**JOURNAL OF AGROMEDICINE AND MEDICAL SCIENCES (AMS) ISSN: 2460-9048 (Print), ISSN: 2714-5654 (Electronic)**

Available online at http://jurnal.unej.ac.id/index.php/JAMS

# Malondialdehyde Expression in HT29 Cells with *Blastocystis sp.* Exposure

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**Article Info**

**Article History:**
*Received: April 02, 2024*
*Accepted: July 24, 2024*
*Published: October 31, 2024*

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**How to cite this article:** :

Rusjdi, SR. Irawati N, Yanwirasti, Hilbertina N (2024). Effect of *Blastocystis sp.* Exposure on Malondialdehyde Expression in HT29 Cells. *Journal of Agromedicine and Medical Sciences. 10(3): 138-143*

*https://doi.org/10.19184/ams.v10i3.47044*

**Abstract**

*Blastocystis sp.* is one of the intestinal protozoa that is frequently found in stool specimens of colorectal cancer patients. The clinical aspects of this protozoan are still very controversial. The debate on *Blastocystis sp.* as a commensal, protecting, or aggravating colorectal cancer still continues. Increased expression of malondialdhyde, a marker of oxidative stress, is associated with the pathogenesis and progressivity of various diseases, including cancer. This study aims to determine the exposure of *Blastocystis sp.* on malondialdehyde expression in colorectal cancer cell line HT29. This research is an experimental study in vitro. HT29 cells were treated with various concentrations of *Blastocystis sp.* antigen (0.005 µg/ml, 0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml and 0.5 µg/ml) for 72 hours. Malondialdehyde expression was examined using spectrophotometric method. The results of statistical analysis using the Kruskall Wallis test showed that there were differences in malondialdehyde expression in HT29 cells after being exposed to *Blastocystis sp.* in various concentration (p=0.007). Post Hoc test showed that significant differences in malondialdehyde expression were found in the exposure groups of 0.1 and 0.5 µg/ml with the control group, 0.005 and 0.01 µg/ml with 0.1 µg/ml and groups of 0.005 and 0.01 µg/ml with 0.5 µg/ml. So it can be concluded that exposure to *Blastocystis sp.* with a certain intensity can suppress oxidative stress so that it can protect and inhibit the progressivity of cancer.

**Keywords**: *Blastocystis sp.*, HT29, malondialdehyde

## Introduction

*Blastocystis sp.* is one of the protozoa that often infects the gastrointestinal tract of children and adults. Prevalence of *Blastocystis sp.* in developing countries ranges from 22.1–100%. This situation is related to the lack of hygiene and sanitation in developing countries. The pathogenic nature of this parasite is still controversial, as it can be asymptomatic and cause mild to severe clinical conditions. Symptoms due to infection can be nausea, vomiting, anorexia, diarrhea, urticaria and arthralgia (Luh et al., 2018; Sylla et al., 2022).

*Blastocystis sp.* is one of the microorganisms that is often found in fecal specimens of colorectal cancer patients which is the fourth most malignant disease and the fourth most cause of death in Indonesia (GLOBOCAN, 2020; Kumarasamy et al., 2022). The prevalence of these protozoan infections was four times higher compared to the control group. Although its existence is often associated with colorectal cancer, its pathogenicity and clinical significance are still debated (Öncü Öner et al., 2022).

Several recent studies state that *Blastocystis sp.* is a eukaryote organism that includes the gut microbiome which can produce substances that are antioxidants and inhibit inflammatory reactions (Aykur et al., 2024).

Studies in several countries contrarily concluded and found that the presence of these protozoa can modify immune response

and oxidative stress, causing exacerbations and increasing the proliferation ability of cancer cells. Epidemiological and experimental studies in vivo on experimental animals show that *Blastocystis sp.* causes oxidative stress (Fujikawa et al., 2019; Kumarasamy et al., 2022).

Oxidative stress is a state of imbalance between oxidative and antioxidant molecules in cells. In this state, there is an overproduction and accumulation of Reactive Oxygen Species (ROS) in cells and tissues. ROS is a free radical and non-radical byproduct of metabolic processes in organelles such as mitochondria, peroxisomes and endoplasmic reticulum.

ROS has an important role in the physiological processes, one of which is as the body's defense against infectious agents. In addition, ROS also plays a role in cellular processes such as mitochondrial energy production, cellular signaling and regulation of gene expression (Gain et al., 2022).

Excessive accumulation of ROS will result in damage to cellular components including lipids, proteins and DNA as well as changes in the immune response resulting in tissue and organ dysfunction. Oxidative stress due to ROS induction has a detrimental impact, one of which can trigger and increase the progression of cancer. This increase in progressivity, which is characterized by increased invasion and metastasis ability, occurs due to ROS-induced inflammation (Arfin et al., 2021; Bardelčíková et al., 2023).

