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Study Role of Leptin, Obestatin, IGFBP2 Biomarkers in Children Infected with *Entamoeba histolytica*

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Abstract

THE protozoan parasite *Entamoeba histolytica* is the causative agent of amebic colitis, an infectious illness that kills over 100,000 people annually throughout the world.. this study aimed to diagnosis of amoebiasis using an smear direct microscope and PCR assay, assessment of some hormone markers includes. Leptin, obesatatin and IGFBP2 by ELISA. the research was conducted from July 2023 to November 2023.Atotal of 563 stool samples for infants and children those who suffered from diarrhea and abdominal pain in Ibn Al-Atheer Hospital, Al-Khansaa Hospital, and Al-Salam Hospital in governorate of Nineveh. Examined using an direct microscope 100 positive samples (18 %) and 463 negative samples (82 %) Detection of the DNA of amoeba by specific genes primers by PCR method. 74 positive samples were observed out of 464 examined. There was a considerable increase in Leptin levels. in patients along with an average concentration of 4.4±1.6 ng/mL, compared to the control group, whose average concentration 1.4±0.4ng/mL, obestatin levels in patients were signified greater with a mean of 188.1±85.04ng/mL, compared to the control group whose average concentration 49.2±11.5 ng/mL, and increase in IGFBP2 levels. in patients along with an average concentration of 49.8± 22.4ng/mL, compared to the control group whose average concentration 14.4±5.3ng/mL.: The study revealed that patients had increased levels of leptin, obestatin, and IGFBP2 when the infection of *E.histolytica* occurred.

Keywords: *Entamoeba histolytica*, ELISA, leptin, obestatin and IGFBP2, PCR.

Introduction

Amoebiasis, a condition characterized by a parasitic infection in the human body impacting the large intestine, is brought about by the presence of a protozoan known as *Entamoeba histolytica*, which primarily resides extracellularly. As per the World Health Organization (WHO), A significant mortality rate is linked to diarrhea, making it a potentially fatal illness, particularly in children under five. Sadly, according to the World Health Organization, two out of every five Africans lack access to a clean drinking water source. Kenya is one of the nations that suffers from poor drinking water quality and inadequate sanitation, particularly in the rural and slum regions. People who practice poor sanitation are more likely to contract cholera, dysentery, typhoid, and polio. Parasitic diseases, such as amoebiasis resulting from Entamoeba histolytica, are among the causes of diarrhea. Amoeba-related diarrhea is a regular occurrence in sub-Saharan African nations, such as Kenya. [1] *Entamoeba histolytica* is widely distributed across the globe and poses a significant concern in nearly all areas where there is a lack of separation between human excrement, food and water[2].

The recent categorization of *E. histolytica* has resulted in the identification of many species., namely the pathogenic species of Entamoeba include *Entamoeba histolytica*, while the non-pathogenic species include *Entamoeba dispar* and *Entamoeba moshkovskii.* The categorization of these three species has added complexity to the epidemiology of amoebiasis. This is because they cannot be distinguished by microscopy, which is the primary diagnostic method in tropical countries with inadequate resources. Instead, it is imperative to utilize molecular techniques, such as polymerase chain reaction-based procedures, to differentiate between these species [3,4]

The life cycle of this parasite is transmitted via the ingestion of fecal matter., which is a straight forward and direct process. It exclusively relies on humans as its host. The clinic pathological consequences of invasive amoebiasis are severe and can be categorized as either intestinal or extra-intestinal, with the latter potentially affecting other

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organs such as the liver, skin, spleen, brain, and lungs. The specifics of these have been emphasized.[4] Approximately 80% to 90% of those who are infected show no symptoms, although the main organ that . The parasite invades and establishes itself in the mucous membrane of the sigmoid and colon. The highly invasive strains of the *E. histolytica* species demonstrate their ability to penetrate by causing tissue damage. [5].

Leptin, which performs a role in regulating the immune system and the gastrointestinal tract [6,7], Babies who are under nourished have low levels of the hormone leptin in their blood, which reduces the production of inflammatory cytokines[8,9]. Intestinal parasites and other infections may lead to a lack of eating[10].

