***Original Research Article***

# Metronidazole induces metazoan death of *Giardia* by the help of Pyruvate


## Abstract:

**Background:** Metronidazole is the most common drug for the treatment of infectious agent *Giardia*. The trophozoites need to fight against oxidative stress generated by metronidazole for their survival. It has been reported that trophozoites have several enzymes involved in response to oxidative stress such as pyruvate ferredoxin oxidoreductase, NADH oxidase, peroxiredoxin, etc. to combat the harsh condition but the actual mechanism of trophozoites death due to metronidazole treatment was still remain unclear.

**Methods:** The present study aims to establish the effects of pyruvate in *Giardia* trophozoites 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.

**Results:** 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 induced lipid peroxidation of stressed *Giardia*.

**Conclusion:** These results suggest that pyruvate is the key regulatory metabolite that helps generation of different radicals to initiate apoptotic like death in *Giardia* trophozoites during metronidazole exposure.

**Keywords: Metronidazole, Oxidative stress, Metazoan, ROS, Pyruvate, apoptosis.**

## 1. Introduction:

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

In the elevated oxygen environment, trophozoites consume oxygen up to a threshold level depending on the Species, above which consumption is arrested due to the production of reactive oxygen species (ROS) (Biagini *et al.*, 1997; Lloyd *et al.*, 2000). There are some

similarities in the energy metabolism of *Giardia lamblia* with bacteria. It 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 pool (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 different from the 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 disulfide reductase, which can reduce cysteine (Brown *et al.*, 1998). It was reported that peroxiredoxins has an important role in the antioxidant defense of *Giardia* (Mastronicola *et al.*, 2014). Intermediary metabolite pyruvate, containing the α-keto carbonyl group, makes it a potential scavenger of reactive oxygen species, particularly $H_2O_2$ (Bunton, 1949; Fink, 2001). There are some reports established that Pyruvate can enters into the cells with the help of a monocarboxylate transporter (Kim *et al*., 2005; Lin *et al*., 1998). In the present study, metronidazole has been chosen to generate oxidative stress in trophozoites in vitro. There are several studies were performed using mass spectrometry to identify the differential metabolites effect by metronidazole to identify potential routes that are essential for parasite survival. Here, we have carried out the role of pyruvate towards metronidazole reduction to produce different nitro radicals which are responsible for apoptotic death in *Giardia*. 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* trophozoites under metronidazole stress.

## 2. Materials and Methods:

### 3.1 Chemicals:

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

## 3.2 *Giardia* trophozoite 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 according to our previous report (Raj *et al*., 2018) with few modifications. Solutions were prepared freshly on the day of the experiment. For experimentation, the same sets of trophozoites were taken for individual experiments. Dose and time kinetics of the oxidative stress by metronidazole have been standardized following the $IC_{50}$ values as reported previously (Raj *et al*., 2014). Finally, from the standardized data, trophozoites were exposed to 1µg/µl metronidazole concentration.

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

Intracellular generation of reactive oxygen intermediates was 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 ($10^7$ cells/ml) incubated with $H_2$DCFDA (1.5 µM) for 15 min at 37 $^{\circ}$C. Consequently, 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 the same gain were observed for each condition.

## 3.3 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 dichlorofluorescein. *Giardia* trophozoites ($10^7$cells/ml) were incubated in the presence and absence of different concentrations of pyruvate (0-5 mM) in TYIS-33 medium under metronidazole treatment. After 1 h of incubation, the 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 the addition of $H_2$DCFDA, fluorescence emission was measured continuously at 530 nm after excitation at 488 nm.

## 3.4 Viability determination by flowcytometry:

Treated and non-treated trophozoites previously incubated with or without pyruvate were harvested and aliquots were made up to $10^7$ cells/100 µL into microcentrifuge tubes. Trophozoites were washed with PBS and centrifuged at 2000 rpm for 10 minutes to obtain the pellet. Then pellet was 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 FACS ARIA-III flow cytometer (BD Biosciences, San Jose, USA) instrument was performed.

## 3.5 Lipid peroxidation assay:

### 3.5.1 Sample preparation

The treated and untreated cells ($10^7$ 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 trophozoites were homogenized in ice-cold PBS in a proportion of $10^7$ no. of cells in 1ml of PBS. The homogenates were centrifuged for 15 min, 10000g at +4 $^{\circ}$C. The 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. The supernatant was collected and mixed with 200 µl of 0.8% thiobarbituric 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.