There are five types of biomarkers for oxidative stress. Type 0 is a biomarker used for direct measurement of ROS in patients in vivo; type 1 measures oxidized lipids, type 2 measures the activation of biochemical pathways that trigger the formation of ROS; type 3 measures factors present in the host such as low-molecular-weight anti-oxidant substances and anti-oxidant enzymes; and type 4 is a biomarker that measures genetic factors and mutations that affect a person's susceptibility to oxidative stress. Malondialdehyde is a type 1 biomarker that is the end product of lipid peroxidation which is known to be one of the best indicators of ROS levels. This compound is often used as a marker of oxidative stress and antioxidant status especially in cancer patients. The role of *Blastocystis sp.* to cancer disease in relation to oxidative stress is still very controversial. This study aims to determine the expression of malondialdehyde in colorectal cancer cell line HT29 with exposure to *Blastocystis sp.* (Sulaiman, 2024; Ghezzi, 2020)

**Methods**

*Research Design*

This study is an experimental study in vitro using HT29 cells and *Blastocystis sp.* isolated from colorectal cancer patients using Post Test Control Group Design.

*Research Sample*

The research sample was colorectal cancer cells (human colorectal adenocarcinoma cell line -HT29) in culture.

*Determination of Research Sample*

The size of the research sample was obtained as many as 5 sub-cultures for each treatment obtained based on the Federer formula (1977) for experimental tests.

*Preparation of Jones Medium*

The materials used to make Jones medium include disodium phosphate (Na2HPO4), monopotassium phosphate (KH2PO4), NaCl, Yeast extract, and aquadest. The solution is put into an autoclave at 1210C for 24 hours. A total of 10 ml of horse serum is added to the sterile medium under UVR-laminar flow and stored at -200C until the time of use. Prior to inoculation of the fecal specimen, the culture medium is re-introduced into the autoclave at $37^0$C (Hassan et al., 2016).

*Preparation of Blastocystis sp. Antigen*

*Blastocystis sp.* isolated from fecal specimens of colorectal cancer patients at Dr. M. Djamil Padang Hospital. Approximately 50 mg of fecal samples were inoculated into 3 ml of Jones medium supplemented with 10% horse serum. The parasite cultures are incubated at 37◦C in a CO2 incubator and screened daily for 5 to 7 days. The specimen is said to be positive if *Blastocystis sp.* microscopic and confirmed by PCR examination using a primer with forward sequence (5'-AGTAGTCATACGCTCGTCTCAAA-3') and reverse sequence (5'-TCTTCGTTACCCGTTACTGC-3'). *Blastocystis sp.* (1 × 105 parasites/ml) were purified from bacteria-contaminated cultures using density gradient centrifugation. Preparation of soluble antigen from *Blastocystis sp.* referring to the procedure carried out by Rajamanikam (2019). Successful isolation of soluble antigen *Blastocystis sp.* confirmed using the copro-ELISA *Blastocystis sp.* Savyon Diagnostics Ltd.

*Preparation of human colorectal adenocarcinoma cell line -HT29 culture with Blastocystis sp. exposure*

Colorectal cancer cells were obtained from the HT29 Sigma-Aldrich colorectal cancer cell line culture. HT29 cells are cultured using DMEM. Cells were cultured in 30 wells (well plates) using exposure to Blastocystis sp with different concentrations, namely 0.005 µg/ml, 0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.5 µg/ml and control with five replications for each treatment concentration.

*Examination of Malondialdehyde*

Malondialdehyde (MDA) in cultured cells was examined after 72 hours using the Placer method at the Biochemistry Laboratory, Faculty of Medicine, Andalas University by centrifuging HT29 cell culture specimens and then separating the serum. The tools and materials provided are in the form of tubes for samples, aquabidest and TBA reagent dis. After that, mixing is carried out using a vortex mixer, incubation and cooling. Centrifugation is performed at 7,000 RPM for 10 minutes later, separate the

filtrate. Adsorben supernatants were measured with a spectrophotometer at a wavelength (λ) of 532 nm.

*Data analysis*

The data obtained from all experimental groups were first tested for normality using the Saphiro Wilk test and the homogeneity test with the Levenne test. The data obtained were not normally distributed so it was continued to be analyzed using non-parametric tests (Kruskall Wallis and post hoc tests).

*Research Ethic*

This study used human colorectal adenocarcinoma cell line - HT29 and soluble antigen *Blastocystis sp.* and has passed the ethics review from the Research Ethics Commission of the Faculty of Medicine, Andalas University No. 513/UN.16.2/KEP-FK/2023.

