Original Research Article

Metronidazole induces the metazoan death of *Giardia* trophozoites with the aid of pyruvate

Abstract:

Background: Metronidazole is the most common drug for the treatment of infectious agent *Giardia*. The trophozoites need to fight against the oxidative stress generated by metronidazole for their survival. It has been reported that trophozoites possesses several enzymes involved in response to oxidative stress like pyruvate-ferredoxin oxidoreductase, NADH oxidase, peroxiredoxin to combat the harsh condition. These enzyme systems generally act on the amitochondriate trophozoites to attenuate the reactive oxygen species generation which causes cytotoxicity but the actual mechanism of trophozoites death due to metronidazole treatment was still not clear.

Methods: The present study aims to establish the effects of pyruvate in *Giardia* trophozoites exposed to metronidazole treatment. Intracellular reactive oxygen species (ROS) production by *Giardia* trophozoite suspension was monitored in the presence and absence of pyruvate with the help of a dichlorodihydrofluoresceine diacetate (H_2DCFDA) based assay. In the present study, we have investigated the effects of pyruvate on DNA damage in the trophozoites during metronidazole stress. We have also looked into the expression levels of some genes to show their relevance to metronidazole stress.

Results: The exogenously addition of physiologically relevant concentration of pyruvate was shown to elevate the rate of ROS generation in *Giardia* suspension under metronidazole stress. Our results provide evidence that exogenously added pyruvate have induced lipid

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peroxidation of stressed *Giardia*. Several known genes are modulated due to the exposure of metronidazole in trophozoites.

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] is 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 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 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 which are 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).

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"In the elevated oxygen environment, trophozoite utilizes oxygen up to a threshold level depending on the Species, beyond that consumption is arrested due to the production of reactive oxygen species (ROS)" (Biagini *et al.*, 1997; Lloyd *et al.*, 2000). *Giardia* has the similar energy metabolism like bacteria. It has the eubacterial like pyruvate: ferredoxin oxidoreductase (Townson *et al.*, 1996) and pyro-phosphate dependent glycolytic enzymes (Mertens, 1990; Phillips *et al.*, 1997). It contains cysteine in place of glutathione as the major intracellular pool (Brown *et al.*, 1993) and it possesses the arginine dihydrolase pathway as a supplementary energy source (Schofield *et al.*, 1990; Dimopoulos, 2000). "The amitochondriate *Giardia* has antioxidant defense systems which are unusual from the eukaryote". [26] The known antioxidant enzyme systems like superoxide dismutase, catalase, and non-specific peroxidase activities are undetectable in *Giardia lamblia* (Brown *et al.*, 1995) but it has a thioredoxin reductase like disulfide reductase, which can reduce cysteine (Brown *et al.*, 1998). "It was reported that peroxiredoxins has a vital 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 a few reports established that Pyruvate can penetrate into the cells with the help of a monocarboxylate transporter" (Kim *et al*., 2005; Lin *et al*., 1998). "In this study, metronidazole has been chosen to generate oxidative stress in trophozoites to mimic our gut environment in vitro. Several studies were reported using mass spectrometry and determined the differential metabolites effect under metronidazole treatment to identify potential routes that are essential for parasite survival" (Popruk *et al*., 2023). In the present study, 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

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intermediary metabolite responsible for the killing of *Giardia* trophozoites under metronidazole stress.

2. Materials and Methods:

1.1 Chemicals:

1.2 All reagents (like medium components, assay components, etc.) were purchased from Sigma-Aldrich (St. Louis, MO, the USA) unless otherwise it was mentioned.

3.2 *Giardia* trophozoite culture:

"The trophozoites of *Giardia lamblia* (Portland1 strain) 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 and performed with trophozoites according to our previous report with few modifications". (Raj et al., 2018) All the required solutions were prepared freshly on the day of the experiment. To perform the experiment properly, 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.

