P., Rao, N. K., Thomas, G. W., Winkler, J. V., Yukl, R. L., Kingston, R. G. and Curtis, C. G. (2001): An analog of the human albumin N-terminus (Asp-AlaHis-Lys) prevents formation of copper-induced reactive oxygen species. Biochem Biophys Res Commun., 284: 856-862.

Biagini, G. A., Park, J. H, Lloyd, D., and Edwards, M. R. (2001): The antioxidant potential of pyruvate in the amitochondriate diplomonads *Giardia intestinalis* and Hexamita inflata. Microbiology., 147: 3359-3365.

Biagini, G. A., Suller, M. T. E., Finlay, B. J. and Lloyd, D. (1997): Oxygen uptake and antioxidant responses of the free-living diplomonad Hexamita sp. J Eukaryot Microbiol., 44: 447-453.

Biagini, G. A., Mclntyre, P. S., Finlay, B. J. and Lloyd, D. (1998): Carbohydrate and amino acid fermentation in the free-living primitive protozoon Hexamita sp. Appl Environ Microbiol., 64: 203-207.

Brown, D. M., Upcroft, J. A., and Upcroft, P. (1993): Cysteine is the major low molecular weight thiol in Giardia *duodenalis*. Mol Biochem Parasitol., 61: 155-158.

Brown, D. M., Upcroft, J. A., and Upcroft P. (1995): Free radical detoxification in *Giardia duodenalis*. Mol Biochem Parasitol., 72: 47-56.

Brown, D. M., Upcroft, J. A., Edwards, M.R. and Upcroft, P. (1998): Anaerobic bacterial metabolism in the ancient eukaryote *Giardia duodenalis*. Int J parasitol., 28: 149-164.

Bruderer, T., Wehrli, C., and Kohler, P. (1996): Cloning and characterization of the gene encoding pyruvate phosphate dikinase from *Giardia duodenalis*. Mol biochem parasitol., 77: 225-233.

Bunton, C. A. (1949): Oxidation of α-diketones and α-keto acids by hydrogen peroxide. Nature., 144-163.

Burton, G. W., and Ingold, K. U. (1989): Vitamin E as an in vitro and in vivo antioxidant. Ann NY Acad Sci., 570: 7–22.

Cillard, J., Cillard, P., Cormier, M., and Girre, L. (1980): Alpha-tochopherol prooxidant effect in aqueous media: increased autoxidation rate of linoleic acid. J Am Oil Chem Soc., 57: 252-255.

Crestanello, J. A., Kamelgard, J., and Whitman, G.JR. (1995): The cumulative nature of pyruvate's dual mechanism for myocardial protection. J Surg Res., 59:198 –204.

Dimopoulos, M., Bagnara, A. S., and Edwards, M. R. (2000): Characterization and sequence analysis of a carbamate kinase gene from the diplomonad Hexamita inflata. J Eukaryot Microbiol., 47: 499-503.

Elias, E.V., Quiroga, R., Gottig, N., Nakanishi, H., Nash, T.E., Neiman, A., Lujan, H.D. (2008): Characterization of SNAREs determines the absence of a typical Golgi apparatus in the ancient eukaryote *Giardia lamblia*. J Biol Chem., 283(51):35996-6010.

Ellis, J. E., Williams, R., Cole, D., Cammack, R. and Lloyd, D. (1993): Electron transport components of the parasitic protozoon *Giardia lamblia*. FEBS Letts., 325, 196-200.

Evans, P.J., Gallesi, D., Mathieu, C., Hernandez, M.J., Felipe, M., Halliwell, B., and Puppo, A. (1999): Oxidative stress occurs during soybean nodule senescence. Planta., 208: 73–79.

Farthing, M. J. (1997): The molecular pathogenesis of giardiasis. J Pediatr Gastroenterol Nutr., 24: 79-88.

Fink, M. P. (2001): Ringer's ethyl pyruvate solution: A novel resuscitation fluid. Minerva Anestesiol., 67: 190-2.

Floriano-Sánchez, E., Villanueva, C., Medina-Campos, O.N., Rocha, D., Sánchez-González, D.J., Cárdenas-Rodríguez, N., and Pedraza-Chaverrí, J. (2006): Nordihydroguaiaretic acid is a potent in vitro scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents in vivo ozone-induced tyrosine nitration in lungs. Free Radic Res., 40 (5):523-33.

Ghosh, E., Ghosh, A., Ghosh, A. N., Nozaki, T., and Ganguly, S. (2009): Oxidative stress-induced cell cycle blockage and a protease-independent programmed cell death in microaerophilic *Giardia lamblia*. Drug Des Devel Ther., 3: 103–110.

Groussard, C., Morell, I., Chevanne, M., Monnier, M., Cillard, J., and Delamarche, A. (2000): Free radical scavenging and antioxidant effects of lactate ion: an in vitro study. J Appl Physiol., 89: 169-175.

Herbener, G. H. (1976): A morphometric study of age-dependent changes in mitochondrial population of mouse liver and heart. J Gerontol., 31: 8-12.

Holleman, M. A. F. (1904): Notice sur l'action de l'eau oxyge´ne´e sur les acides αce´toniques et sur les dice´tones 1.2. Recl Trav Chim Pays Bas Belg., 23:169– 171.

Husain, A., Jeelani, G., Sato, D. and Nozaki, T. (2011): Global analysis of gene expression in response to L-Cysteine deprivation in the anaerobic protozoan parasite Entamoeba histolytica. BMC Genomics., 12: 275-293.

Husain, K., Scott, B. R., Reddy, S. K., and Somani, S. M. (2001): Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. Alcohol., 25: 89-97.

Jaroll, E. L., and Paget, T. A., (1995): Carbohydrate and amino acid metabolism in *Giardia*: a review. Fol Parasitologica., 42: 81-89.

Kim, J.B., Yu, Y. M., Kim, S. W., and Lee, J. K. (2005): Anti-imflammatory mechanism is involved in ethyl pyruvate-mediated efficacious neuroprotection in the postischemic brain. Brain Res., 1060: 188-92.

Knodler, L.A., Schofield, P.J., and Edwards, M.R. (1995): L-Arginine transport and metabolism in *G. intestinalis* support its position as a transition between prokaryotic and eukaryotic kingdoms. Microbiology., 149, 2063–2070.

Lane, S. and Lloyd, D. (2002): Current trends in research into the waterborne parasite *Giardia*; Crit. Rev. Microbiol., 28: 123-147.

Lin, R. Y., Vera, J. C., Chaganti, R. S., and Golde, D. W. (1998): Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. J Biol Chem., 273: 28959-65.

Lindmark, D. G., (1980): Energy metabolism of the anaerobic protozoan *Giardia lamblia*. Mol Biochem Parasitol., 1: 1-12.

Lloyd, D., Harris, J. C., Maroulis, S., Biagini, G. A., Wadley, R. B., Turner, M. P. And Edwards, M. R. (2000): The microaerophilic flagellate *Giardia intestinalis*: oxygen and its reaction products collapse membrane potential and cause cytotoxicity. Microbiology., 146: 3109-3118.

Mastronicola, D, Falabella, M, Testa, F, Pucillo, LP, Teixeira, M, Sarti, P, Saraiva, LM, and Giuffrè. (2014): Functional characterization of peroxiredoxins from the human protozoan parasite *Giardia intestinalis*. PLoS Negl Trop Dis., 9;8(1):e2631

Mastronicola, D, Giuffrè, A, Testa, F, Mura, A, Forte, E, Bordi, E, Pucillo, LP, Fiori, PL, and Sarti, P. (2011): *Giardia intestinalis* escape oxidative stress by colonizing the small intestine: a molecular hypothesis. IUBMB Life., 63 (1): 21-25.

Mead, J. M. (1976): Free radical mechanisms of lipid damage and consequences for cellular membranes. London: Academic Press., 51-68.

Mendis, A., Thompson, R., Reynoldson, J., Armson, A., Meloni, B., and Gunsberg, S. (1992): The uptake and conversion of 1-[U^{14}C-] aspartate and L-[U^{14}C-] to $^{14}CO_2$ by intact trophozoites of *Giardia duodenalis*. Comp Biochem Physiol; 102: 235-239.

Mertens, E. (1993): ATP versus pyrophosphate glycolysis revisited in parasitic protest. Parasitol Today., 9: 122-126.

Mertens, E. (1990): Occurrence of pyrophosphate: fructose 6-phosphate 1-phosphotransferase in *Giardia lamblia* trophozoites. Mol Biochem Parasitol., 40: 147-149.

Paget, T. A., Kelly, M. L., Jarrol, E. L., Lindmark, D. G. And Lloyd D. (1993): The effects of oxygen on fermentation in *Giardia lamblia*. Mol Biochem parasitol., 57: 65-72.

Park, S. M., Sinskey, A. J., and Stephanopoulos, G. (1997): Metabolic and physiological studies of Corynebacterium glutamicum mutants. Biotechnol Bioeng., 55: 864-879.

Phillips, N. F. B., Li, Z., and Lindmark, D. G. (1997): Isolation of a pyrophosphate dependent phosphofructokinase from Hexamita inflata. Mol Biochem Parasitol., 90: 377380.

Rael, L. T., Thomas, G. W., Craun, M. L., Curtis, C. G.,Or, R. B. and Or, D. B. (2004): Lipid Peroxidation and the Thiobarbituric Acid Assay: Standardization of the Assay When Using Saturated and Unsaturated Fatty Acids. Journal of Biochemistry and Molecular Biology., 37: 749-752.

Raj, D., Ghosh, E., Mukherjee, K. A., Nozaki, T., and Ganguly, S. (2014): Differential gene expression in *Giardia lamblia* under oxidative stress: Significance in eukaryotic evolution. Gene., 535: 131-139.

Sandhu, S. K., and Kaur, G. (2003): Mitochondrial electron transport chain complexes in aging rat brain and lymphocytes. Biogerontology., 4: 19-29.

Schofield, P. J., Costello, M., Edwards, M. R., and O'Sullivan, W. J. (1990): The arginine dihydrolase pathway is present in *Giardia intestinalis*. Int J Parasitol., 20: 697-699.

Schuessel, K., Frey, C., Jourdan, C., Keil, V., Weber, C. C., Muller, F., Muller, W. E., and Echest, A. (2006): Aging sensitizes toward ROS formation and lipid peroxidation in PSIM146L transgenic mice. Free Radic Biol Med., 40: 850-862.

Scott, K. G., J. B. Meddings, D. R. Kirk, S. P. Lees-Miller, and A. G. Buret. (2002**):** Intestinal infection with *Giardia* spp. reduces epithelial barrier function in a myosin light chain kinase-dependent fashion. Gastroenterology., 123**:**1179-1190.

Singer, S. M., and Nash T. E. (2000): The role of normal flora in *Giardia lamblia* infections in mice. J. Infect. Dis., 181**:**1510-1512.

Townson, S. M., Hanson, G. R., Upcroft, J. A. and Upcroft, P. (1996): Characterization and purification of pyruvate: ferredoxin oxidoreductase from *Giardia* dudenalis. Mol Biochem Parasitol., 79: 183-193.

Vorbeck, M., Martin, A., Park, J., and Townsend, J. (1982): Aging related decrease in hepatic cytochrome oxidase of the Fischer 344 rat. Arch Biochem Biophys., 214: 67–79.