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ORIGINAL ARTICLE

The Correlation between Heat Shock Protein and Oxidative Stress in Entamoeba Histolytica Patients

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Key words:
Entamoeba histolytica*; heat shock protein; Malondialdehyde; Glutathione; ROC; serum*

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ABSTRACT

Background: Entamoeba histolytica *causes amebiasis, a dangerous diarrheal disease. This is a major health problem in areas with poor sanitation and overcrowding, leading to many infections and deaths worldwide. HSP70, a key protein for protein management in all organisms, handles folding and transport.* ***Objectives****: This study aimed to determine the relationships between* heat shock protein and entamoeba histolytica patient. ***Methodology:*** A case-control study to measure heat shock protein levels in Entamoeba histolytica-infected patients was conducted *at Al-Amin center between September 2024 and January 2025 to measure heat shock protein levels in* Entamoeba histolytica *infected patients. 110 blood samples were collected from multiple hospitals within Al-Najaf, and 50 tested positive for the parasite. 3mL of serum was extracted from each positive sample, and stored at -80°C for later heat shock protein analysis.* ***Results****: heat shock protein levels were markedly higher (4.552±0.807) in* E. histolytica*-infected patients than in controls (1.959±0.236).* ***Conclusions:*** *The research demonstrated that individuals infected with* Entamoeba histolytica *had considerably higher levels of heat shock proteins70 compared to those in the control group.*

INTRODUCTION

Entamoeba histolytica, a single-celled parasite, is the causative agent of amebiasis, a gastrointestinal disorder marked by amoebic diarrhea[1]. Despite the existence of other closely related, non-disease-causing parasites, *E. histolytica* remains a significant public health challenge, primarily in tropical and subtropical zones with compromised hygiene and dense populations, leading to substantial global infection and mortality rates[2]. Infection is particularly common in developing countries, including Iraq, where clinical studies have reported a notable prevalence among symptomatic patients[3]. While less frequent in industrialized countries, it affects older adults and certain high-risk groups[4]. Proteins known as heat shock proteins (HSPs) are critical for maintaining cellular well-being by ensuring proteins operate correctly. These proteins function as chaperones, assisting in the proper formation of proteins and preventing damaging clumps. Furthermore, they participate in cellular communication and control[5]. Under stress, cells increase HSP production as a protective measure. When HSPs malfunction, they are associated with diseases such as cancer and neurodegeneration[6]. HSP70 (DnaK in prokaryotes) is a key protein for protein management in all organisms. Humans have diverse HSP70 genes, some stress-activated, others always on. These proteins, located throughout cells, control protein folding, breakdown, and transport[7]. Co-chaperones regulate their functions, including preventing protein clumping and guiding protein fate. HSP70 levels increase in amebiasis, particularly severe cases, suggesting its role in infection and inflammation response [8].

METHODOLOGY

This case-control study investigated heat shock protein levels in patients with *Entamoeba histolytica* infection. The research was conducted at the Advanced Research Laboratory in the Al-Amin center for advanced biotechnology and research between September 2024 and January 2025. Researchers collected 110 samples, with 50 confirmed cases of *E. histolytica* infection. Blood samples were taken from patients at several hospitals in Al-Najaf, including Al-Manathera General Hospital, Al-Hakeem General Hospital, Al-Sader Medical City, Al-Zahraa Teaching Hospital, and Al-Najaf Al-Ashraf Teaching Hospital. From these 110 samples, 50 were confirmed positive for the parasite. Three milliliters of venous blood were collected from each of the 50 confirmed positive cases. The blood was processed by centrifuging to separate the serum, which was then stored at -80°C for later analysis of heat shock protein levels.