Acute-phase responses to infections might include symptoms like malnutrition. According to the available data, organisms have developed dietary restrictions as a biological response to combat pathogenic invasions[11]. A Th1 response, specifically interferon-γ, is a critical component of amebiasis-protective immunity. [12]; thus, this becomes more important when *E. histolytica* is in the picture. Because the leptin receptor is also present in the intestinal mucosa[13], leptin may have an additional protective function against amebiasis via mucosal immunity[14]. Because of its proproliferative and antiapoptotic activities, leptin regulates the operation of the intestinal barrier, aids in maintaining the structural integrity of the lining of the intestines, and increases the production of mucin in response to injury to the mucosa[15].

Obestatin, is an endocrine hormone present in humans, synthesized by the stomach and small intestine[16]. In 2005, researchers discovered a peptide called obestatin. This peptide is made up of 23 amino acids and is generated from the C-terminal region of a larger protein called preproghrelin. The discovery of obestatin was made by the use of bioinformatic analysis of the genomic sequence of preproghrelin in several species[17], Obestatin, a term derived from combining "obese" with "statin" to indicate suppression, was originally shown to reduce hunger and prevent weight gain[18]

Insulin-like growth factors (IGFs) namely IGF-I and IGF-II, are single chain polypeptides composed of ٧٦ and ٧٠amino acids, respectively. They have molecular weights of 7.65 and 7.47 kDa, respectively[19]. These growth factors, in combination with growth hormone, insulin, sex steroids, and other growth factors, have immediate anabolic effects on protein and carbohydrate metabolisms, and also have a lasting influence on cell proliferation, differentiation, and death[20]. Furthermore, IGFs have strong mitogenic properties towards several types of cancer cells[21]. IGFs exert their effects by binding to the insulin-like growth factor-I receptor (IGF-IR), a specific receptor located on the cell membrane. This receptor is capable of phosphorylating tyrosine residues.

Material and Methods

Specimens collection

A research was undertaken from July 2023 to November 2023, comprising the collection of fecal and serum samples from 563 children, aged between 1 day and 10 years, who had diarrhea. The study included children of both genders. The specimens were collected from Ibn al-Ather hospital, El Khansa Educational Hospital for Births and Children, and Al salam Educational Hospital in Nineveh Governorate.

Microscopic Examination

The fecal samples were visually inspected to detect the hue, odor, and presence of mucus and/or blood. The parasite was seen under the microscope at 10X and 40X magnification using direct smear techniques with normal saline and Lugol iodine stain. The presence of one to four nucleus cysts and/or trophozoites of amoeba verified the diagnosis of *E. histolytica*. [22].

Molecular Assay:

DNA Extraction

DNA extractions were performed with genomic DNA mini kits(Genaid) .The first technique used by was to physically use 2 small glass beds to distract the parasite membranes, The second method, We follow the steps of the kit. The process involved enzymatically disrupting the membrane of the parasites using proteinase K. [23] The precipitate is stored at -20 ℃.

Assessment of Concentration and Purity of DNA extracted

Modern molecular laboratories place a premium on both efficient DNA extraction methods and the precise measurement of nucleic acid concentration and purity, to conduct the DNA quantification and purity tests, a fraction was taken from the final amount of 100 µl of extracted and processed DNA. Spectrophotometric analysis was performed on the isolated genomic DNA to evaluate The DNA yield is measured in nanograms (ng) and the purity is determined by the absorbance ratio at 260/280. (mcrodigital, Nabi,Korea) according to the guidelines laid forth[24] .The highest concentration was taken along with the lowest concentration and also for purity (Table 1).

PCR assay

Primers for polymerase chain reaction (PCR) assays targeting certain *Entamoeba* genera were designed using nucleotide sequences derived from the small-subunit rRNA gene. The *Entamoeba* genus-specific primers was prepared from (Microgen

Company, Korea) The primers used were gen target 18S ribosomal RNA gene Entamoeba histolytica,primers names(EntaF,EhR) Sequence (5'-3') size for EntaF:5´-GTT GAT CCT GCC AGT ATT ATA TG- 3´and) Sequence (5'-3') size for EhR:5-´CAC TAT TGG AGC TGG AAT TAC-3´ which generate 550 bp[25].PCR amplifications unique to each genera were carried out in a final volume of 25 includes 12.5 µl of PCR buffer, 0.5 µl of each forward and reverse primer Entam1, 0.5 µl of DNA material, and 8.5 µl of free water nuclease. A thermocycler (Px2 Thermal Cycler, thermoHybaid, UK) PCR System was used to conduct the reactions. Samples were first denatured for five minutes at 94˚C. They were then heated in 35 cycles for one minute at 94˚C, one minute at 55˚C, and one minute at 72˚C. Finally, they were extended for seven minutes at 72˚C.