### 3.5.2 Measurement of MDA concentration by spectrophotometer:

The process of lipid peroxidation results in the formation of malondialdehyde (MDA). This is a secondary product in the sequence of lipid peroxidation reactions (Evans *et al*., 1999; Rael *et al*., 2004). The MDA assay was used to assess the MDA concentration as described by Bar-Or *et al*., (2001) with few modifications. The absorbance of the chromophore was taken at 535 nm. The MDA concentration is presented as nmoles of MDA produced/mg protein using a molar extinction coefficient of 1.56 x$10^5$ $M^{-1}cm^{-1}$.

## 3.6 Estimation of intracellular pyruvate concentration at different time points under metronidazole stress

Intracellular pyruvate concentration was quantified under metronidazole stress conditions by using Pyruvate Assay Kit (ab65342). Trophozoites ($10^7$cells/ml) were incubated in TYIS-33

medium and exposed to metronidazole for 0 to 8 h. the homogenized mixture was centrifuged for 15 min, 10000g at +4 °C. Perchloric acid (6%, PA) was used to lyse trophozoites and inactivate the enzyme. The supernatant was collected and used for pyruvate assay according to the manufacturer's protocol. The pyruvate concentration was determined according to a standard curve established between 0 and 0.5 mM pyruvate (Raj *et al*., 2018).

## 3.7 DNA fragmentation assay:

Pellets of *Giardia* trophozoites ($5x10^6$/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. To remove RNA contamination DNA samples were treated with DNase-free RNase (0.1 mg/ml) for 1 h at 37 °C. 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 (Metronidazole free) after 24 h.

## 3.8 Gene expression studies:

To study gene expression by real-time PCR (qRT–PCR), trophozoites were grown and 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. 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. All the primer sequences were taken from Raj *et al*., 2014. 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 combated DNase I digestion. 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 'housekeeping' gene actin.

## 3.9 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.

# 4 Results:

### 4.1 Observation of intracellular fluorescence in trophozoites of *Giardia* under metronidazole treatment

The $H_2$DCFDA, a non-fluorescent molecule, can enter the cells. After getting entry into the cytosol of the cell, esterase activity renders the indicator, non-permeable by forming fluorescent product dichlorofluorescein 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.1**).