3.3 Visualization of intracellular ROS generation in *Giardia* trophozoites under confocal microscopy:

The production of intracellular ROS was measured by using dichlorodihydrofluoresceine diacetate (H_2DCFDA) fluorescent probe according to the

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Schuessel *et al*., (2006) with few modifications. "For the estimation of the intracellular reactive oxygen species (ROS) generation, treated and untreated cells were (10^7 cells/ml) incubated with H_2DCFDA (1.5 µM) for 15 min at 37 °C. Consequently, observations were made with a confocal microscope (LSM510, Meta; Carl Zeiss, Thornwood, NY, USA). It should be noted that at least the 100 cells/group with identical morphology and with the same gain were observed under each condition". **[26]**

3.4 Estimation of total intracellular ROS by using spectrofluorometer:

"To determine the total intracellular oxidant generation which is based on the oxidation of H_2DCFDA to the fluorescent molecule dichlorofluorescein, trophozoites (10^7cells/ml) were incubated in the presence and absence of different concentrations of pyruvate (0-5 mM) in fresh TYIS-33 medium under metronidazole exposure. After 1 h of incubation, the medium was removed and trophozoites were washed with PBS. After that, ROS levels under treated and non-treated samples were measured. The total ROS productions were observed by a spectrofluorometer (QuantaMaster30, Photon Technology International), using H_2DCFDA. The fluorescence emission was monitored continuously at the 530 nm wavelength after excitation at the 488 nm wavelength". [26]

3.5 Viability determination by flowcytometric analysis:

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 tube. Trophozoites were washed with PBS and centrifuged at 2000 rpm for 10 minutes to obtain the pellet. Then pellet was resuspended in the 100 µL of flow cytometry staining buffer. To adjust flowcytometer settings for propidium iodide (PI), the 5 µL of PI staining solution was added to a control tube of otherwise unstained cells. All tubes were shaken gently and

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incubated for 1 minute in the dark. Propidium iodide (PI) fluorescence was measured for each tube immediately with a Becton-Dickinson FACS ARIA-III flow cytometer instrument (BD Biosciences, San Jose, USA).

3.6 Lipid peroxidation assay:

3.6.1 Sample preparation

The treated and untreated cells (10^7cells/ml) were harvested according to the previous protocol (Raj *et. al*, 2018). Trophozoites were washed properly and homogenized in ice-cold phosphate buffer saline (PBS) in a proportion of the 10^7cells/ml of PBS. The homogenates were spun for 15 min, 10000g at +4 °C. After that, 125 µl of 20% trichloroacetic acid was added to the supernatant and mixed properly, then centrifuged at 15000g for 10 min at +4 °C. After centrifugation, 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. Then the mixture was kept at room temperature for spectrophotometric analysis.

3.6.2 Measurement of MDA concentration by spectrophotometer:

"The progression of lipid peroxidation accomplishes with 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 assay was done to measure the MDA concentration as described by Bar-Or *et al.*, (2001) with few modifications. The absorbance of the chromophore was taken at 535 nm and the MDA concentration was represented as nmoles of MDA produced/mg protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$". [26]

3.7 Estimation of intracellular pyruvate level at different time points under metronidazole stress

The concentration of Intracellular pyruvate was measured under metronidazole stress conditions by using Pyruvate Assay Kit (ab65342). Trophozoites (10^7cells/ml) were

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incubated in fresh TYIS-33 medium and it was treated with metronidazole for 0 to 8 hrs. After that, trophozoites were homogenized and the 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).

3.8 DNA fragmentation assay:

"Pellets of *Giardia* trophozoites ($5x10^6$/ml) from untreated and metronidazole treated were collected. Trophozoites were pre-incubated in TYIS-33 medium with or without pyruvate for 8 h at 35.5 °C. After that it was harvested and resuspended in digestion buffer, containing 10 mM EDTA, 50 mM Tris, 0.5% SDS Sarcosine, pH=8.0) containing 0.5 mg/ml proteinase K. DNase-free RNase (0.1 mg/ml) were used to remove RNA contamination from DNA samples incubated for 1 h at 37 °C. DNA extraction, precipitation and washing were performed according to the" (Raj *et. al*., 2014). DNA fragmentation assay was also performed with 8 h stress-induced trophozoites reseeded in fresh TYI-S-33 medium (Metronidazole free) after 24 h.

