ASSESSMENT OF SOME IMMUNOLOGICAL AND PHYSIOLOGICAL BIOMARKERS IN PATIENTS WITH CELIAC DISEASE IN WASIT PROVINCE \ IRAQ

Rehab Abdul-Razzaq Abdul-Hassan1, Mohammed R. S. AL-Attabi *1, MuntatharAliAbdul-Redha2

1. Department of Biology, college of Science, Wasit University, Iraq
2. Department of microbiology, college of medicine, Wasit University, Iraq

Email: dr.mohammedrijy1@gmail.com

Abstract:

Background: As consequence of stress several immunological and molecules processes inCoeliac disease (CD) become activated.Molecular chaperones (called as HSPs), α-gliadin peptide p31-43, can elicit an IL-15-mediated innate immune response in the small intestine of coeliac disease patients, leading to intestinal epithelial damage.

Aim of Study: to assess the oxidative stress status in the celiac disease and its relation to HSPs and innate immune response.

Materials and Methods: study included 40 CD patients, and 20 healthy control. Serum HSP70, serum IL-15, and serum MDA investigated to confirm the relationship and activity of it in celiac disease.

Results: from our study results indicates that HSP 70 and IL-15 increased in CD and observed highly significant (sig.0.0001) between patients and control group, but no significant (sig.0.707) between patients and control when show the MDA results.also the HSP-70 have greatly Standard Deviation that result from the variance between patients and control very high.

In conclusion, from experiments of prior researchers and during our study results, they confirmed that the important factors that play the main role in the diagnosis of celiac disease are oxidative stress. The common effect due to oxidative stress is the overexpression of heat shock protein due to the temperature above optimum. As well as, gluten exposure effect in susceptible individual leads to increase free radical production and stimulate the innate immune response in the intestinal epithelial cell, then triggers cytokines production.Likewise, the best treatment against this disease is GFD.

Keywords: celiac disease, MDA, IL-15, HSP-70

http://doi.org/10.36295/ASRO.2021.24503

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Volume/Issue: Volume: 24   Issue: 05

Introduction:

Beginning, Coeliac disease is a complex and common inflammatory disorder that occurs in the small intestine, it have autoimmune features in genetically predisposed individuals, moreover, stimulates by chronic exposure to gluten of wheat, also in barley and/or rye (1). On the other hand, increased prevalence of over the last decades are not understood, but similar to some immune-
mediated diseases, for example, allergy, and asthma, this shows the tendency that confirms the important role of environmental stress factors as well as the genetic predisposition (2).

On the same side, Different chemical and environmental stress factors to stimulate oxidative damage in the cells (3). The main factors that play an important role in Oxidative stress is a reactive oxygen species, which are produced continuously during the respiration process. Furthermore, levels of ROS are markedly increased due to different stress factors that lead to oxidative deterioration of proteins, lipids, and DNA (4). Additionally, an increase of temperatures above the optimum defined as heat stress sensed by living organisms, which extend to disturbs cellular homeostasis and may result in severe retardation in growth and development, in addition to death (5).

The cell response to heat stress represented by overexpression of heat stress proteins (6). On the same side, HSPs acts as extrinsic chaperones or “molecular chaperones” are a suite of more highly conserved proteins with different molecular weights, they are produced in all organisms during exposure to cellular stress (7). Furthermore, the discovery of HSPs begins in the early 1960s (8), other functions of HSPs are in protein degradation as fundamental components of the ubiquitin-dependent degradative pathway (9). On the other hand, The non-immunogenic peptides, that called toxic gliadin (p31-43) represent major factors that cause and stimulate increased production of interleukin 15 (IL-15), this cytokine plays important role in the pathogenesis of the coeliac disease (10,11). Moreover, Increased production of IL-15 leads to activates and overexpression of IELs (12), then observe the cytolytic effects of activated IELs on intestinal epithelial cells (11). Overexpression of IL-15 acts as a persistent activator to the adaptive immune system (10). Serum malondialdehyde level, a biomarker of lipid peroxidation process.

**Patient and Methods:**

Studies included 40 case CD patients with 20 healthy controls. Serum patients and control examination by use ELISA technique in al-Zahraa hospital.

**HSP-70**

Assay procedure (A brief assay procedure is on the 11th page)

1. Add the Standard working solution to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 μL for each well). Add the samples to the other wells (100 μL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Remove the liquid out of each well, do not wash. Immediately add 100 μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.

3. Aspirate or decant the solution from each well, add 350 μL of wash buffer to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.