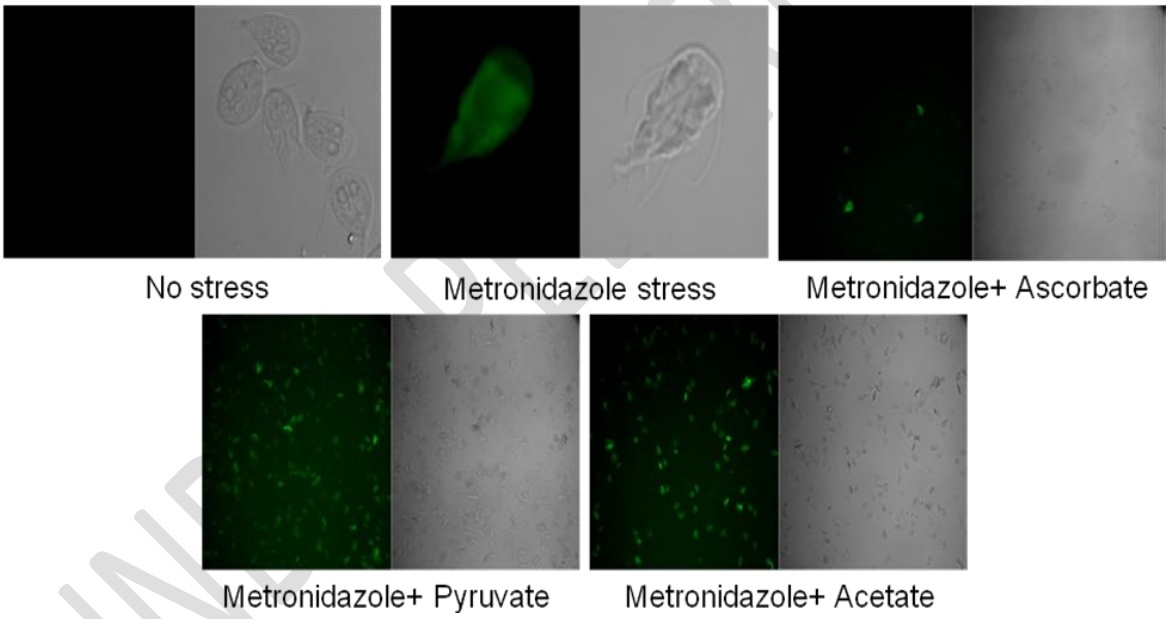


**Figure 1: $H_2$DCFDA-loaded cells under confocal microscope after metronidazole stress.** Increases in fluorescence are representative of increase in the rate of oxidative species generated. Fluorescence was monitored from the suspension of live cells after the addition of metronidazole (1 µg/µl) in 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

### 4.2 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.2**).

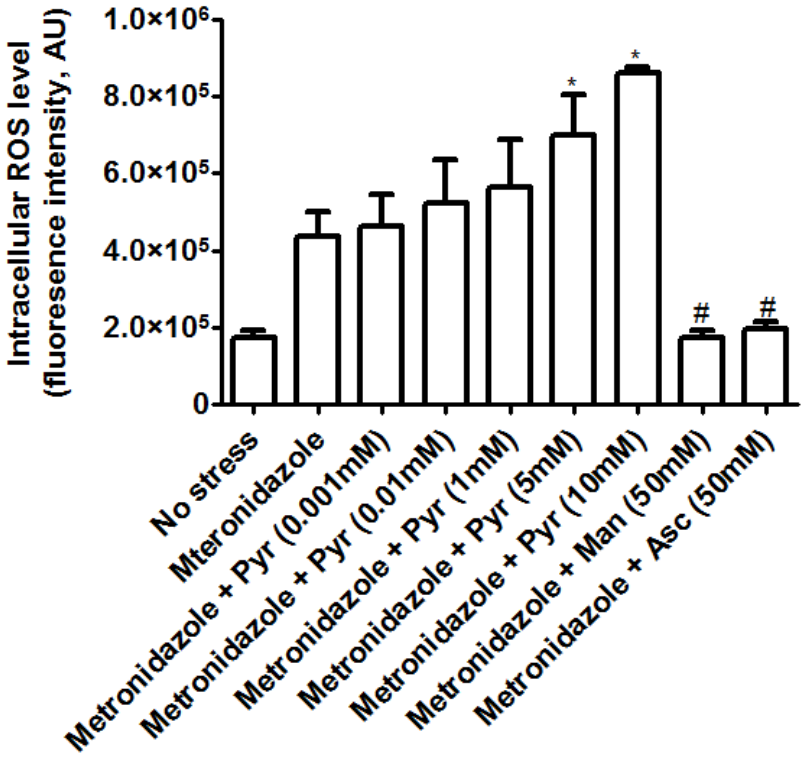


**Figure 2: Pyruvate increases the level of ROS in *Giardia lamblia* trophozoites exposed to metronidazole.** *Giardia* trophozoites were incubated in TYIS-33 medium under metronidazole (1µg/µl) exposure and exposed to increasing doses of pyruvate (from 0-10mM). Levels of ROS were estimated by spectrofluorometry using 2´, 7´-dichlorodihydrofluoresceine diacetate. The data are from three representative experiments.

### 4.3 Pyruvate accelerates cytotoxicity by inducing ROS generation

Flow cytometry was performed to confirm the antioxidant activity of pyruvate in *Giardia* trophozoites under metronidazole treatment. Trophozoites were incubated for 1 h with an increasing concentration of sodium pyruvate under metronidazole treatment for 3 h. Metronidazole exposure reduces trophozoite viability to 39.53%, which was significantly

lower than the untreated trophozoites (88%, P < 0.001) (**Fig.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.