3.9 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 with some modifications. 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 used as described in the" (Raj *et al*., 2014). "Quantitative PCR was

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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) confirms 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 genes were given as values in arbitrary units relative to the amount of constitutively expressed 'housekeeping' gene actin" (Raj *et al*., 2015).

3.10 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". [26]

4 Results:

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

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The 2´, 7´-dichlorodihydrofluoresceine diacetate (H_2DCFDA), a non-fluorescent molecule, can penetrate into the cell easily. In the cytoplasm of the cell, esterase activity renders the indicator, non-permeable by forming fluorescent product dichlorofluorescein. The fluorescence intensity of the dye is proportional to the rate of oxidation by reactive oxygen species. Cellular fluorescence intensity in the trophozoites was observed by confocal microscopy under metronidazole treatment. The results have shown that exogenously added physiological concentrations of pyruvate did not attenuate fluorescence, generated by reactive oxygen intermediates (**Fig.1**).

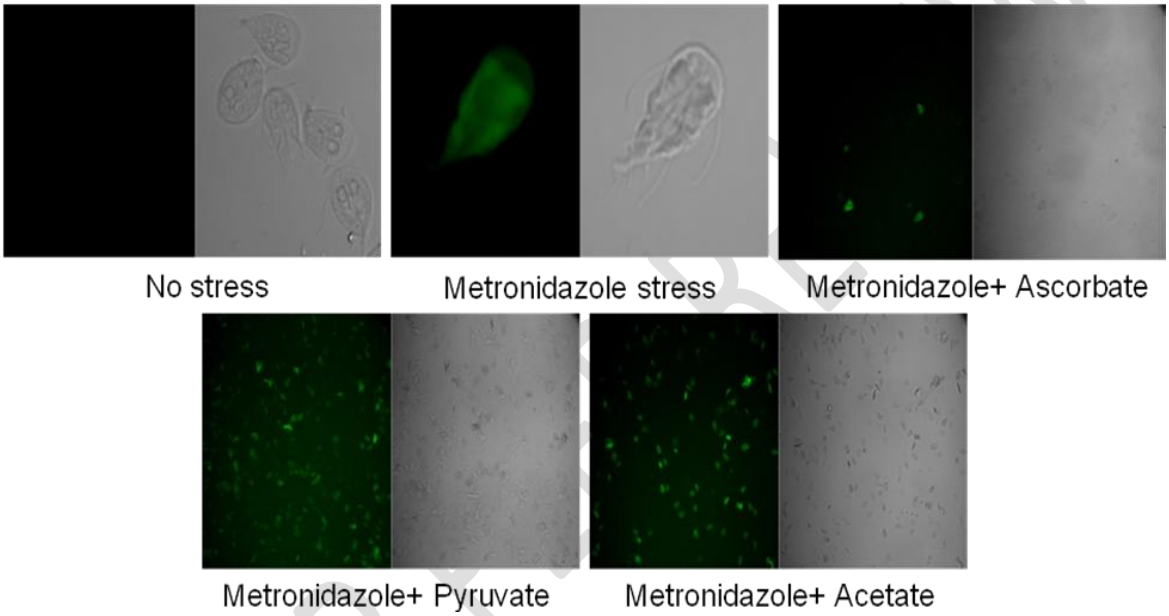

Figure 1: H_2DCFDA-loaded cells under confocal microscope after metronidazole stress. Increases in fluorescence are representative of increase in the rate of oxidative species generated. Fluorescence was monitored from the suspension of live cells after the addition of metronidazole (1 µg/µl) in the absence and presence of pyruvate (2 mM) and acetate (2 mM). We have used ascorbate (2 mM) as a positive control and without metabolite as a negative control. Fluorescence intensity was increased in the trophozoites pre-incubated with pyruvate.

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 under metronidazole treatment. The level of ROS was determined in *Giardia* trophozoites with or without pyruvate by spectrofluorometry. The ROS level elevated significantly ($P < 0.01$) under metronidazole treatment than H_2DCFDA-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**).