Leptin, Obestatin and IGFBP2 Elisa test

The leptin, obestatin and igfbp2 serum concentrations were quantified by the enzyme-linked immunosorbent assay. Were taken 60 serum patients and 30 serum control the plates of 96 well (ELISA, BT LAB China). The ELISA plates were precoated with leptin, obestatin and igfbp2 antibodies. The samples were added to the plates, and the color development in the substrate solution correlated with the level of leptin, obestatin and IGFbp2. After applying a stop solution, the process was halted, and the absorbance was measured at 450 nm.

statistical analysis

To perform statistical analysis, IBM SPSS software program, version 26.0 was used. Frequencies and percentages were used to present categorical information. The format for continuous variables was mean ± SD.

Values of $P \leq 0.05$ were regarded as significant by using the t test, The program Graph Pad Prism 10.a was used to create the graphs.

Results

Diagnosis of Entamoeba by Microscopic Examination

The total number of this study was 563, 100 sample (18%) test result was positive for microscopic light infection of Entamoeba, Uninfected samples (82%) Table 2, Fig.1. A. and B.

Diagnosis of Entamoeba histolytica by PCR assay:

The PCR technique on 100 samples diagnosed with an optical microscope, only 74 samples were found to contain

DNA of the specific genus Entamoeba. Table 3 and Fig.2

Serum Leptin level in patients and comparison groups:

The study revealed a significant rise in the mean concentration of **Leptin** in children who were infected.

(Mean ± SD 4.41±.1.69 ng/mL) compared to the control group (1.47± 0.46ng/mL) Table.4 and Fig.3

Serum obestatin level in patients and comparison groups:

The study revealed a significant rise in the mean concentration of obestatin in children who were infected.

(Mean ± SD 188.8±85.04 ng/mL) compared to the control group (49.2± 11.50 ng/mL) Table.5 and Fig.4

Serum IGFBP2 level in patients and comparison groups

The study revealed a significant rise in the mean concentration of IGFBP2 in children who were infected. (Mean ± SD 49.8±22.4ng/mL) compared to the control group (14.4± 5.3 ng/mL) Table.6 and Fig.5

Discussion

The present study documented a 12% infection rate of the E.histolytica parasite in 563 fecal samples examined microscopically by direct wet mount smear, as illustrated in Table No. (1), which is consistent with previous studies [26][27] and contrasted with others studies [28][29]. Discrepancies in the recorded percentages in this study compared to the afore mentioned ones could be attributed to various factors such as personal hygiene, population density, sanitation levels, geographical location, climate conditions, economic and social status, as well as the total sample size, laboratory precision and expertise, and fecal sample examination techniques.

Using PCR assay to diagnose the species of parasite, it revealed a rate of infection with the *E.histolytica* amounting to 74% out of a total of 100 samples that were positive by microscopic examination, as in Table (2), as it agreed with what was recorded in[26, 27] , and it did not agree with what was recorded by both [28, 29] , as it was The difference in the results of the PCR technique is due to the difference in methods for extracting DNA from stool samples, the type of primers and genes used, and the type of PCR technique, as well as the difference in the amount of parasite in the stool samples. The *E. histolytica* is spread and distributed irregularly among countries of the world due to differences in climatic conditions, customs and traditions. Negative sample may be due

to other non-pathogenic amoeba species, for example *E.dispar* or *E.moshkivishi*, and perhaps a mistake in diagnosis or the presence of contaminating inhibition materials in the stool samples for the PCR technique, which is characterized by very high sensitivity and specificity.