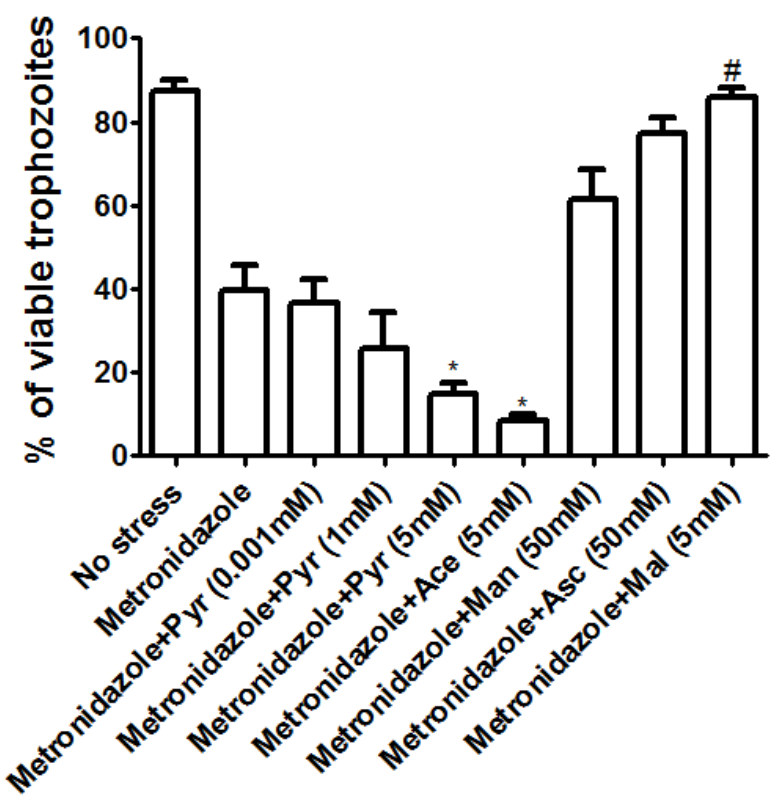


**Figure 3: Pyruvate cannot protect *Giardia* trophozoites from metronidazole induced cytotoxicity.** Cultured *Giardia* trophozoites were incubated at 35.5 °C under metronidazole treatment (1 µg/µl) for 3 h with increasing concentration of pyruvate. Pyruvate and metronidazole were simultaneously applied 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 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. $^{*}P<0.001$, compared with control; $^{\#}P<0.001$, compared with the stressed sample.

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

The degree of lipid peroxidation has been determined based on 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.4**).

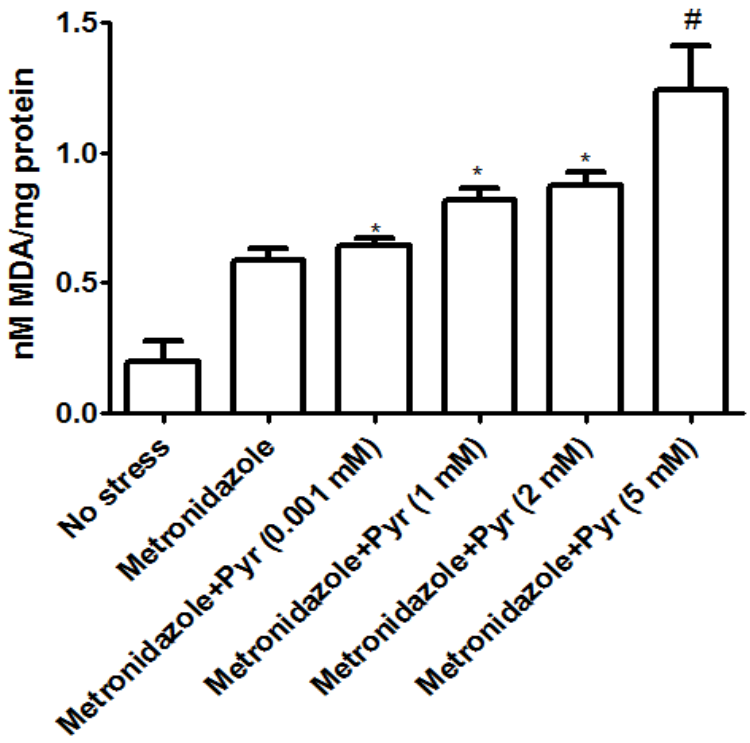


**Figure 4: Effect of pyruvate on the degree of lipid peroxidation in cultured *Giardia* trophozoites upon metronidazole exposure: (A)** MDA concentration in metronidazole treated *Giardia* trophozoites after 8 h incubation. Values are mean ± SEM of three independent experiments, each performed in triplicate. $^{*}P<0.05$, compared with control; $^{\#}P<0.001$, compared with the stressed sample.