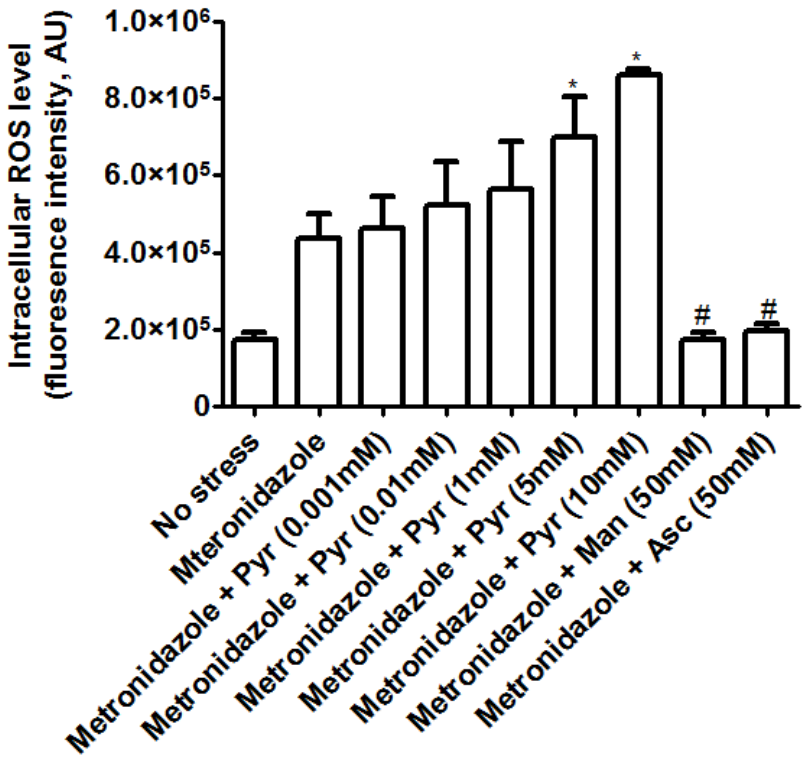

Figure 2: Pyruvate increases the ROS level in the trophozoites under metronidazole exposure. *Giardia* trophozoites were incubated in TYIS-33 medium under metronidazole (1µg/µl) treatment and exposed to increasing concentrations of pyruvate (from 0-10mM). ROS level was estimated by using 2´, 7´-dichlorodihydrofluoresceine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

4.3 Pyruvate accelerates cytotoxicity by inducing ROS generation

Flow cytometry was performed to validate the antioxidant activity of pyruvate in *Giardia* trophozoites under metronidazole treatment. Trophozoites were previously incubated with an increasing concentration of sodium pyruvate under metronidazole treatment for 3 h at 35.5 °C. Metronidazole exposure reduces trophozoite viability to 39.53%, which was considerably lesser than the untreated trophozoites (88%, $P < 0.001$) (**Fig.3**). The trophozoites previously incubated with pyruvate did not protect trophozoites from metronidazole toxicity. Viability was decreasing with increased concentrations of pyruvate in the trophozoites under metronidazole exposure. Acetate, produced from pyruvate, did not vary the viability of *Giardia* either in control conditions or treated with metronidazole.