This study recorded a highly significant enhance in the mean concentration of leptin in the serum of patients compared to the control group, as it agreed with Suha and Yahya 2019[30] and with a study conducted by Duggal and Guo 2011[31] . While you did not agree with Salem in 2013 in Najaf Al-Ashraf / southern Iraq[32] and also with Salem and Kazem in 2019 in Kufa[33].Leptin is an adipocytokin that plays an important role in the relationship between nutrition and immunity. It is a hormone protein that contributes and controls food intake and energy metabolism, It rises due to the host's immunological response to parasitic intestinal infections. [34].Intestinal parasitic infections caused anorexia, which is a management strategy for patients. It is an aspect of the immune system's response to detecting a parasite invasion, resulting in alterations and adjustments to the host's dietary habit. Therefore, loss of appetite results in a decrease in energy levels in children. At the same time, the level of some cytokine compounds rises, and thus the level of Leptin in the serum will rise. Likewise, Leptin can be considered a Cytokine that induces and stimulates a strong immune response to T.helper1 and is therefore considered an proinflammation[35]. Likewise, the reason for the rise in leptin may be attributed to infection with the *E. histolytica* parasite, as the parasite invades the tissue and causes damage and damage to the intestinal mucosal epithelium on the surface. For example, ulceration, inflammation, and changes in the viability of epithelial cells during the acute phase of infection lead to Reducing of intestinal villi and expansion of crypts, which may activate the mesenteric lymph nodes, which may activate nearby adipose tissue to secrete leptin[36].

The present investigation observed a statistically important increase in the average level of obestatin in the blood serum of patients compared to the control group. I agreed with Al-Hadrawi and others 2019 in Kufa, southern Iraq[37]. and with Al-Awadi et al. 2022 in Najaf Al-Ashraf[38] and also agreed with other studies conducted for patients with diabetes and pancreatitis [39]that the reason for the increase in Obestatin may be due to infection with E. histolytica, which causes damage to the intestinal mucosa and damage to the villi of the stomach and intestines, especially during In the acute phase of infection, the hormone Obestatin is secreted in large quantities from the epithelial cells and lining of the stomach and intestines, where it inhibits the movement of the digestive canal, causes loss of appetite, weight loss, and prevents stomach emptying and jejunum movement [40-42].

The present investigation observed a statistically important increase in the average level of IGFBP2 in the blood serum of patients compared to the control group. No research similar to this study was found, but Ohedi et al., 2003, mentioned in a study they conducted on echinococcis, trichinolosis, and toxoplasosis, where a lowers in the level of IGFBP2 in the serum patients compared to the control group, as a result of a lower level of immunity and lack of nutrition[43].

Conclusion

The study revealed that patients had increased levels of leptin, obestatin, and IGFBP2 when the infection occurred of *E .histolytica*.

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Declaration of Conflict of Interest:

The author declares that there is no conflict of interest.

Ethical of approval

Following all applicable ethical standards, the study was conducted. The participants were informed verbally of the purpose of this study. Patients were chosen for participation in the study and ethical clearance was acquired prior to sample collection. In addition to providing patients with normal instructions and help for filling out the survey, the researcher did a good job of communicating the purpose and process of the survey. The local Ethics Committee, 26953, gave its stamp of approval to the study's design, patient data, and consent form on July 10, 2023.

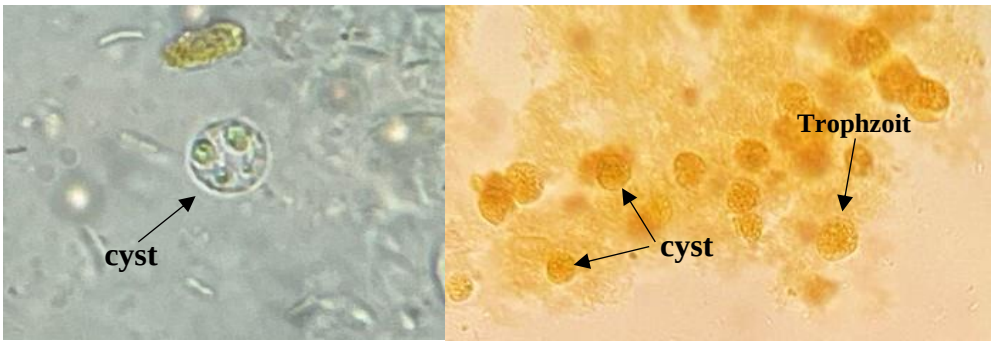

Fig . 1. A. show cyst of by wet smear normal saline(0.9%) under microscopic examination with 40X Magnification. B. cyst &Trophzoite by Lugol's iodine (1%) examination with 40X Magnification.