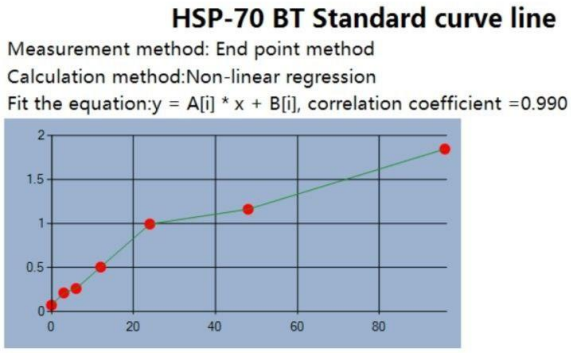
PROCEDURE of Human Heat Shock Protein 70 ELISA Kit

The experiment (Human Heat Shock Protein 70 ELISA Kit) began with bringing all reagents, standards, and samples to room temperature. Test strips were

placed in the provided frames, and any extra strips were refrigerated. Next, 50 µl of the standard solution (containing biotinylated antibody) was added to the appropriate wells. For the sample wells, 40 µl of sample and 10 µl of anti-HSP70 antibody were added, followed by 50 µl of streptavidin-HRP to all standard and sample wells. The plate was then mixed, sealed, and incubated at 37°C for 60 minutes. After incubation, the plate was washed five times with wash buffer, allowing a short soak time for each wash, and then dried. Subsequently, 50 µl of substrate A and then 50 µl of substrate B were added to each well. The plate was resealed and incubated in the dark at 37°C for 10 minutes. The reaction was stopped by adding 50 µl of Stop Solution, which caused the color to change from blue to yellow. Finally, the absorbance of each well was measured at 450 nm using a microplate reader within 10 minutes of stopping the reaction.

HSP70 Standard Curve Analysis

As illustrated in Figure 1, the HSP70 standard curve was produced using computer software. This involved graphically representing the average OD values of each standard on the Y-axis in relation to their concentrations on the X-axis, and then employing regression analysis to determine the most appropriate line to represent the data.



ABS	Concentration
0.073	0.000
0.212	3.000
0.262	6.000
0.505	12.000
0.994	24.000
1.162	48.000
1.846	96.000

Fig. 1: HSP-70 BT standard curve line

Malondialdehyde and Glutathione quantification

The levels of **malondialdehyde (MDA)** and **glutathione (GSH)** were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Specifically, the **Human Glutathione ELISA Kit** and the **Human Malondialdehyde ELISA Kit**, both sourced from BT LAB Bioassay Technology Laboratory (501 Changsheng S Rd, Nanhu Dist, Jiaxing, Zhejiang, China), were employed. All assays were conducted in strict adherence to the manufacturer's provided instruction protocols.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software, USA). Normality of continuous variables was tested and, the data were reported as mean ± standard error. Group comparisons were conducted using independent-sample t-tests. Pearson correlation coefficients were calculated. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Comparison of HSP70(ng/ml) between control and patients

Figure 2 demonstrates that heat shock protein levels were markedly higher (4.552±0.807) in *E. histolytica*-infected patients than in controls (1.959±0.236), with a statistically significant difference (P<0.0001).

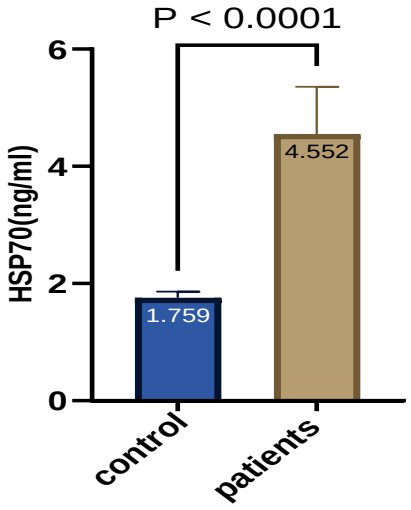


Fig. 2: Comparison of HSP70(ng/ml) between control and patients.

Comparison of MDA (nmole/ml) between control and patients

Figure 3 demonstrates a significant elevation in **malondialdehyde (MDA)** levels within the patient group. Specifically, patients exhibited an MDA level of 2.574±0.409, which is statistically higher (P=0.0065) than the control group's level of 1.534±0.052. This indicates a notable increase in MDA in patients compared to healthy controls.