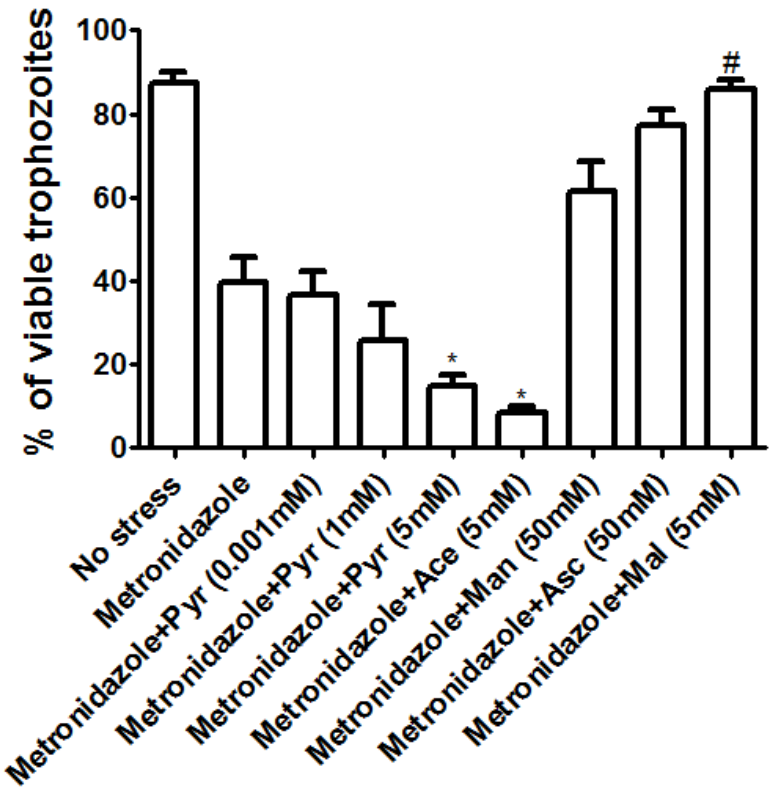

Figure 3: Pyruvate unable to protect trophozoites from metronidazole induced cytotoxicity. Trophozoites were previously incubated with increasing concentration of pyruvate under metronidazole treatment (1 µg/µl) for 3 h at 35.5 °C. Stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (metronidazole free) and their viability was determined after 24 h by using flow cytometry. Acetate was unable to decrease the rate of ROS generation in *Giardia* trophozoites. Results are expressed as the percentage of surviving trophozoites compared with untreated trophozoites. 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) production. We have estimated lipid peroxidation status in *Giardia* under metronidazole stress with or without pyruvate supplementation. Lipid peroxidation was observed to be increased by 30% in trophozoites under metronidazole compared to the untreated trophozoites. Pyruvate administration in the trophozoites suspension 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**).

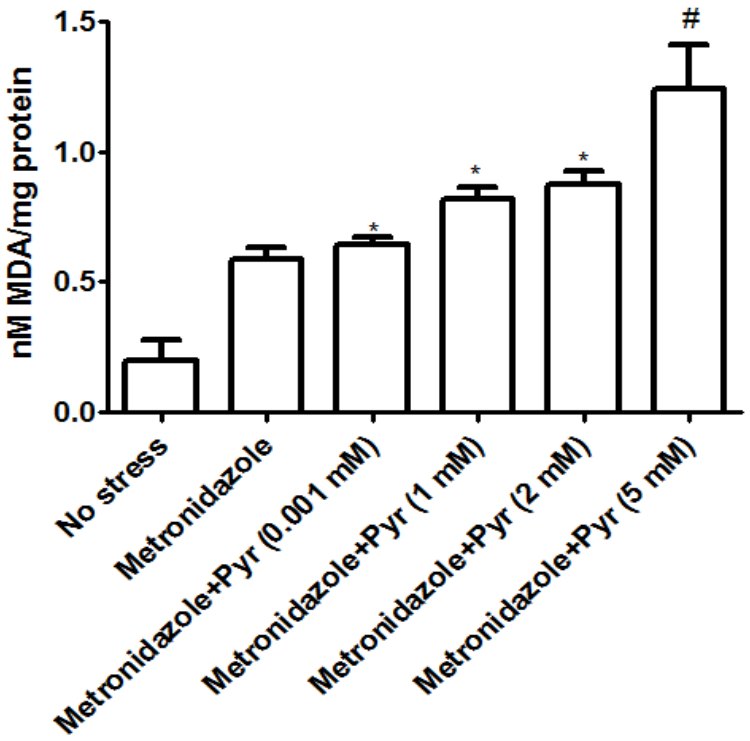

Figure 4: The role of pyruvate on the degree of lipid peroxidation in *Giardia* trophozoites upon metronidazole exposure: MDA concentration in *Giardia* trophozoites under metronidazole treatment pre-incubated with different concentration of pyruvate for 8 h. Values are mean ± SEM of three independent experiments, each performed in triplicate. $^{*}P<0.05$, compared with control; $^{\#}P<0.001$, compared with the stressed sample.

