Study of Some Parameter in Men Infected With
Entamoeba Histolytica in Al-Najaf Al-Ashraf Province

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Abstract
The study was conducted on 60 out patients and 30 healthy men to determine the influences of infected with Entamoeba histolytica on levels of iron, melatonin and prolactin in men infected with E. histolytica in compared with healthy group. Who have visited Al-Sadder medical city and Al-Hakeem Hospital in Al- Najaf Province during the period from January till August 2012. Diagnosis infection with this parasite by using the wet amount microscope for stool from patients. The results showed significant decrease (P<0.05) in iron and ferritin in E. histolytica infection patients in compared to control group. Furthermore the results showed serum melatonin and prolactin were significant increased (P< 0.05) in E. histolytica infection patients in compared to control group.

Key words: parasitology . E.histolytica. physiological effect

Introduction
Entamoeba histolytica is an enteric protozoan parasite that infects 500 million people, causes amoebiasis in 50 million and kills 100 000 individuals annually, thus constituting a serious health public problem (WHO 1997; Tankyuksel &William, 2005). In industrialized countries most of the infected patients are immigrants, institutionalized people and those who have recently visited developing countries (Fotedar et al. 2007). Severe infections inflame the mucosa of the large intestine causing amoebic dysentery. The parasites can also penetrate the intestinal wall and travel to organs such as the liver via blood stream causing extraintestinal amoebiasis (Stanley, 2001). Symptoms of these more severe infections include anemia (Kaeni, 2003). E. histolytica is morphologically identical species. In bright-field microscopy, E. histolytica cyst is spherical and usually measure 12 to 15 μm (range may be 10 to 20 μm). A mature cyst has 4 nuclei while an immature cyst may contain only 1 to 3 nuclei. Peripheral chromatin is fine, uniform, and evenly distributed (Azam, 1996). Elongated, chromatoid bodies with bluntly rounded ends may sometimes be found. Glycogen can be diffuse or absent in mature cysts while clumped in immature cysts. Wet mount preparations and trichrome stained smears of stool specimens are the recommended procedures for identification of E. histolytica. All organisms possess mechanisms for maintaining perfectly balanced iron levels in the body (Sharp and Srai, 2007). This delicate equilibrium is mainly accomplished via protein-mediated iron manipulations that include absorption, transport, recycling, storage and utilization. Iron (Fe) is a transition trace element that is crucial for life and participates in the cellular metabolism of virtually all living organisms (Fernando, 2009). Both haemophores and hemoglobin receptors bind the iron source using the observed motifs FRAP (Phe-Arg-Ala-Pro) and NPNL (Asn-Pro-Asn-Leu) (Simpson, 2000). However, haemophores present higher affinity for the iron source than receptors, since they have to scavenge iron during starvation, whereas receptors only have to wait for the iron supply
(Izadi-Pruneyre et al., 2006). The molecular mechanisms of iron acquisition have been studied in bacteria; however, in protozoa, these processes are still poorly understood. Iron storage is extremely important to all forms of life, thus ferritin is found in the three domains of living beings, Archaea, Bacteria, and Eukarya. However, some eukaryotic cells like yeasts and protozoa apparently do not have a ferritin molecule, but have evolved other ways to store and maintain their own iron homeostasis (Suchan et al., 2003; Koorts and Viljoen, 2007; Arosio et al., 2009).

E. histolytica is a protozoan that infects humans. Under laboratory conditions, this pathogen can grow axanically and maintain its growth using different provisions of iron such as: free iron, lactoferrin, transferrin, ferritin or human hemoglobin (Leon-Sicairos et al., 2005).

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine synthesized from tryptophan. The physiological properties of melatonin are not limited to its neuroendocrine role in controlling circadian rhythms (Cassone, 1990).

The pineal gland is an important mediator of the photoperiodic time cues through which seasonal breeders respond to the annual cycle in day length, the pineal secretes melatonin, the blood concentrations of which increase during the hours of darkness.

Melatonin affects plasma cortisol concentration but it seems that its effects are due to some particular physiological state like blindness in individuals; in normal subjects these results are not reported. So our data confirms the previous reports findings that melatonin the pineal hormone has no effect on cortisol concentration and there lies no physiological coupling of these two hormones in mammals because melatonin is the hormone of darkness its peak has been observed during night and cortisol the hormone associated with stress and wakefulness has its peak observed during active period of day in healthy subjects (Zehra et al., 2010). The aim of this study to estimate the levels of iron, ferritin, melatonin and prolactin in serum of men infected with E. histolytica parasite.

**Subjects and Methods**

**Specimens**

From January till August, 2012, 60 samples were collected from patients and 30 healthy male who attended the clinics in AL-Sadder Teaching Hospital and AL-Hakeem Hospital in AL-Najaf province, Stool samples were collected into clean, wide-mouth specimen bottles, from male patients and blood samples were also drawn from the same patients by vein-puncture into specimen tubes and remains for 30 minutes at room temperature. After that the samples were centrifugation at 3000 rpm for 5 minutes (Backman/counter, Germany) to separate the serum and collected in other sterile tubes, each sample of serum was divided into four parts; each of them was kept in deep freeze at -20°C till used for the determination of iron, melatonin, ferritin and Prolactin.