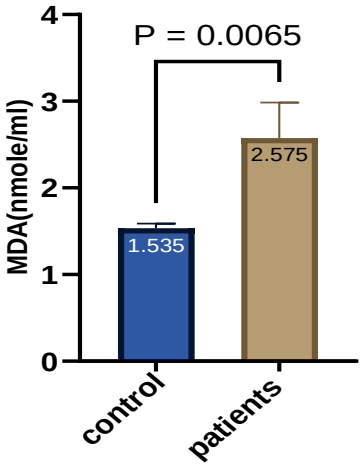


Fig. 3: Comparison of MDA (nmole/ml) between control and patients

Comparison of GSH levels (ng/ml) between control and patients

Figure 4 clearly illustrates a significant reduction in **glutathione (GSH)** levels among patients. The patient group exhibited a mean GSH level of 40.643±1.621, which is remarkably lower than the control group's mean of 50.963±1.022. This difference is highly statistically significant, with a P-value of less than 0.0001.

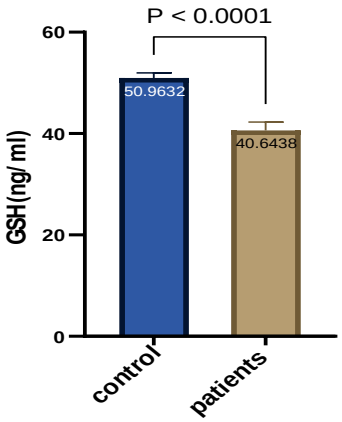


Fig. 4: Comparison of GSH levels (ng/ml) between control and patients.

Comparison of biomarker according to age between control and patient groups

Table 1 details glutathione (GSH) levels across three distinct age groups. In individuals aged ≤30 years, the patient group's average GSH was 42.96±3.11, while the control group's was 49.52±3.67. This difference was not statistically significant (p=0.202). However, a marked and statistically significant decrease in GSH was observed in older patient cohorts. For those aged 31−49 years, patients had a mean GSH of 39.77±1.63 compared to 50.48±1.21 in controls, a highly significant difference (p=0.002). Similarly, in the ≥50 years age group, patients showed an average GSH of 38.71±2.73 versus 51.266±2.597 in controls, which was also a highly significant finding (p=0.001).

For individuals aged ≤30 years, the patient group exhibited a mean MDA value of 2.98±0.89, while the control group showed 1.60±0.16. This difference was not statistically significant (p=0.375). Similarly, in the 31−49 years age range, the patient group's mean MDA was 2.71±0.90 compared to 1.55±0.06 in controls, which was also not statistically significant (p=0.218). However, a statistically significant increase in MDA was observed in the oldest age group. For those aged ≥50 years, the patient group's mean MDA was 2.20±0.33, while the control group exhibited 1.45±0.14. This difference was statistically significant (p=0.05).

In an analysis of HSP70 levels across different age groups, the patient group consistently demonstrated higher mean values than the control group, though the statistical significance varied. For individuals aged 30 years or younger, the patient group exhibited a mean HSP70 value of 4.63±1.54 compared to the control group's 1.68±0.33, but this difference was not statistically significant (p=0.082). However, for the 31-49 year age range, the patient group showed a statistically significant difference with a mean of 5.03±1.92 against the control group's 1.75±0.12 (p=0.021). In the oldest group, aged 50 years or more, the patient group's mean HSP70 level was 3.33±0.80 while the control group's was 2.66±0.92, a difference that was again not statistically significant (p=0.59) as show in table 1.

Table 1: Comparison of biomarkers according to age between control and patient groups.