4.5 Determination of intracellular pyruvate content in *Giardia* trophozoites under metronidazole treatment

The concentration of intracellular pyruvate in *Giardia lamblia* trophozoites was evaluated in normal and oxidative stress condition. It was then investigated whether *Giardia lamblia* can regulate the intracellular levels of pyruvate in response to metronidazole treatment or it had other fate. Under metronidazole stress the intracellular level of pyruvate 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 increased to 3.95 µmol/mg proteins ($P < 0.05$) than the control set.

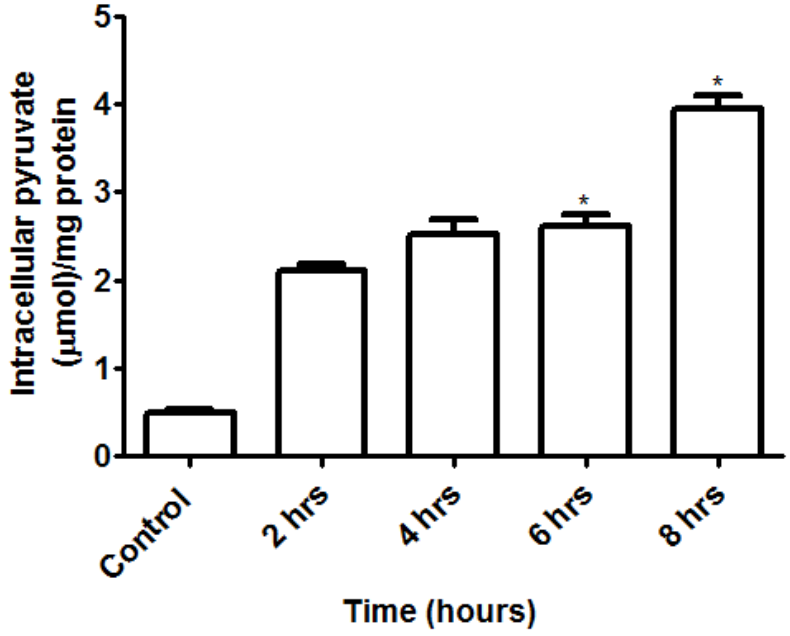

Figure 5: Intracellular pyruvate content in *Giardia* trophozoites under metronidazole treatment. Intracellular pyruvate content was determined in *Giardia* under metronidazole treatment. The quantification of intracellular pyruvate content was measured at every two hours of interval. Values are means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

4.6 DNA laddering assay

The fragmentation of genomic DNA in the mammalian cell is the hallmark of apoptosis (Popruk, S. *et. al*., 2023). Hence, we have examined the DNA degradation 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 with pyruvate. The ladder pattern was not clear as a metazoan DNA ladder and showed some degree of smearing, identified by electrophoresis on the 1.5% agarose gel (**Fig.6**).

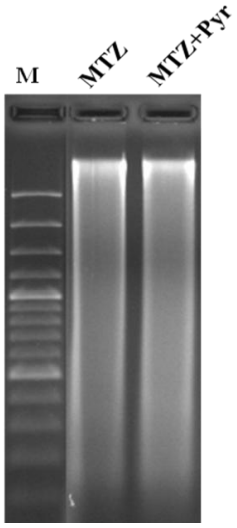

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Figure 6: Role of pyruvate on DNA degradation. Electrophoretic analysis of DNA fragmentation on the 1.5% agarose gel for *Giardia* trophozoites treated with metronidazole in the presence and absence of pyruvate. (M=Marker, MTZ=Metronidazole, Pyr=Pyruvate)

4.7 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 have performed a time-course investigation of gene expression of the pyruvate metabolic pathway under metronidazole stress using a quantitative real-time polymerase chain reaction (RT-PCR). We have selected 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 treatment (Raj *et. al*., 2014). Our present study has observed that the arginine deiminase (ARGD)-encoding gene was down-regulated in *Giardia* trophozoites under metronidazole exposure which suggests that the blockage of arginine dihydrolase pathway which used to play a significant role in energy metabolism by providing a site for anaerobic substrate level phosphorylation. In *Giardia lamblia*, pyruvate can be formed by three different pathways. The malate dehydrogenase (MDH) gene was upregulated at one time point upon metronidazole exposure. The gene showed a down-regulation from the 6th hour of metronidazole treatment. In response to metronidazole treatment, NADH oxidase remained down-regulated after 6th hour time point (**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 always remained up-regulated during metronidazole stress.

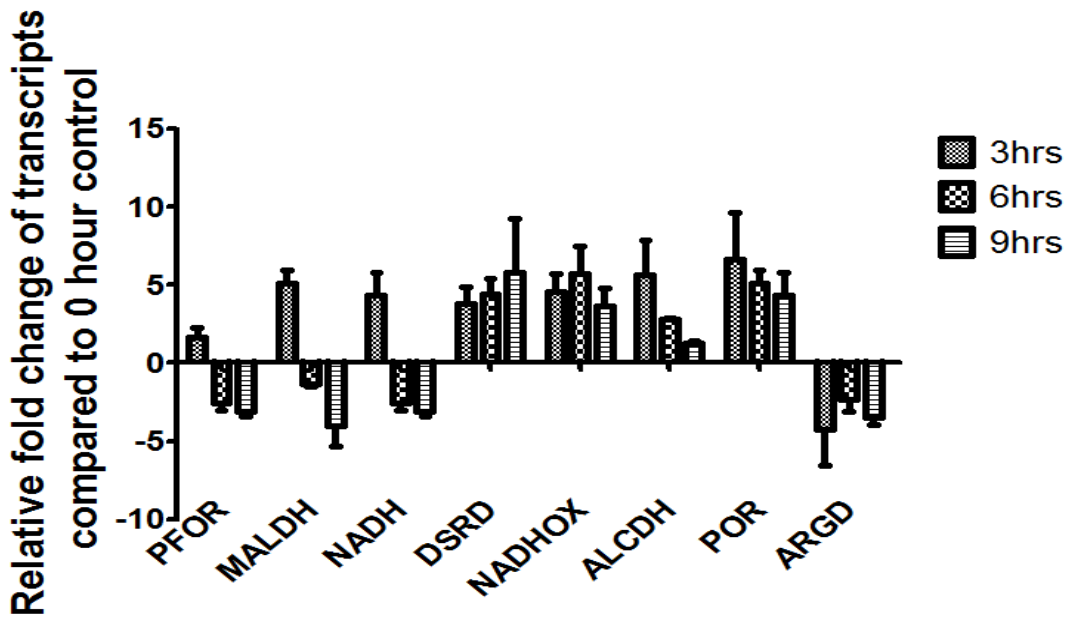

Figure 7: The effect of metronidazole treatment on gene expression studies in *Giardia lamblia.* Gene expression (fold change) studies were performed under metronidazole stress. The data are represented as fold change in relative expression compared with actin on the basis of comparative C_t ($2^{-\Delta\Delta Ct}$) method. Values are shown as mean ± SEM of three independent experiments, each performed in triplicate. **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. Several anaerobic parasitic infections caused by different protozoan parasites respond well enough 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 the oxidative stress generated by this known anti-parasitic drug, metronidazole. Its reduction was initiated by pyruvate in the presence of pyruvate-ferredoxin oxidoreductase enzyme, but progressive damage in the trophozoites was observed by the radical generating system. The data that we have found suggests that different enzymes involved in response to metronidazole stress in *Giardia* such as pyruvate ferredoxin oxidoreductase, NADH oxidase, and peroxiredoxin. The present study aims to establish the effects of pyruvate in *Giardia* trophozoites exposed to metronidazole treatment.