5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.

6. Add 90 μL of Substrate Reagent to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.

7. Add 50 μL of Stop Solution to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.

8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

IL-15

Assay procedure:

1. Add the Standard working solution to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Remove the liquid out of each well, do not wash. Immediately add 100 μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.

3. Aspirate or decant the solution from each well, add 350 uL of wash buffer to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.

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7. Add 50 μL of Stop Solution to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.

8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

MDA assay:

Assay Protocol:
1. All reagents (except samples) must be equilibrated to RT. Shake the unknown sample for homogenation well.
2. Add 100μL standards/samples to related name test tubes.
3. Add 100μL R4 reagent (if it is cloudy, warm until to become a clear solution).
4. Add 200μL ready Chromogenic solution.
5. Heat the above mixture for one hour at boiling water bath (pink color formation).
6. Cool the above tests tube in ice bath and centrifuge them 10 min around 10,000 rpm.
7. Pipette 200μL of pink color supernatant into the microplate.
8. Read the absorbance at 535nm.
9. Calculate MDA level in unknown samples based on standard curve which drown using standard points’ absorbance.

**Statistical Analysis**

Data were organized and analyzed by using SPSS V 0.25. Descriptive statistics (frequency distribution and percentages with tables and figures, and mean± standard deviation) and inferential statistics (Independent t-test, Mann-Whitney U test, and Pearson/ Spearman Bivariate correlations) were used. P-value of 0.05 was considered statistically significant.

**Results and discussion:**

**Table 1:** Serum level of HPS70, MDA and IL-15 in celiac disease patients and healthy control

<table>
<thead>
<tr>
<th>Markers ▼</th>
<th>Sample (n=60)</th>
<th>Independent T-test and Mann-Whitney U test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients (n=40)</td>
<td>controls (n=20)</td>
</tr>
<tr>
<td>HPS70</td>
<td>123.84 (35.87)*</td>
<td>37.13 (20.97)</td>
</tr>
<tr>
<td>MDA</td>
<td>29.90**</td>
<td>31.70</td>
</tr>
<tr>
<td>IL-15</td>
<td>40.10</td>
<td>10.90</td>
</tr>
</tbody>
</table>

* mean (SD). ** mean rank. *** Mann Whitney U Test

**Table 2:** Bivariate Correlation of all indicators

<table>
<thead>
<tr>
<th>Correlation Coefficient (r)</th>
<th>Age</th>
<th>IL-15</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td></td>
<td></td>
<td>.280*</td>
</tr>
<tr>
<td>Sig.</td>
<td></td>
<td>.030</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>IL-15 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>.318*</td>
<td>.072-</td>
<td></td>
</tr>
<tr>
<td>Sig.</td>
<td>.013</td>
<td>.584</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>MDA (um)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>-.208-</td>
<td>.705**</td>
<td>.088</td>
</tr>
<tr>
<td>Sig.</td>
<td>.111</td>
<td>.000</td>
<td>.502</td>
</tr>
<tr>
<td>N</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>
* Correlation is significant at the 0.01 level (2-tailed).
** Correlation is significant at the 0.05 level (2-tailed).

Independent T-test: $t_{11.782}$, df 56, Sig. 0.0001.

Figure 1: Mean difference of HPS70 level in patients (n=40) and controls (n=20).

Mann Whitney U Test: 376.0, Sig. 0.707.

Figure 2: Mean rank difference of MDA level in patients (n=40) and controls (n=20).

Mann Whitney U Test: 8.0, Sig. 0.0001.

Figure 3: Mean rank difference of IL-15 level in patients (n=40) and controls (n=20).
From table 2 we concluded:

Correlation of Age and parameters:

Age and IL-15 (inversive correlate), with sig. Age and MDA (extrusive correlate), with sig. Age and HSP-70 (inversive correlate), with non significant. Moreover, IL-15 and MDA (inversive correlate), with non sig. IL-15 and HSP-70 (extrusive correlate), with high sig. Additionally, MDA and HSP-70 (weak correlate), with non sig.