Biomarker	Mean ± S.E.		p-value*
	Control group	Patient group	
GSH(ng/ml)			
(≤30) years	49.52 ± 3.67	42.96 ± 3.11	0. 202
(31-49) years	50.48 ± 1.21	39.77 ± 1.63	0.002
(≥50) years	51.266 ± 2.597	38.71 ± 2.73	0.001
p-value#	0.905	0.682	-
MDA(nmole/ml)			
(≤30) years	1.60 ± 0.16	2.98 ± 0.89	0.375
(31-49) years	1.55± 0.06	2.71± 0.90	0.218
(≥50) years	1.45± 0.14	2.20± 0.33	0.05
p-value#	0.681	0.739	-
HSP70(ng/ml)			
(≤30) years	1.68 ± 0.33	4.63 ± 1.54	0.082
(31-49) years	1.75 ± 0.12	5.03 ± 1.92	0.021
(≥50) years	2.66 ± 0.92	3.33 ± 0.80	0.59
p-value#	0.257	0.670	-

*Independent samples- T test #One-Way ANOVA test, S.E: standard error, P≤0.05.

Comparison of biomarkers according to sex between control and patient groups

Table 2 demonstrates a significant reduction in **Glutathione (GSH)** levels within patient groups when compared to their healthy controls. In male patients, the mean GSH level was 41.238±2.053, which was significantly lower than the 49.313±1.372 observed in male controls (p=0.002). This decrease was even more pronounced in female patients, who showed a mean GSH level of 39.488±2.682 compared to 51.937±1.600 in female controls (p<0.001). The lower p-value for the female group indicates a higher level of statistical significance, suggesting a more substantial reduction in GSH levels among female patients.

Table 2 presents **Malondialdehyde (MDA)** levels, a key indicator of oxidative stress, in both patient and control groups, separated by sex. For male patients, the MDA level was 2.537±0.505, compared to 1.576±0.066 in male controls, with a p-value of 0.068. In female patients, the MDA level was 2.699±0.713, while female controls showed 1.48±0.083, resulting in a p-value of 0.051. These findings suggest a consistent trend toward elevated MDA levels in both male and female patient groups, implying increased oxidative stress. However, it's worth noting that the p-values for both sexes are close to, but do not quite reach, the conventional threshold for statistical significance (p<0.05).

Table 2 details the levels of **Heat Shock Protein 70 (HSP70)** , differentiating between patient and control groups based on sex. Among male participants, the patient group displayed a mean HSP70 level of 3.552±0.888, which was higher than the 1.92±0.133 observed in male controls. While this indicated a trend toward increased HSP70 in male patients, it did not reach statistical significance (p=0.078). In contrast, female patients showed a statistically significant elevation in HSP70 levels, with a mean of 5.666±1.72, substantially greater than the female control group's mean of 1.584±0.155 (p=0.018). This notable increase in female patients suggests a more pronounced cellular stress response within this demographic.

Table (2): Comparison of biomarkers according to sex between control and patient groups

Biomarker	Mean ±S.E.		p-value*
	Control group	Patient group	
GSH (ng/ml)			
Male	49.313± 1.372	41.238±2.053	0.002
Female	51.937± 1.6	39.488± 2.682	<0.001
p-value	0.217	0.614	-
MDA (nmole/ml)			
Male	1.576± 0.066	2.537± 0.505	0.068
Female	1.48± 0.083	2.699±0.713	0.051
p-value	0.373	0.853	-
HSP70 (ng/ml)			
Male	1.92± 0.133	3.552±0.888	0.078
Female	1.584± 0.155	5.666±1.72	0.018
p-value	0.236	0.104	-

*Independent samples- T test, S.E: standard error, P≤0.05.

Correlation of HSP70 levels with demographic and oxidative stress markers

The table summarizes correlations between **HSP70** levels and various demographic and oxidative stress indicators. A strong positive correlation was observed between **HSP70** and **MDA** (r=0.819, P=0.0001), while a significant negative correlation was found between **HSP70** and **GSH** (r=−0.398, P=0.004).

Conversely, no significant correlations were identified between **HSP70** and demographic factors such as age (r=−0.081, P=0.575), sex (r=0.067, P=0.643), residency (r=−0.037, P=0.800), or education level (r=−0.237, P=0.097).