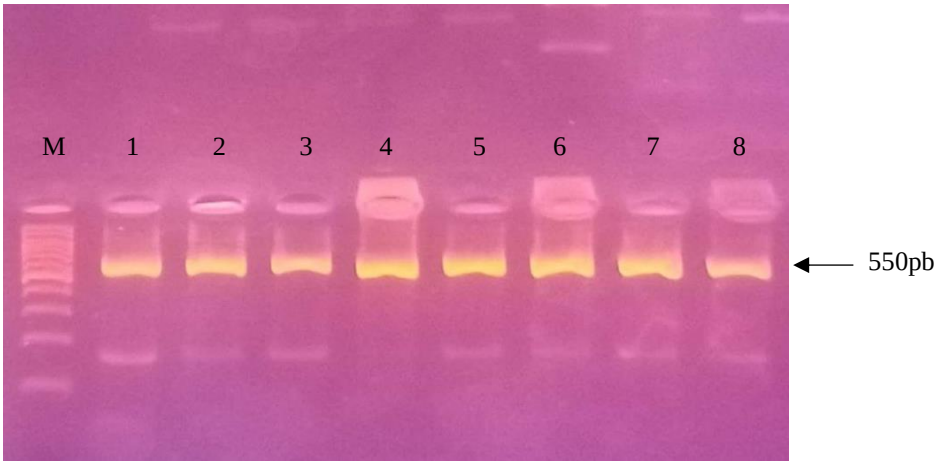

Fig. 2. PCR product is subjected to electrophoresis in a gel made of 2% agarose.M: DNA size marker (100 bp ladder) is used to denote the size of DNA. Bands in lanes (1-8) indicate the presence of Entamoeba histolytica.

TABLE 1. Data on DNA Isolation Results

	Nanophotometer Analysis	
Number sample	**High**	**Low**
Concentration (ng/µL)	584.8	3.9
Purity (A260/A280)	1.9	1.4

TABLE 2. Diagnosis of Entamoeba by Microscopic Examination

Total number examined	**Positive**		**Negative**	
	NO.	**%**	**NO.**	**%**
563	100	12%	463	82%

TABLE 3. Diagnosis of *Entamoeba histolytica* by PCR assay

Total number examined	**Positive**		**Negative**	
	NO	%	**NO**	%
100	74	74%	26	26%

TABLE 4. Serum Leptin level in patients and control groups

leptin ng/mL	Patients NO= 60	Control NO= 30	P .value
Mean± SD	4.41±1.69	1.47±0.46	0.001*

P. value is high significant at P≤ 0.05*

*Mean values of logarithmic count for different products with different superscript letters in the same rows are significantly different at (P<0.05).

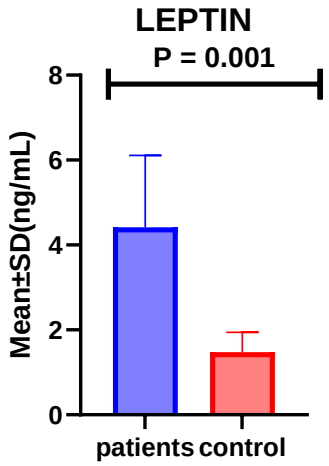

Fig.3. Concentration serum leptin in patients and control groups

TABLE 5. Serum obestatin level in patients and control groups:

Obestatin ng/mL	Patients NO= 60	Control NO= 30	P. value
Mean± SD	188.8±85.04	49.2±11.50	0.001*

P. value is high significant at P≤ 0.05*

*Mean values of logarithmic count for different products with different superscript letters in the same rows are significantly different at (P<0.05).