**Result**

The results showed that there was a difference in malondialdehyde expression in HT29 cells after being exposed to *Blastocystis sp.* with various concentrations (p=0.007). In some specific antigen concentrations, a decrease in malondialdehyde levels was seen, namely at concentrations of 0.005 µg/ml, 0.01 µg/ml, 0.05 µg/ml and 0.1 µg/ml. In the control group and the highest antigen concentration of 0.5 µg/ml showed high levels of malondialdehyde. From these results, it can be seen that *Blastocystis sp.* at certain concentrations can suppress the occurrence of oxidative stress. The optimal protective effect is found in the concentration of antigen exposure of 0.01 µg/ml. In other words, it can be concluded that at a certain intensity *Blastocystis sp.* has a protective effect to overcome excessive ROS production and accumulation which is characterized by a decrease in malondialdehyde levels. The Post Hoc pairwise comparison test showed differences in malondialdehyde expression in the 0.1 and 0.5 µg/ml exposure groups with the control group, 0.005 and 0.01 µg/ml with 0.1 µg/ml and 0.005 and 0.01 µg/ml with 0.5 µg/ml groups.

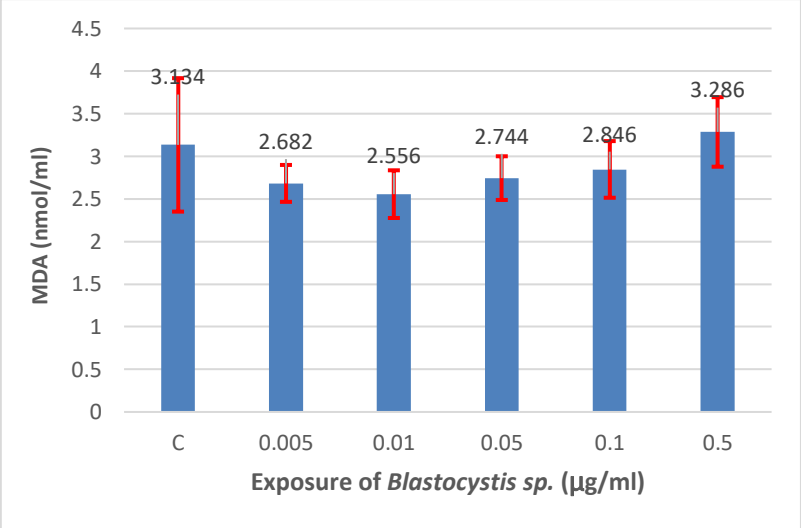


**Figure 1**. Malondialdehyde levels in human colorectal adenocarcinoma cell line - HT29 with various concentrations of exposure to *Blastocystis sp.* The control group was an HT29 cell culture without exposure to *Blastocystis sp.* The value is expressed in the form of an average level of malondialdehyde ± standard deviation. With the Kruskal Wallis test, it was concluded that there was a difference in the average level of malondialdehyde in HT29 cells with various exposure concentrations with a p value of 0.007. (P<0.05).

**Table 1**. Post hoc Test of Malondialdehyde Expression

| group | significance value |
|---|---|
| 0.01 µg/ml - 0.005 µg/ml | 0.801 |
| 0.01 µg/ml - control | 0.746 |
| 0.01 µg/ml - 0.05 µg/ml | 0.418 |
| 0.01 µg/ml - 0.5 µg/ml | 0.011* |
| 0.01 µg/ml - 0.1 µg/ml | 0.006* |
| 0.005 µg/ml - control | 0.943 |
| 0.005 µg/ml - 0.05 µg/ml | 0.577 |
| 0.005 µg/ml - 0.5 µg/ml | 0.022* |
| 0.005 µg/ml - 0.1 µg/ml | 0.012* |
| control - 0.05 µg/ml | 0.627 |
| control - 0.5 µg/ml | 0.027* |
| control - 0.1 µg/ml | 0.014* |
| 0.05 µg/ml - 0.5 µg/ml | 0.084 |
| 0.05 µg/ml - 0.1 µg/ml | 0.05 |

| | | | | | |
|---|---|---|---|---|---|
| 0.5 | µg/ml | - | 0.1 | µg/ml | 0.815 |

**Discussion**

*Blastocystis sp.* is an intestinal protozoa that is often found in colorectal cancer patients, especially in advanced stages. This protozoa has six stages, namely amoeboid, vacuolar, vacuole, multi vacuole, granular and cyst. The cyst stage is the infective stage and the amoeboid stage is the most pathogenic stage. The vacuolar stage is the stage that is most often found in the examination of specimens in the laboratory. The pathogenicity of these protozoans is still a matter of debate around the world because of its very wide clinical spectrum ranging from asymptomatic, mild symptoms to severe symptoms and can even cause death. Symptoms can be abdominal pain, bloating, nausea, vomiting, diarrhea and extra intestinal symptoms in the form of urticaria (Galindo et al., 2023; Lepczyńska et al., 2017).