Table 3: Correlation of HSP70 levels with demographic

Correlation of serum HSP70 with	Correlation coefficient(r)	p-value
Age(year)	-0.081	0.575
Sex(M/F)	0.067	0.643
Residency (U/R)	-0.037	0.800
Education(0,1,2,3)	-0.237	0.097
GSH(ng/ml)	-0.398*	0.004*
MDA(nmole/ml)	0.819*	0.0001*

*: Correlation is significant at the 0.05 level . r: correlation coefficient. f: female, M: male.

Receiver Operating Characteristic Curve Analysis of HSP70 between control and patients

Figure 3 illustrates the **diagnostic accuracy of serum HSP70 levels** for detecting *E. histolytica* infection, as determined by **receiver operating characteristic (ROC) curve analysis**. The analysis yielded an **Area Under the Curve (AUC) of 0.721** (with a 95% confidence interval of 0.624–0.817) and a **p-value of less than 0.001**, suggesting a **moderate level of diagnostic performance**. An **optimal cut-off value of 2.281 ng/ml** was identified, indicating that serum HSP70 levels could potentially serve as a **confirmatory biomarker** for *E. histolytica* infection.

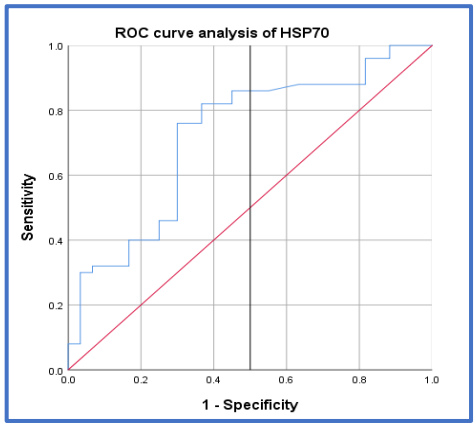


Fig. 5: Receiver Operating Characteristic (ROC) curve statistics for serum HSP70(ng/ml) levels in diagnosis of *E. histolytica* in the patients.

Research by Chen et al. [9] explored the utility of HSP70 levels in diagnosing acute angle-closure glaucoma. Their ROC curve analysis revealed that HSP70 demonstrated a sensitivity of 79.79% and a specificity of 74.29% for identifying the condition, suggesting its potential as an additional diagnostic tool for clinicians. Similarly,[10] investigated the prognostic value of extracellular heat shock protein 70 (eHsp70) in septic shock. While eHsp70 exhibited a moderate predictive ability for mortality, with an AUC of 0.63 in ROC analysis, Kaplan-Meier analysis indicated that elevated eHsp70 levels were associated with an increased risk of early death. This suggests that eHsp70 could, in conjunction with other markers, serve as an indicator of septic shock severity.

DISCUSSION

Infection with *Entamoeba histolytica* induces stress in both the host and the parasite, leading to an increase in Heat Shock Protein (HSP) production due to inflammation, tissue damage, and altered cellular conditions[11,12]. Host HSPs play a role in maintaining cellular balance, repairing damage, and activating the immune system, which could enhance the body's defense against the parasite [13,14]. Oxidative stress, a known inducer of HSP expression, has been shown to negatively affect tissues and disrupt physiological balance in human and animal models [3,15,16].

However, *E. histolytica* may also utilize host HSPs for its own survival, and the parasite itself produces HSPs that can stimulate the host's immune response. This leads to a complex mixture of host and parasite HSPs in patient samples, reflecting the intricate interaction between stress responses and immune modulation during infection [17,18].

A study by Kammanadiminti & Chadee[19] demonstrated that soluble proteins from *E. histolytica* can protect intestinal epithelial cells (IECs) by inducing HSP production. Importantly, this protective effect was only observed in IECs that had been exposed to macrophage secretions for 24 hours, and not in untreated IECs. This research highlighted the protective role of amebic proteins and the ability of macrophage secretions to prepare IECs for stress responses. Furthermore, the ATP binding of Hsp90 is crucial for its function, making it a potential target for amebiasis treatment [20].