**Specimen processing**

Freshly voided stool specimens were processed and examined microscopically using X40 objective lens for intestinal parasites as described by Paniker (1989). Ten X40 objective fields of the stool smears were examined before a slide was considered negative.

**Serum Iron (Colorimetric Test)**

The colorimetric test method was used to estimate the serum of iron via RANDOX reagents, code HB012. (RANDOX Kit, U.K) by cypress diagnostics biochemistry analyser.

**Assay Procedure**

1- The solution was brought at room temperature for 10 minutes.

2- The standard and a reagent blank were prepared for each series of determination into identified test tube as follow
3- Mix and wait 5 minutes at 37°C or 10 minutes at room temperature.
4- Read absorbance of standard and sample against standard / sample blank. The colour is stable for at least 30 min.
5- The final absorbance was red at 590 nm.

**Calculation of Results Iron (mg/dl)**

\[
\text{Serum Iron concentration} = \frac{\text{Abs.sample} - \text{Abs.samleblank}}{\text{Abs.standrad} - \text{Ab standard blank}} \times 100 \text{(Stand. Con.).}
\]

**Ferritin ELISA**

This test was intended to quantify the serum levels of ferritin through the immunoenzymatic technique Enzyme-Linked Immunosorbent Assay (ELISA) using bio Elisa reader ELx 800 (bio kit, U.S.A.) .The human Accu Bind ferritin ELISA kit was achieved according to the manufacturing company (Monobind Inc, U.S.A.) .

**Assay Procedure**

1- The components of the kit were equilibrated at the room temperature before use.
2- 25 μl of standard, controls and sample was added per well.
3- 100 μl of Biotinylated ferritin Antibody was added to each well. Wells were covered with a sealing tape and incubated for 30 minutes. The timer was started after the last sample addition.
4- The micro plate was washed six times with 300 ml of wash buffer using bioeliser washer ELx 50 (bio kit, U.S.A.).
5- 100 μl of ferritin Enzyme Reagent was added per well and incubated for 30 minutes. The bio Elisa reader ELx 800 (bio kit, U.S.A.) was turned on and set up the program in advance.
6- The micro plate was washed as described above.
7- 100 μl of working substrate solution was added per well and incubated for about 15 minutes or until the optimal blue colour density develops.
8- 50 μl of stop solution was added to each well. The colour will change from blue to yellow.
9- The absorbance on bio Elisa reader EL x 800 was read at a wave length of 450 nm immediately. Results were provided within 1 minute on the LCD display and printed out.

**Serum Prolactin estimation**

This assay executed with specific kit for test, supplied by (Immuno - Biological Laboratories, IBL-America. RE52131).

**Assay procedure**

1. Secure the desired number of coated Microtiter Wells in the holder.
2. Dispense 25μl Prolactin Standards (0; 5; 20; 50; 100; 200ng/ml), controls and serum specimen with new disposable tips into appropriate wells.
3. Dispense 100μl anti- Prolactin Enzyme-Conjugate into each well.
4. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
5. Incubate for 30 minutes at room temperature
6. Briskly shake out the contents of the wells.
7. Rinse the wells with 300μl of Aqua dest. 5 times.
8. Strike the wells sharply on absorbent paper to remove residual water droplets.
9. Add 100μl of Substrate Solution to each well, at timed intervals.
10. Incubate for 10 minutes at room temperature.
11. Stop the enzymatic reaction by adding 50μl of Stop Solution to each well, at the same timed intervals as in step 9.
12. Read the OD at 450 ±10nm with a microtiterplate reader.

**Assay procedure**

1. Pipette 50 IL of each extracted Standard, extracted Control and extracted sample into the respective wells of the Microtiter Plate.
2. Pipette 50 IL of Melatonin Biotin into each well.
3. Pipette 50 IL of Melatonin Antiserum into each well.
4. Cover plate with adhesive foil. Shake plate carefully. Incubate 14-20 h at 2-8°C.
5. Remove adhesive foil. Discard incubation solution. Wash plate 3 x with 250 IL of diluted Assay Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
6. Pipette 150 IL of freshly prepared Enzyme Conjugate into each well.
7. Cover plate with new adhesive foil. Incubate 120 min at RT (18-25°C) on an orbital shaker (500 Melatonin ELISA (40-371-25005) Gen Way Biotech Inc. 5 / 6 rpm).
8. Approx. 10 min before end of incubation prepare PNPP Substrate Solution.
10. For adding of Substrate and Stop Solution use, if available, an 8-channel Micropipettor. Pipetting should be carried out in the same time intervals for Substrate and Stop Solution. Use positive displacement and avoid formation of air bubbles.
11. Pipette 200 IL of freshly prepared PNPP Substrate Solution into each well.
12. Incubate 20 - 40 min at RT (18-25°C) on an orbital shaker (500 rpm).
13. Stop the substrate reaction by adding 50 IL of PNPP Stop Solution into each well. Briefly mix contents by gently shaking the plate.
14. Measure optical density with a photometer at 405 nm (Reference-wavelength 600- 650 nm) within 60 min after pipetting of the Stop Solution.

**Calculation of Results**

1. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
2. Calculate the average absorbance values for each set of reference standards, controls and patient samples.
3. Using the mean absorbance value for each sample determine the corresponding concentration of Proloactin in ng/ml from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

**Statistical analysis**

Data were analyzed using the software packages Graphpad prism for Windows (5.04, Graphpad software Inc. USA), Data are presented as the mean ± standard error (SE). The comparison between the patients and healthy groups were analyzed by one-way ANOVA. A p-value < 0.05 was considered significant.

**Results**

**Relation between Melatonin of amoebiasis patients and healthy group.**

Figure (1) showed comparison between amoebiasis patients and healthy group. This result revealed the significant increased p<0.05 in serum Melatonin 21.931 ± 0.872 (pg/ml), renal failure patients in compared to healthy group 9.543± 0.444 (pg/ml).
**Relation between prolactin of amoebiasis patients and healthy group.**

The result in figure (2) showed comparison between amoebiasis patients and healthy group where as significant increased (p<0.05) of serum prolactin concentration in amoebiasis patients 31.348 ± 0.215 ng/ml as compare with HT group 13.777 ± 0.909 ng/ml.

**Relation between Iron of amoebiasis patients and healthy group.**

The result of figure (3) shows comparison between renal failure patients and healthy group where as significant decrease (p<0.05) of serum Iron concentration in amoebiasis patients 34.111 ± 0.221 Ug/dL as compare with HT group 60.127± 3.654 Ug/dl.
Figure (3) comparison between Iron level of amoebiasis patients and Healthy group.
*Significant difference (P<0.05) between control group and patients

Relation between ferritin of amoebiasis patients and healthy group.
The Result of figure (4) showed comparison between renal failure patients and healthy group where as significant decrease \( p<0.05 \) of serum ferritin concentration in amoebiasis patients \( 11.541 \pm 0.592 \) ng/ml in compared to HT group \( 25.653 \pm 1.120 \) ng/ml.

Discussion
The current study has revealed that the serum iron and ferritin significantly decrease in *E. histolytica* infection patients compared to healthy group. While serum melatonin and serum prolactin were significantly increase in *E. histolytica* infection patients compared to healthy group. The decrease in iron level in patients with *E. histolytica* maybe due to the pathogenicity of this parasite dependent on the relationship between iron concentration and adhesion of parasite on epithelial cell (Garcia et al. 2003). Whereas the source of iron maybe from the hemolysis of red blood cells from lesion occur by the parasite. The consuming of iron by *E. histolytica*
may cause a decrease in the iron levels. The decrease in ferritin levels maybe due to an increase in consuming iron by this parasite and this leads to decrease in the storage of iron as ferritin or increased utilized by parasite whereas some studies describing E. histolytica as an iron source (Lehker & Alderete, 1992). As confirmed by the data from Fernando et al. (2009) that E. histolytica use the ferritin as an iron source; therefore, ferritin decreased when an infectious process occur. The current study agrees with the study of Weinberg (1999) who recorded that the E. histolytica uses the ferritin as source of iron and these lead to decrease in the ferritin in serum of men infected with E. histolytica compared to healthy group. The present study has revealed that the serum melatonin and prolactin significantly increase in E. histolytica infection patients compared to healthy group. The increase in melatonin level in patients with E. histolytica maybe due to the Phagocytosis plays an important role in E. histolytica pathogenicity, making it an object of investigations.

A comprehensive evaluation of the phagocytic capacity of amoebae involves leukophagocytosis because amoebae are constantly in contact with leukocytes in vivo and must be able to destroy them to survive. However, the increase levels of melatonin maybe due to leukocytosis caused by E. histolytica, interactions between amoebiasis and white blood cells lead to increase in melatonin and this known immunomodulator (Aline, 2011). The increase in levels of prolactin in patients with E. histolytica maybe due to the decrease in levels of iron and these lead to anemia, serum prolactin levels and liver prolactin-binding sites were increased in iron deficient rats (Barkey, 1985 and Barkey, 1986). From this study conclusion that serum levels of prolactin increase in patients infected with E. histolytica due to effects of early iron deficiency on the dopamine system.

References

Aline, C. França-Botelho; Juliana, L.; França, Fabricio, MS.; Oliveira, Eduardo, L. Franca; Adenilda, C. Honório-França; Marcelo, V. Caliari and Maria, A. Gomes2011. Melatonin reduces the severity of experimental amoebiasis. Parasites & Vectors, 4:62


Izadi-Pruneyre, N.; Huche, F.; Lukat-Rodgers, G. S.; Lecroisey, A.; Gilli, R.; Rodgers,K. R.; Wandersman, C. and Delepelaire, P. 2006 The heme transfer from the solubleHasA
hemophore to its membrane-bound receptor HasR is driven by protein–protein interaction from a high to a lower affinity binding site. J. Biol. Chem. 281, 25541–25550.