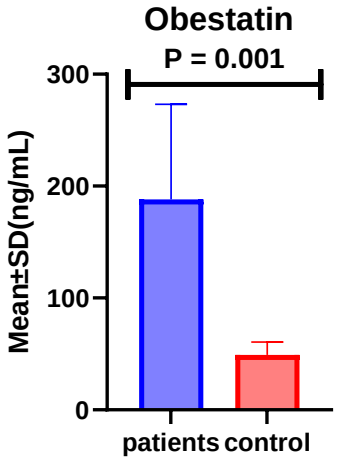

Fig. ٤. Concentration serum obestatin in patients and control groups

TABLE 6. Serum IGFBP2 level in patients and control groups:

P-selectin ng/mL	Patients NO= 60	Control NO= 30	P. value
Mean± SD	49.8±22.4	14.4±5.3	0.001*

P. value is high significant at P≤ 0.05*

*Mean values of logarithmic count for different products with different superscript letters in the same rows are significantly different at (P<0.05).

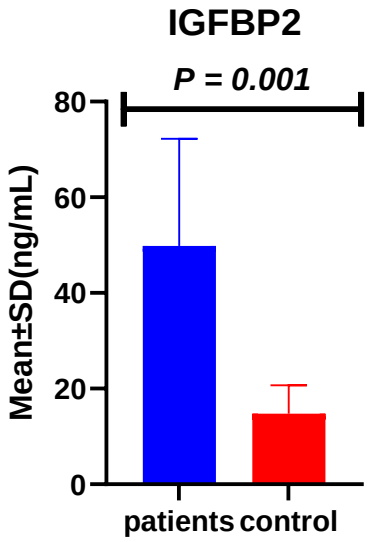

Fig. ٥. Concentration serum IGFBP2 in patients and control groups

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دراسة دور المؤشرات الحيوية اللبتين وأوبستاتين وIGFBP2 في الأطفال المصابين بطفيلي الاميبا الحالة للنسيج

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الملخص

الاميبا الحالة للنسيج: هو طفيل أولي يسبب التهاب القولون الأميبي، وهو مرض غازي مسؤول عن ما يصل إلى ١٠٠٠٠٠ حالة وفاة سنويًا على مستوى العالم. هدفت هذه الدراسة إلى تشخيص داء الأميبات باستخدام المجهر المباشر وفحص PCR، ويتضمن تقييم بعض العلامات الهرمونية. اللبتين والأوبساتاتين وIGFBP2 بواسطة الاليزا. أجري البحث في الفترة من تموز ٢٠٢٣ إلى تشرين الثاني ٢٠٢٤. بلغ عدد عينات البراز للرضع والأطفال الذين يعانون من الإسهال وآلام البطن ٥٦٣ عينة في مستشفى ابن الأثير ومستشفى الخنساء ومستشفى السلام في محافظة نينوى. تم فحصها بالمجهر المباشر ١٠٠ عينة إيجابية (١٨%) و٤٦٣ عينة سلبية (٨٢%) الكشف عن الحمض النووي للأميبا بواسطة بادئات جينية محددة بطريقة PCR. وقد لوحظت ٧٤ عينة إيجابية من أصل ٤٦٤ عينة تم فحصها. كانت هناك زيادة كبيرة في مستويات اللبتين. في المرضى الذين لديهم متوسط تركيز ٤,٤ ± ١,٦ نانوغرام / مل، مقارنة بالمجموعة الضابطة، التي متوسط تركيزها ١,٤ ± ٠,٤ نانوغرام / مل، كانت مستويات الأوبيستاتين في المرضى أكبر بمتوسط ١٨٨,١ ± ٨٥,٠٤ نانوغرام / مل، مقارنة إلى المجموعة الضابطة التي كان متوسط تركيزها ٤٩,٢ ± ١١,٥ نانوغرام / مل، وزيادة في مستويات IGFBP2. في المرضى مع متوسط تركيز ٤٩,٨ ± ٢٢,٤ نانوجرام/مل، مقارنة بالمجموعة الضابطة التي متوسط تركيزها ١٤,٤ ± ٥,٣ نانوجرام/مل.: كشفت الدراسة أن المرضى لديهم مستويات متزايدة من اللبتين والأوبستاتين وIGFBP2 عند حدوث العدوى. من E.histolytica.

الكلمات الدالة : الاميبا الحالة للنسيج ،الاليزا،اللبتين ،الاوبيستاتين، IGFBP2،PCR.