"Reactive oxygen species (ROS) production by *Giardia* suspension was monitored intracellularly, in the presence and absence of pyruvate with the help of dichlorodihydrofluoresceine diacetate (H_2DCFDA) fluorescent based assay. In the present study, we have examined the effects of pyruvate addition during metronidazole stress on DNA damage in *Giardia* and we have observed that unlike other oxidative stress condition pyruvate didn't have protected DNA of the trophozoites from reactive oxygen intermediates" (Raj *et al*., 2015). "We have also investigated the expression levels of some genes to show their relevance to metronidazole stress. Metronidazole radicals cause damage in a non-targeted manner and in the presence of oxygen, radical formation is reduced by futile cycling" (Krakovka S *et. al.*, 2022).

"The 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 also induced

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 down-regulated during metronidazole treatment. The results obtained from gene expression studies suggest that these genes are involved in combating metronidazole stress.

In this study, we have demonstrated 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 these free radicals can arrive at the organelle membrane and produce lipid radicals by lipid peroxidation which undergoes apoptotic death.

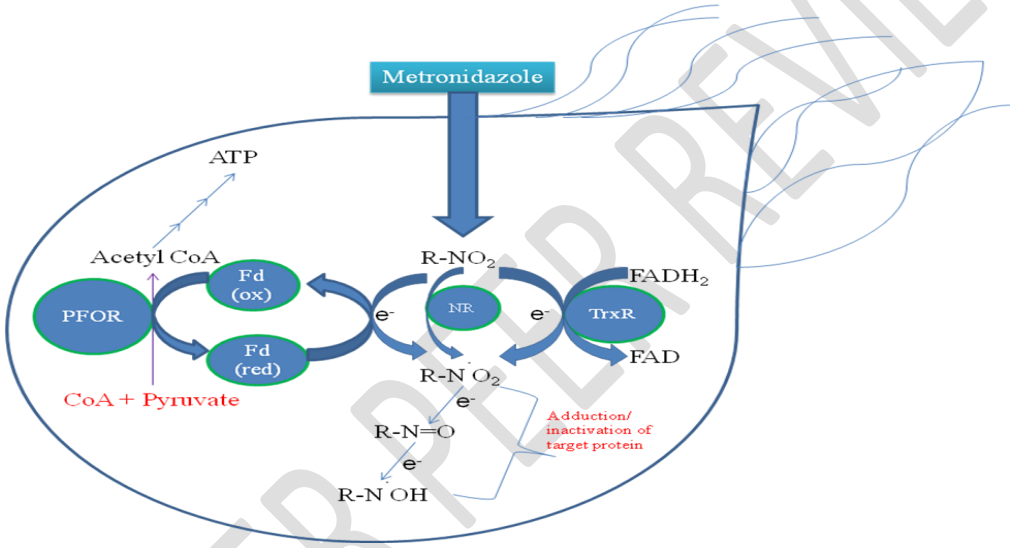

Figure 8: Mode of action of metronidazole in parasite *Giardia lamblia*.

In the case of metronidazole, reduced ferredoxin appears to be the primary electron donor responsible for its reduction. Different nitro radicals ($R\text{-}NO_2$) are activated by the parasite via the reduction to an anion radical which is highly reactive and then it will start damaging DNA and proteins resulting in parasite's death.

6. Conclusion:

Pyruvate, a so called antioxidant has augmented ROS generation in *Giardia* trophozoites, previously treated with metronidazole. Intracellular ROS generation increases in *Giardia* trophozoites by metronidazole treatment. Reduction of metronidazole was initiated by pyruvate and different metronidazole radical anions are generated in the cytoplasm of

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trophozoites and then radicals are spread rapidly to the membrane to damage the membrane lipid layers that causes apoptotic like metazoan death of the trophozoites. The gene expression studies have revealed that different metabolic genes are up or down regulated under metronidazole stress. These enzymes are the representative of different biochemical pathways which indicates that these pathways are involved directly or indirectly in the oxidative stress management system of *Giardia*. There are several enzymes like NADH oxidases, pyruvate-ferredoxin oxidoreductases, disulfide reductases, etc. have also found that up-regulated due to metronidazole stress and may have participated in stress regulation of *Giardia lamblia*. So, the results found that have unveiled some new avenue for further investigations on oxidative stress management in *Giardia lamblia*.

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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_2DCFDA**: 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.

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