Present study results observed that HSP-70 and IL-15 show highly significant difference between patients and control (0.0001), but MDA results shown non-significant difference. From this maybe conclude that gluten immunogenic peptides (p31-43) are very active and cause stimulate of IL-15 production. IL-15 play important role in stimulates the IELs effector functions of as well as induce their cytotoxicity against epithelial cells in celiac disease patients, this done by increasing their expression of activating NK receptors in addition to synergizing with the NK receptor signaling cascade (13,14). On the same side, other studies confirm that the IL-15 contributes to increasing survival and subsequent accumulation of non-transformed IELs in CD (10) (15). Other evidence similarly to our study result that shown increase concentration of IL-15 in CD patients (figure three) prior studies indicates that the upregulation of IL-15 synthesis in intestinal tissues of CD occurs at high levels in patients that exposure to gluten peptides (16, 17, 18). On the other hand, it is unclear whether the role of gluten to stimulate stress or tissue inflammation occurs directly or indirectly. The source of IL-15 production include various cells of the innate immune system as macrophages and dendritic cells, in addition to different of structural cells including fibroblasts and epithelial cells.

Through recent years, IL-15 considered as complex regulatory cytokine has emerged through recent years as a more complex regulatory cytokine, in the mediation between innate and adaptive immunity as well as have a major role in the control of homeostasis and immune responses within specific environments (19,20). Furthermore, it shows that the fail and dysregulation of responses controlled by IL-15 could be an important component that results in certain human immune pathologies. However, the causative role for IL-15 was first elicited for diseases such as rheumatoid arthritis and psoriasis, but more recently IL-15 has a major important role and consider as main implicated in gastrointestinal disease (21,22,23). Interleukin-15 (IL-15) essential for many biological functions to the maintenance and function of multiple cell types. In addition, the expression of IL-15 is tightly regulated, as well as, IL-15 upregulation occurs in many organ-specific autoimmune disorders. In celiac disease that represents as intestinal inflammatory disorder cause due to the high gluten exposure observed that the upregulation of IL-15 expression in the intestinal mucosa has become a hallmark of the disease. On the same side, due to the overexpressed of IL-15 both in the gut epithelium and in the lamina propria, this results in IL-15 acts on various cell types and affects distinct immune components and pathways to damage intestinal immune homeostasis (24).

From our results indicates to activity of free radicals due to gluten exposure, furthermore, the lipid peroxidation process in an organism generating due to the effect of the free radicals. As well as, Malondialdehyde (MDA) represents one of the final products of polyunsaturated fatty acids peroxidation in the cells. Moreover, increased levels of the free radical result in the overproduction of
MDA, depending on this, our studies show positive correlated between the MDA and HSP-70. On the same side, the malondialdehyde level acts as a marker of oxidative stress, in addition, the antioxidant status in cancerous patients (25). Maybe conclude that increased ROS lead to increased free radicals production in untreated CD and results in lipid peroxidation but in patients on GFD, the activity of free radicals reduce due to gluten absence. Additionally, In celiac disease, the studies confirm that endogenous intoxication occurs in the small intestine. the difference observed in the first and the second phases antioxidant protection is a fundamental factor of the disease pathogenesis, studies revealed the correlation between MDA and intoxication index (r=0.67; p=0.006) (26).

On the other hand, HSP, define as stress-inducible proteins are recently referred to as molecular chaperones (27). Depending on the HSP results observed highly significant between patient and control, from this maybe conclude that ROS activity increased which leads to stimulating the HSP expression. In fact, when the temperature is above the optimum this may be sensed as heat stress by living organisms, this result in disturbs cellular homeostasis and can extend to severe retardation in growth and development, then death (28). In Figure one show increased HSP levels in patients than in control, refer to previous studies which have same result, prior studies results indicate that HSP-70 mRNAs were significantly elevated in the celiac groups whether the patient on diet or untreated compared with controls, about other studies, they show that increased intestinal expression of HSP-70 despite GFD, this indicates that GFD only partially reduced oxidative stress (29). On the other hand, the overexpression of IL-15 in patients with active celiac disease and refractory celiac sprue occurs not only in lamina propria but also in the intestinal epithelium. Additionally, IL-15 transport at the surface of enterocytes. Functionally, this cytokine play induction role in the extending and survival of the clonal abnormal intraepithelial lymphocytes which recognize refractory celiac sprue and stimulate their secretion of interferon-gamma as well as their cytotoxicity against intestinal epithelial cells (30).

Conclusion:

In conclusion, the important factors that play the main role in the diagnosis of celiac disease are oxidative stress. The common effect due to oxidative stress is the overexpression of heat shock protein due to the temp. above optimum. As well as, gluten exposure effect in susceptible individual leads to increase free radical production and stimulate the innate immune response in the intestinal epithelial cell, then triggers cytokines production. Likewise, the best treatment against this disease is GFD.

References:


