Chapter 7

Pyruvate induces ROS generation in the microaerophilic enteroparasite *Giardia lamblia* under metronidazole stress
7.1 Introduction:

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is the most commonly used drugs against parasitic infections worldwide. It prevents colonization to the gastrointestinal mucosa, an important criteria for the establishment of the diseases (Busatti et al. 2007). The nitroimidazole metronidazole is evidently the well studied compound affecting the intermediary metabolism. When trophozoites are exposed with metronidazole the cell loses motility within a few hours (Müller et al., 2006). It is well-known that the drug accumulates within the protozoon by a passive diffusion process. A specific drug reduction to an active product maintains the concentration gradient necessary for continued drug uptake, and that drug reduction necessitates the presence of electron donors of adequately powerful reducing ability (Muller & Lindmark, 1976). Metronidazole (as a prodrug) is reduced to a series of metronidazole reduction products (nitro radical anion, nitroso and hydroxylamine derivatives) by electrons coming from the enzyme pyruvate:flavodoxin/ferredoxin oxidoreductase (PFOR), a protein missing in higher eukaryotic cells (Horner et al., 1999). These radicals cause irreversible damages. It binds to the sulfhydryl (-SH) group in the active center of various enzymes, including thioradixin reductase thereby impairing essential cellular functions (Leitsch et al., 2009).

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 thioradixin 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). The α-keto carbonyl in pyruvate makes it a potent scavenger of reactive oxygen species, particularly H₂O₂ (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, metronidazole has been chosen to generate oxidative stress in the trophozoites in vitro. However, the effect of pyruvate in *Giardia* has not been depicted during metronidazole stress. In this study, we revealed that pyruvate is an important intermediary metabolite responsible for the killing of *Giardia* under metronidazole stress.

### 7.2 Materials and Methods:

#### 7.2.1 Chemicals:

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

#### 7.2.2 *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 K₂HPO₄ and 1.8 mM KH₂PO₄. Pyruvate (sodium salt), menadione, H₂O₂, 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. Dose and time kinetics of the oxidative stress by metronidazole have been standardized following the IC₅₀ values as reported previously (Raj *et al.*, 2014). Finally, from the standardized data, 1µg/µl metronidazole was exposed during the study.

#### 7.2.3 Imaging of ROS production in *Giardia* trophozoites using confocal microscopy

Intracellular generation of reactive oxygen intermediates were evaluated by using dichlorodihydrofluoresceine diacetate (H₂DCFDA) fluorescent probe according to Schuessel *et al.*, (2006) with some modifications. For monitoring intracellular ROS production, treated and untreated cells were (2x10⁶ cells/ml) incubated with H₂DCFDA (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). H₂DCFDA 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.

7.2.4 Measurement of total ROS 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 metronidazole 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.

7.2.5 Viability determination by 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.

7.2.6 Lipid peroxidation assay:

7.2.6.1 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$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$. 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.

7.2.6.2 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 x10^5 M⁻¹cm⁻¹.

7.2.7 Estimation of intracellular pyruvate concentration at different time points under metronidazole stress
Intracellular pyruvate concentration was quantified under metronidazole stress condition by using Pyruvate Assay Kit (ab65342). Trophozoites (4x10⁶ cells/ml) were incubated in TYIS-33 medium and exposed to metronidazole 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.

7.2.8 DNA fragmentation assay
Pellets of Giardia trophozoites (5x10⁶/ml) from untreated, metronidazole treated were taken. Trophozoites 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 (H₂O₂ free) after 24 h.
7.2.9 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 ºC. 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 Table7.1). 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 ºC (15 min). Subsequent DNA amplification was performed in 40 cycles including denaturation (94 ºC for 15 s); annealing (60 ºC for 30 s); and extension (72 ºC for 30 s). Fluorescence was measured at 72 ºC 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.

7.2.10 Statistical analysis:

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.

7.3 Results:

7.3.1 Observation of intracellular fluorescence in trophozoites of *Giardia* under metronidazole treatment

The H₂DCFDA, a non-fluorescent molecule, is able to enter the cells. After getting entry into the cytosol of the cell, esterase activity renders the indicator, non-permeable by forming
fluorescent product dichlorofluoresceine and the fluorescence intensity of the dye is proportional to the rate of oxidation by reactive oxygen species. Observation of cellular fluorescence in the trophozoites was examined by confocal microscopy under metronidazole treatment. Our results have shown that exogenously added physiological concentrations of pyruvate did not attenuate fluorescence, produced by reactive oxygen species (Fig. 7.1).

Figure 7.1: H$_2$DCFDA-loaded cells under confocal microscope after metronidazole 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 metronidazole (1 µg/µl) in the absence and presence of pyruvate (2 mM) and acetate (2 mM). We used ascorbate (2 mM) as a positive control and without metabolite as a negative control. Pyruvate increases the fluorescence intensity.

7.3.2 Pyruvate induced total ROS production in trophozoites under metronidazole exposure
Trophozoites were previously incubated with increasing concentrations of pyruvate from 0.001 to 10 mM and then treated with metronidazole. The level of ROS was measured in Giardia trophozoites with or without pyruvate. The ROS level increased significantly (P < 0.01) by metronidazole treatment than H2DCFDA-loaded untreated trophozoites. The fluorescence intensity was increased significantly (P < 0.05) in the presence of pyruvate for the range of concentrations from 5 to 10 mM (Fig. 7.2).
Figure 7.2: Pyruvate increases the level of ROS in *Giardia lamblia* trophozoites exposed to metronidazole. *Giardia* trophozoites were incubated in TYIS-33 medium under metronidazole (1µg/µl) exposure and exposed to increasing doses of pyruvate (from 0-10mM). Levels of ROS were estimated by spectrofluorometry using 2', 7'-dichlorodihydrofluoresceine diacetate. The data are from three representative experiments.

7.3.3 Pyruvate accelerates cytotoxicity by inducing ROS generation
Flowcytometry was performed to confirm the antioxidant activity of pyruvate in *Giardia* trophozoites under metronidazole treatment. Trophozoites were incubated for 1 h with increasing concentration of sodium pyruvate under metronidazole treatment for 3 h. Metronidazole exposure reduces trophozoites viability to 39.53%, which was significantly lower than the untreated trophozoites (88%, P < 0.001) (Fig. 7.3). The trophozoites previously incubated with Pyruvate did not protect trophozoites from metronidazole toxicity. When treated with metronidazole, trophozoites viability was decreasing by increasing concentrations of pyruvate. Acetate, produced from pyruvate, did not vary the viability of *Giardia* either in control conditions or treated with metronidazole treatment.
Figure 7.3: Pyruvate cannot protect *Giardia* trophozoites from metronidazole induced cytotoxicity. Cultured *Giardia* trophozoites were incubated at 35.5 °C under metronidazole treatment (1 µg/µl) for 3 h with increasing concentration of pyruvate. Pyruvate and metronidazole were simultaneously applied to the trophozoites. Stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (metronidazole 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*<0.001, compared with control; #*P*<0.001, compared with stressed sample.

### 7.3.4 Determination of lipid peroxidation by measuring malondialdehyde (MDA)

The degree of lipid peroxidation has been determined on the basis of malondialdehyde (MDA) formation. We have measured lipid peroxidation status in *Giardia lamblia* under metronidazole stress with or without pyruvate supplementation. Lipid peroxidation was found to be increased by 30% in trophozoites under metronidazole compared to the untreated trophozoites. Supplementation of pyruvate ranging from 0.001mM to 5mM significantly increased the lipid peroxidation from 34% (*P*<0.05) to 63.15% (*P*<0.05) in the trophozoites under metronidazole stress compared to the stressed trophozoites without pyruvate incubation (Fig.7.4).
7.3.5 Measurement of intracellular pyruvate concentration in *Giardia* trophozoites under metronidazole treatment

The intracellular pyruvate concentration in *Giardia lamblia* trophozoites were measured during oxidative stress condition. It was then examined whether *Giardia lamblia* have the ability to regulate intracellular level of pyruvate in response to metronidazole stress. Under metronidazole stress the intracellular pyruvate level raised linearly up to 2.1 µmol/mg proteins after 2 h (Fig.7.5). 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 2.6 µmol/mg proteins ($P < 0.01$). Finally, at the end of 8 h pyruvate levels in metronidazole-treated trophozoites were significantly increases (3.95 µmol/mg proteins, $P < 0.05$) than the trophozoites without treated.
**Figure 7.5:** Intracellular pyruvate concentration in *Giardia* trophozoites during metronidazole stress. Intracellular pyruvate content was measured in *Giardia lamblia* under metronidazole 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.

### 7.3.6 DNA ladder assay

The hallmark of apoptosis in mammalian cell is the degradation of genomic DNA. Therefore, we examined the DNA fragmentation pattern for untreated and stressed-induced trophozoites and also in metronidazole-induced trophozoites previously supplemented with pyruvate. The stressed-induced trophozoites showed a DNA fragmentation pattern after 8 h exposure to pyruvate. The ladder pattern was not clear as a metazoan DNA ladder and showed some degree of smearing with fragmented DNA in the low molecular weight region, identified by electrophoresis on a 1.5% agarose gel (**Fig.7.6**).
Figure 7.6: Effect of pyruvate on DNA fragmentation. Electrophoretic analysis of DNA fragmentation on a 1.5% agarose for Giardia trophozoites treated with metronidazole in the absence and presence of pyruvate.

7.3.7 Transcriptional regulation of gene expression in trophozoites of Giardia lamblia upon metronidazole exposure

To understand the effect of metronidazole stress in transcriptional regulation of gene expression in Giardia lamblia, we performed a time course analysis of gene expression of pyruvate metabolism pathway under metronidazole stress using a quantitative RT-PCR. We have chosen eight genes, related to the oxidative stress metabolism of Giardia lamblia modulated by at least 2 fold at one or more time points in response to metronidazole. In our study, we have shown that arginine deiminase (ARGD)-encoding gene was down-regulated in Giardia trophozoites during metronidazole stress. In Giardia lamblia, pyruvate can be produced by three different pathways. Malate dehydrogenase (MDH) gene was up regulated at one time points upon metronidazole exposure. The gene showed a down-regulation from 6th hour of metronidazole exposure. In response to metronidazole stress NADH oxidase remained down-regulated after 6th hour time points (Fig.7.7). In our study, PFOR-encoding gene was up-regulated during the first couple of hours under metronidazole stress. The
enzyme disulfide reductase, NADH oxidoreductase, alcohol dehydrogenase and peroxiredoxin transcript was always up-regulated during metronidazole stress.

Figure 7.7: The effect of metronidazole stress in transcriptional regulation of gene expression in *Giardia lamblia*. Modulation of transcripts encoding enzymes involved in oxidative stress metabolism. **A.** Gene expression (fold change) under metronidazole 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: **Antioxidant enzymes:** PFOR: Pyruvate-ferredoxin oxidoreductase, MALDH: Malate dehydrogenase, NADH: NADH ferredoxin oxidoreductase, DSRD: Disulfide reductase, NADHOX: NADH oxidase, ALCDH: Alcohol dehydrogenase, POR: Peroxiredoxin, ARGD: Arginine deiminase.)
Table 7.1: List of genes and their respective primers used in Real time PCR

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<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Size (bp)</th>
<th>Code</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Pyruvate ferredoxin-oxidoreductase</td>
<td>GI50803_114609</td>
<td>3762</td>
<td>PFOR</td>
<td>Forward: 5'-ATCCAACGCGACCCAGAAG-3</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5'-GTTCAGCTGCTACTCCGAGC-3</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>GI50803_3331</td>
<td>996</td>
<td>MALDH</td>
<td>Forward: 5'-GAGACATGCTGGGCTACGA-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5'-CGGCAGGAACCTCAAGCATA-3</td>
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<tr>
<td>NADH oxidase</td>
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<td>1290</td>
<td>NADHOX</td>
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<td></td>
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<td>Reverse: 5'-TGCGTGCTGCTTTTTCG-3'</td>
</tr>
<tr>
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<td>NADH</td>
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<td></td>
<td></td>
<td></td>
<td>Reverse: 5'-CACCCCCCTGCCCATTCTAT-3'</td>
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<tr>
<td>Disulfide reductase</td>
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<td>945</td>
<td>DSRD</td>
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<td></td>
<td></td>
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<td>Reverse: 5'-GAACTTGTGCCTGAGA-3'</td>
</tr>
<tr>
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<td>ARGD</td>
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<td></td>
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<td>Reverse: 5'-GGGACTGCTGTAACCATT-3'</td>
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<tr>
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<td>POR</td>
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<td>ALCDH</td>
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<td></td>
<td></td>
<td></td>
<td>Reverse: 5'-AAGCAGTCCGACGTGAT-3'</td>
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</tbody>
</table>
7.4 Discussion:

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

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

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

In this study, we demonstrate that metronidazole radical anions are generated in the cytoplasm of *Giardia lamblia* under metronidazole exposure previously incubated with pyruvate as a source of reducing power (*Fig.7.8*), and that these free radicals can arrive at the organelle membrane and produce lipid radicals by lipid peroxidation and undergoes apoptotic death.
Figure 7.8: Mode of action of metronidazole in parasite *Giardia lamblia*.

In the case of metronidazole, reduced ferredoxin appears to be the primary electron donor responsible for its reduction. (R-NO₂) is activated by the parasite via the reduction to an anion radical. This highly reactive anion radical will then damage DNA and proteins resulting in parasite death.