The prevalence rate of infection due to this parasite is very high in developing countries including Indonesia. *Blastocystis sp.* it is also the most commonly found protozoa compared to other intestinal protozoa such as *Giardia lamblia*, *Entamoeba* and *Crytosporidium sp.* The difference in the incidence of this infection is related to the quality of hygiene and sanitation, history of contact with animals, consumption of contaminated food and beverages in the infectious stage (Ali et al., 2022; Lepczyńska et al., 2017). Several studies reported that there was a difference in the incidence of Blastocystosis in colorectal cancer patients compared to the control group. Most of the infection events occur in patients with advanced stages (Sulżyc-Bielicka et al., 2021).

This discovery supports the hypothesis that under certain conditions the gut microbiome is able to produce antioxidant substances that can reduce oxidative stress and prevent overproduction of ROS (Uchiyama et al., 2022). Peroxidation lipids are one of the best indicators to determine systemic biological damage due to oxidative stress in parasitic infections. Malondialdehyde is the end product of this process (Cordiano et al., 2023).

In this study, it was found that there was an effect of differences in the concentration of exposure to *Blastocystis sp.* on malondialdehyde levels in human colorectal adenocarcinoma cell line - HT29. This indicates that the soluble antigen *Blastocystis sp.* can suppress the occurrence of oxidative stress which is characterized by a decrease in malondialdehyde levels in the exposure group compared to the no exposure group. The ability to suppress oxidative stress was optimal at exposure concentrations of 0.01 µg/ml and the suppression effect began to decrease at concentrations of 0.05 µg/ml and 0.1 µg/ml. At the highest exposure concentration of 0.5 µg/ml, *Blastocystis sp.* further triggering oxidative stress which is characterized by high levels of malondialdehyde compared to the group without exposure. The results of this study imply that high infection intensity has a tendency to increase progressivity.

Gut microbiome, which is classified as commensal, is able to produce antioxidants and can suppress inflammatory reactions. This is in accordance with the opinion of several experts who state that *Blastocystis sp.* is one of the commensal microorganisms and is often found in the intestinal tract of healthy individuals. Regarding this antioxidant substance, it is still under further research (Kunst et al., 2023; Zmora et al., 2023).

In addition to being commensal, *Blastocystis sp.* this is also thought to have a relationship with the diversity of intestinal bacteria. The existence of these protozoa is related to the population of intestinal good bacteria compared to intestinal pathogenic bacteria (Kodio et al., 2019). This state is caused by various mechanisms; one of them is the interaction between *Blastocystis sp.* with intestinal bacteria either directly or indirectly through the host's immune response induced by exposure to *Blastocystis sp* (Yason et al., 2019).

Some studies show a different thing. In vivo studies on experimental animals show that *Blastocystis sp.* can result in severe oxidative damage characterized by ROS overproduction. (Fahmy MA, 2019; Sulżyc-Bielicka et al., 2021)

The same results were also found in an in vivo study using rats injected with intraperitoneal azoxymethane (AOM) to induce colorectal malignancies in mice. In the group of AOM-induced rats who were exposed to *Blastocystis sp.* showed an increase in oxidative stress which was characterized by an increase in markers, namely advanced oxidative protein product (AOPP) and hydrogen peroxide in rat urine and an increase in damage to the intestinal mucosal epithelium marked by an increase in markers, namely advanced oxidative protein product (AOPP) and hydrogen peroxide in rat urine as well as an increase in damage to the intestinal mucosal epithelium which is characterized by epithelial hypertrophy, intestinal gland deformity and the formation of aberrant crypt foci (ACF) (Kumarasamy et al., 2017).

The study was conducted on normal rats inoculated with *Blastocystis sp.* showed an increase in advanced oxidative protein product (AOPP) and Lipid hydroperoxide (LHP) and a decrease in ferric-reducing antioxidant power (FRAP) and Glutathione peroxidase (GPx). This indicates that under certain conditions, this protozoan infection can also cause oxidative stress characterized by increased oxidation of proteins and lipids accompanied by decreased levels of antioxidant enzymes (Chandramathi et al., 2014; Pawłowska et al., 2023).

**Conclusion**

There was a significant difference in malondialdehyde expression of HT29 cells after exposure *to Blastocystis sp.* with different concentrations. Under certain conditions and intensities, exposure to *Blastocysts sp.* can suppress oxidative stress so that it can protect and inhibit the progression of cancer.

**Conflict of Interest**

There was no conflict of interest in this study.

**Author's Contribution**

Author 1 drafts the concept, designs the research design, collects and analyzes data, interprets the data, and prepares the manuscript. Authors 2 and 3 drafted the draft, providing input on the revision of the manuscript. The authors 4 developed the concept, interpreted the data and determined the research methodology.

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