### 4.5 Measurement of intracellular pyruvate concentration in *Giardia* trophozoites under metronidazole treatment

The intracellular pyruvate concentration in *Giardia lamblia* trophozoites was measured during oxidative stress condition. It was then examined whether *Giardia lamblia* can regulate the

intracellular levels 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.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.

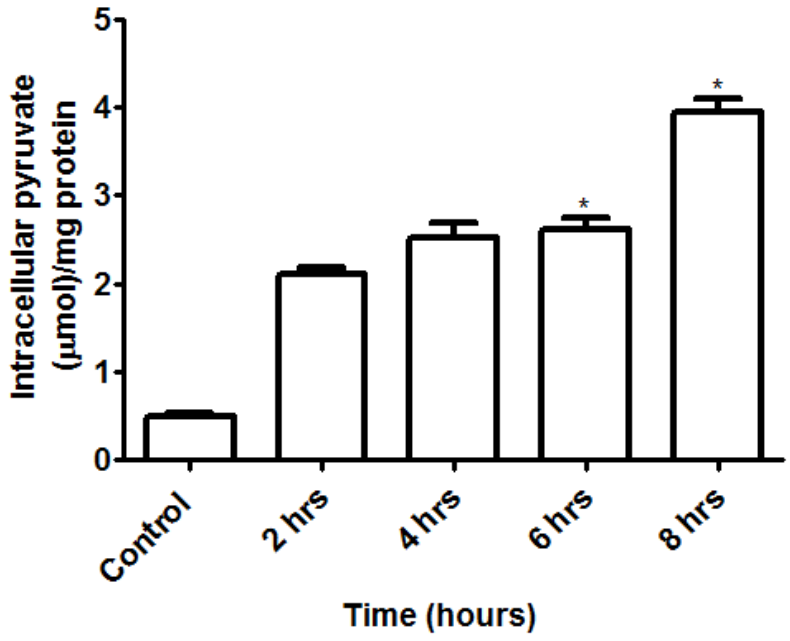


**Figure 5: Intracellular pyruvate concentration in *Giardia* trophozoites during metronidazole stress.** Intracellular pyruvate content was measured in *Giardia lamblia* under metronidazole treatment. The level of intracellular pyruvate was quantified every two hours interval. Values are means ± SEM of three independent experiments, each performed in triplicate. $^{*}P<0.05$, compared with control.

### 4.6 DNA laddering assay

The hallmark of apoptosis in the mammalian cells is the degradation of genomic DNA (Popruk, S. *et. al.*, 2023). 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.6**).

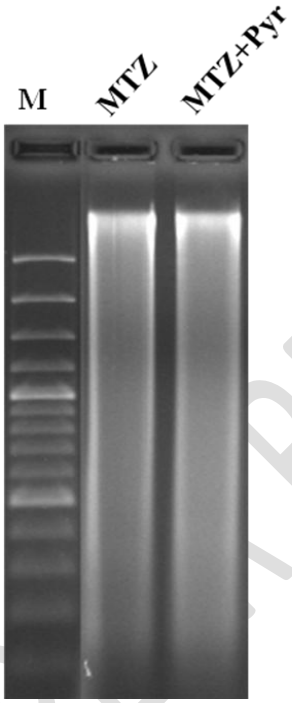


**Figure 6: Effect of pyruvate on DNA fragmentation.** Electrophoretic analysis of DNA fragmentation on a 1.5% agarose for *Giardia* trophozoites treated with metronidazole in the absence and presence of pyruvate. (M=Marker, MTZ=Metronidazole, Pyr=Pyruvate)



### 4.7 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 the 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. The Malate dehydrogenase (MDH) gene was upregulated at one time points upon metronidazole exposure. The gene showed a down-

regulation from the 6th hour of metronidazole exposure. In response to metronidazole stress, NADH oxidase remained down-regulated after 6th hour time points (**Fig.7**). In our study, the 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 remain always up-regulated during metronidazole stress.