Disrupting this binding has shown to be lethal across various organisms. While existing drugs require further evaluation, they provide a foundation for the development of new therapeutic agents [21]. Infection with Entamoeba histolytica triggers an inflammatory response in the host, leading to elevated malondialdehyde (MDA) levels. This process involves the activation of immune cells, which subsequently

release reactive oxygen species (ROS)[22]. While these ROS are crucial for combating the parasite, their excessive production results in oxidative stress, causing damage to host tissues. Specifically, ROS induce lipid peroxidation, a detrimental process that impairs cell membrane lipids and leads to the formation of MDA [23].

Consistent with this mechanism, Talib and Hamad (2022) observed a highly significant increase ($p<0.001$) in MDA levels in infected individuals (13.09±4.016 pg/ml) compared to healthy controls (9.435±2.225 pg/ml), underscoring the substantial oxidative stress associated with *E. histolytica* infection. *Entamoeba histolytica* infection significantly increases oxidative stress in the host. The parasite's invasion, coupled with the host's immune response, generates an abundance of reactive oxygen species (ROS). As a primary antioxidant, glutathione (GSH) is consumed to neutralize these excess ROS, leading to its depletion. Furthermore, the infection triggers an inflammatory response, which intensifies oxidative stress by releasing cytokines that contribute to GSH depletion. *E. histolytica* also possesses virulence factors that compromise the host's antioxidant defenses, either by directly interfering with GSH synthesis or impairing its function. While host immune cells like macrophages and neutrophils produce ROS to fight the parasite, this defense mechanism inadvertently adds to the overall oxidative burden and further depletes GSH levels.

A study by Mohsin[24] revealed a significant reduction in serum glutathione (GSH) levels in patients afflicted with E. histolytica infection. The concurrent decrease in **GSH** and increase in **HSP70** within the 31−49 age group likely indicates a significant physiological response to cellular stress or metabolic disruption. The reduction in GSH suggests a diminished antioxidant capacity, possibly due to elevated oxidative stress or its utilization in detoxification pathways. Conversely, **Malondialdehyde (MDA)** levels were elevated specifically in patients aged ≥50 years, pointing to increased oxidative stress within this older demographic. Interestingly, **GSH** levels were found to be decreased in both the older (≥50 years) patient group and the 31−49 age group, suggesting a nuanced relationship between oxidative stress and the depletion of antioxidants across varying age ranges. It is well-established that aging is associated with heightened oxidative stress, and a decline in GSH is a significant indicator of this process, with blood GSH levels generally decreasing with advancing age[25].

In the 31-49 age group, the observed increase in **Heat Shock Protein 70 (HSP70)** points to a significant physiological response, likely reflecting considerable cellular stress or metabolic disruption. The rise in HSP70 indicates active cellular responses and protective mechanisms. HSP70, a molecular chaperone, is upregulated to mitigate cellular damage and maintain protein homeostasis under stress[26]. This combination suggests a dynamic period where the body is actively responding to stressors, potentially metabolic or immune-related, leading to increased demand and consumption of GSH, while simultaneously upregulating NO and HSP70 for defense and repair[27]. *E. histolytica* infection significantly elevates oxidative stress in the host [28].

This occurs because the parasite induces **reactive oxygen species (ROS)** production, which overwhelms the host's antioxidant defenses, particularly **glutathione (GSH)**. Both cellular damage from the parasite and the host's inflammatory response further contribute to GSH depletion. Notably, females show a more pronounced reduction in GSH, likely due to a combination of hormonal, immunological, and metabolic differences [29].