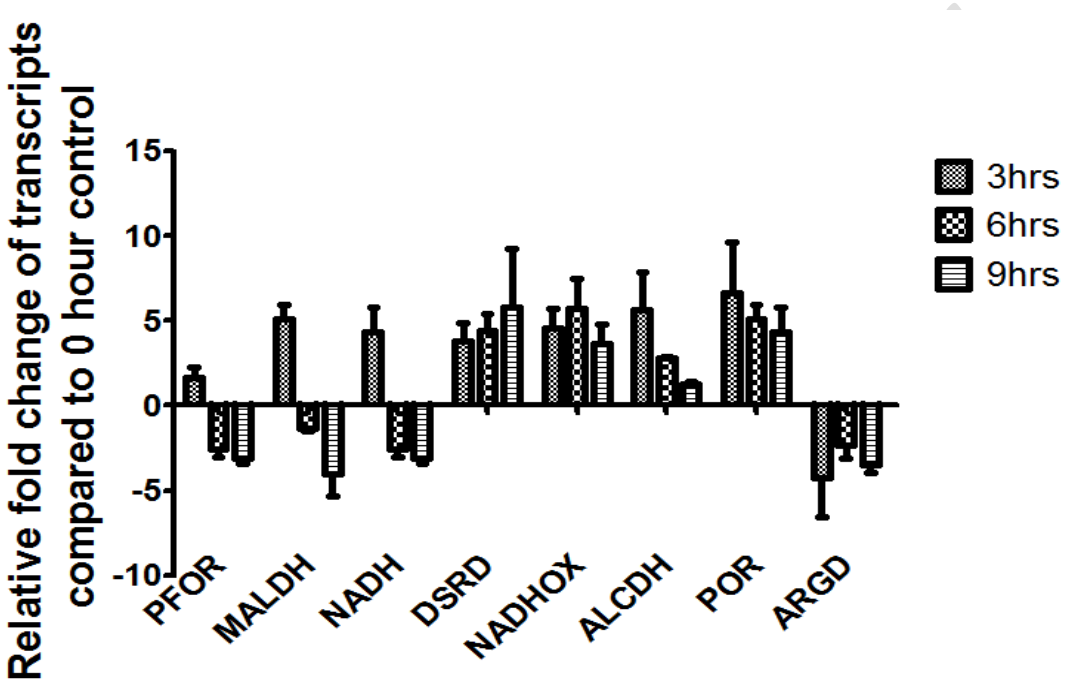


**Figure 7: The effect of metronidazole stress in transcriptional regulation of gene expression in *Giardia lamblia.*** Modulation of transcripts encoding enzymes involved in oxidative stress metabolism. **A.** Gene expression (fold change) under metronidazole stress. Data are shown as fold change in 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 oxidoreductade, **MALDH:** Malate dehydrogenase, **NADH:** NADH ferredoxin oxidoreductase, **DSRD:** Disulfide reductase, **NADHOX:** NADH oxidase, **ALCDH:** Alcohol dehydrogenase, **POR:** Peroxiredoxin, **ARGD:** Arginine deiminase.

## 5. Discussion:

Metronidazole, a 5-nitroimidazole drug has been used to treat giardiasis. Anaerobic parasitic infections caused by different protozoan parasites respond favorably to metronidazole therapy (Ganguly & Raj, 2016). It is an inactive prodrug at the time of administration but activated to

its cytotoxic form via the transfer of an electron to the nitro group of the compound, which converts it to the different nitro derivatives (Land & Johnson, 1999). The trophozoites must fight against oxidative stress generated by metronidazole. Metronidazole reduction was initiated by pyruvate, but progressive damage in the trophozoites by 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 peroxiredoxin, 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 induces lipid peroxidation of stressed *Giardia*. pyruvate can reduce metronidazole and form different types of nitroso radical derivatives which can damage DNA (Popruk, S. *et. al*., 2023). We have shown that expression levels of different metabolic genes are significantly up or downregulated during metronidazole treatment. This suggests that these genes are involved in combating 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.8**) and that these free radicals can arrive at the organelle membrane and produce lipid radicals by lipid peroxidation and undergoes apoptotic death.