The observed increase in **Malondialdehyde (MDA)** levels in both male and female patient groups suggests a trend towards elevated oxidative stress, as MDA is a recognized marker of **lipid peroxidation**. While the mean MDA levels were higher in patients compared to controls, the p-values (0.068 for males and 0.051 for females) did not reach the conventional statistical significance threshold ($p<0.05$). Despite this, the near-significant p-values and the consistent trend, coupled with the decreased GSH levels, collectively reinforce the hypothesis of increased oxidative stress associated with the patient condition. Differences in MDA levels between male and female patients could stem from variations in hormones, distinct oxidative stress or inflammatory responses, metabolic disparities, and sample variability, highlighting the complex interplay of physiological factors influencing MDA production[30] research also confirms the observed trend of females exhibiting higher GSH levels compared to males.

Cruikshank et al.[31] emphasize the crucial role of **glutathione (GSH)** as a primary antioxidant in bodily detoxification processes. Their research also corroborates the consistent finding that females typically exhibit higher GSH levels compared to males. The significantly higher HSP70 levels in female patients suggest a stronger cellular stress response compared to males. This difference may stem from hormonal influences, varied inflammatory responses, or distinct oxidative stress levels between sexes [32]. Potential factors also include disease severity and genetic predispositions, leading to a more pronounced HSP70 upregulation in females[33].

De Oliveira et al.[34] conducted Western blot analysis on rat aortas and found that female animals possess notably lower baseline levels of HSP70 compared to their male counterparts. This finding implies that the amount of HSP70 could be a key mechanism affecting how the aorta contracts in different sexes. The correlations observed suggest a complex interplay between **heat shock protein 70 (HSP70)** and markers of oxidative stress. The strong positive correlation between HSP70 and malondialdehyde (MDA) (r=0.819,

P=0.0001), a marker of lipid peroxidation, indicates that as oxidative damage increases, so too does HSP70. This points to a potential role for HSP70 in responding to or resulting from heightened oxidative stress. Conversely, a significant negative correlation was found between HSP70 and **glutathione (GSH)**, a crucial antioxidant (r=−0.398, P=0.004). This suggests that when the body's antioxidant defenses are robust with sufficient GSH, the need for HSP70, a protein involved in cellular stress response, may diminish. Research supports these findings:[35] revealed that HSP70, a key player in cellular stress responses, is regulated by reactive oxygen species (ROS), which affects its function and production, thereby influencing cell survival through redox balance.

Lubkowska et al.[36] further demonstrated that HSP70 can mitigate oxidative stress damage in kidney cells by enhancing the function of glutathione peroxidase and glutathione reductase in response to hypoxic conditions. The relationship between HSP70 and oxidative stress can vary depending on the context.

Aengwanich and Wandee[37] showed that in broiler blood cells, initial increases in HSP70 and SOD at moderate temperatures offer a protective response, while higher temperatures lead to increased oxidative stress and apoptosis. De Oliveira et al.[38] observed that middle-aged animals have reduced HSP70 in their aortas, correlating with weaker vascular responses and suggesting age-related vascular dysfunction. Furthermore, De Oliveira et al. [34] found that female rats naturally express less aortic HSP70 than males. Blocking HSP70 had a more significant negative impact on female rat blood vessel contraction, implying a greater importance of HSP70 in female vascular function, which might contribute to females' increased protection against cardiovascular diseases. Environmental factors also play a role;[39] determined that urban residents, exposed to greater heat stress, exhibited significantly higher HSP70 levels compared to rural residents.

CONCLUSION

This study unequivocally demonstrated significant alterations in key biomarkers of cellular stress and antioxidant status in patients suffering from *Entamoeba histolytica* infection. Specifically, we observed a notable elevation in both **heat shock protein 70 (HSP70)** and **malondialdehyde (MDA)** levels, alongside a substantial decrease in **glutathione (GSH)**, when compared to the healthy control group.

Ethical approval declaration

The procedures followed in this study were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki). In addition, each participant provided written consent following a concise overview of the project.

Acknowledgment(s)

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Conflict Of Interest

The authors declare no conflict of interest.

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