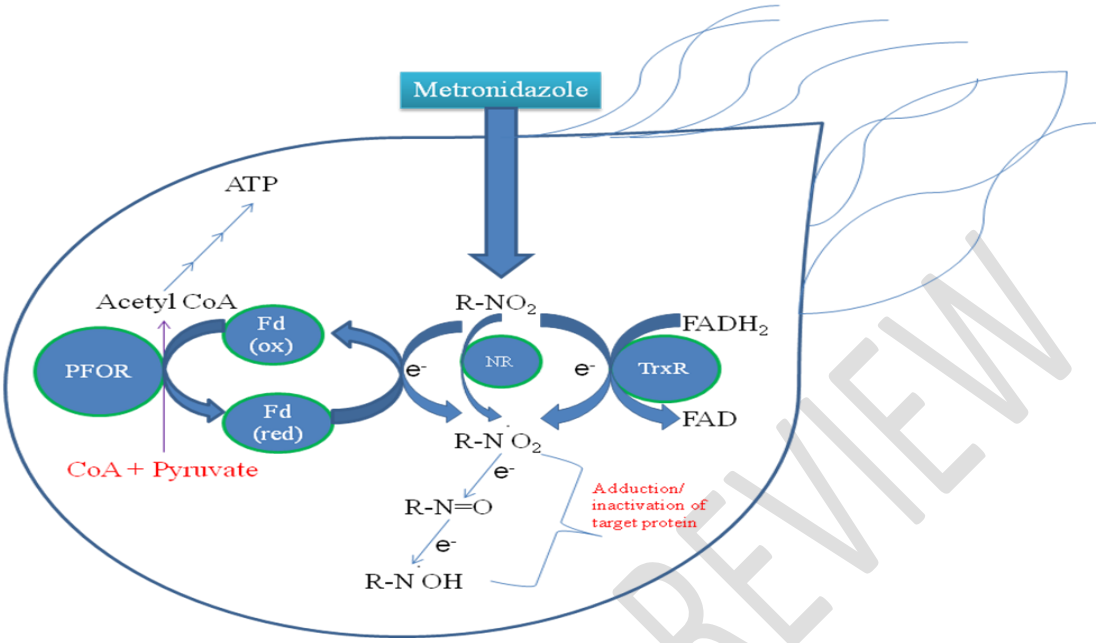


**Figure 8:** Mode of action of metronidazole in parasite *Giardia lamblia*.

In the case of metronidazole, reduced ferredoxin appears to be the primary electron donor responsible for its reduction. ($R\text{-}NO_2$) 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.

## Highlights:

1. Intracellular ROS generation increases in *Giardia* trophozoites by metronidazole treatment.
2. Pyruvate, a so called antioxidant augmented ROS generation in *Giardia* trophozoites previously treated with metronidazole.
3. Metronidazole reduction was initiated by pyruvate and different metronidazole radical anions are generated in the cytoplasm of *Giardia lamblia.*
4. DNA laddering assay proved that trophozoites treated with metronidazole undergoes apoptotic like metazoan death with the aid of pyruvate.
5. Gene expression studies revealed that different metabolic genes are induced under metronidazole stress.

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**Abbreviations:**

**ROS:** reactive oxygen species, **NADH**: (reduced) nicotinamide adenine dinucleotide, **PCD**: programmed cell death protein like protein, **TYIS-33**: tryptone-yeast extract-iron-serum-33, **PBS**: phosphate buffered saline, **PCR**: polymerase chain reaction, **TBS**: Thiobarbituric acid, **MDA**: Malondialdehyde **SDS**: Sodium dodecyl sulphate, **EDTA**: Ethylenediaminetetraacetic acid**, Pyr:** Pyruvate**, Man:** Manitol**, Asc:** Ascorbate**, Ace:** Acetate**, Mal:** Malate**, MTZ:**

Metronidazole**, ARGD:** Arginine deiminase**, NADPH**: (reduced) nicotinamide adenine dinucleotide phosphate, **ATP**: adenosine tri-phosphate, **$H_2$DCFDA**: 2', 7'-dichlorodihydro fluorescein diacetate, **DCF:** 2',7'-dichlorofluorescein, **RT-PCR**: real time PCR, **PFOR:** Pyruvate-ferredoxin oxidoreductade, **MALDH:** Malate dehydrogenase, **NADH:** NADH ferredoxin oxidoreductase, **DSRD:** Disulfide reductase, **NADHOX:** NADH oxidase, **ALCDH:** Alcohol dehydrogenase, **POR:** Peroxiredoxin.