IMMUNOMODULATORY EFFECT OF LACTOFERRIN ON IMMUNOSUPPRESSED RATS BY CYCLOPHOSPHAMIDE

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ABSTRACT

Eighty male albino rats (350±10 g,) 10 to 12 weeks old were conducted in our study. Rats were randomly divided into four, equal groups. The groups treated as following. 1st control group (Gp. A) was given intraperitoneal normal saline (1 mL). 2nd group (Gp. B) was given a single intraperitoneal dose (250 mg/kg body weight) of Cyclophosphamide (CP) on the first day of the experimental period. 3rd group (Gp. C) CP and lactoferrin (LF) treated group. 4th group (Gp. D) administrated LF only (0.5%) in drinking water. Two separate blood samples were collected from heart puncture at end of 1st and 3rd week post treatment for hematological, biochemical, immunological and pathological studies.

The leukogram of CP treatment group showed severe leucopenia (lympho-penia, neutropenia as well as esinopenia.) decrease total protein and albumin blood level. Immunosuppressive effect of CP is documented in our work by decrease gamma globulin and elevation tumor necrosis factor Alfa (TNF α). This study revealed that oral treatment with LF can partially reconstitute humoral and cellular immune response in rats given a sub lethal dose of CP.

INTRODUCTION

LF is one of the most exciting immune stimulants drug to come along in nutritional form in recent years. LF is predominantly neutrophil derived but indications are that it may also be produced by other cells. Receptors for LF can be found on intestinal tissue, monocytes/macrophages, neutrophils, lymphocytes, platelets, and on certain bacteria. (Levay and Viljoen 1995).

CP, a bifunctional alkylating agent, is extensively used as an anticancer chemotherapeutic drug in childhood and adult malignancies, as well as an immunosuppressive agent for autoimmune disorders and other benign diseases (Dollery, 1999). CP metabolites targets rapidly dividing cells, disturbing
cell growth, mitotic activity, differentiation, and functions via alkylation of DNA (Colvin, 1999) and preferentially suppress the immune responses mediated by B-lymphocytes.

The aims of this study are: evaluation of immunomodulatory effect of LF in rat treated with CP through measurement of hematological, immunological and pathological changes.

MATERIAL & METHODS

MATERIALS:

1. Experimental Animals (Rats)
   Eighty male albino rats of We-star strain (350±10 g,) procured from faculty of veterinary Medicine, Zaga-zig University were used for the study. Animals were fed with commercially available standard and balanced rat ration and water was provided ad libitum. The rats were housed under controlled conditions of humidity, temperature (25±2 °C) and light (12 h light/12 h dark) and had free access to water and food. All animals were acclimatized for 1 week before experimentation and the experiment extended for 21 days.

2. Bovine lactoferrin (bLF) was purchased from (Symbiotics Colostrum U.S.A. (lot no. MLF160996;), with a purity of 100% Blf.

3. Cyclophosphamide (CP) was purchased from Baxter Oncology GmbH, Frankfurt am Main Germany, in the form of dry powder substance , under trade name Endoxan 1gm. Lot # 5D178B.

METHODS

Rats were randomly divided into four groups; each is consisting of twenty rats. Each group was separated in metal cages. The groups treated as following. 1st control group (Gp. A) was given I/P normal saline (1 mL).

2nd group (Gp. B) was given a single I/P dose (200 mg/kg body weight) of CP on the first day of the experimental period according to Mythili et al. (2004).

3rd group (Gp. C) CP and bovine LF treated group. 4th group (Gp. D) administered LF only (0.5%) in drinking water according to Zimecki et al. (2005) during the 21 days.

Blood Sampling

Five random blood samples were taken from five rats. Samples were collected from heart puncture at end of 1st, and 3rd week post treatment. Two separate blood samples were collected from each rat, the first sample was taken in epindorf tubes at which mixed with EDTA for hematological examination. Total leukocytic count and differential leukocytic count were measurement according to Feldman et al., (2000), Van-Kampen and Zijlstra (1961), Wintrobe (1967), Coles (1986) and Latimer et al., (2003) respectively. The second blood samples were taken in test tube without anticoagulant. The samples were centrifuged at 3000 rpm
for 10 minutes and the clear serum was separated carefully for determination of some biochemical parameters (total protein and albumin) by using commercial diagnostic kits which were obtained from Human-Germany and Spinreact Spanish).

Immuno-electrophoresis of serum protein has been done using cellulose acetate according to Henry et al., (1974).

Tumor necrosis factor – α (TNF α) was measurement by Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microplate.

The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of TNF-α according to Beutler and Cerami (1987).

**Histopathological Studies**

Animals that died were necropsies immediately after their discovery. After external inspection spleen and liver tissues were immediately excised after sacrifice (at the end of 1st and 3rd weeks) and rinsed with ice-cold physiological saline for histological studies according to Bancroff et al., (1990).

**Statistical Analysis:**

The present results were analyzed by analysis of variance (ANOVA) using SPSS.10 for window. Two groups were significantly different if P was statistically lower than 0.05.

**RESULTS & DISCUSSION**

CP, a bi-functional alkylating agent, is extensively used as an anticancer chemotherapeutic drug in childhood and adult malignancies, as well as an immunosuppressive agent for organ transplantation and other benign diseases (Dollery, 1999).

CP known as immunosuppressive drug, so the cyclophosphamide group was showed highly significant decrease of (TLC) allover the experiment period comparing with control group. This could be attributed to severe depression of bone marrow that manifested by significant decrease of all types of blood cells, lymphopenia, neutropenia, esinopenia and monocytopenia. The histopathology results showed severe lymphoid depletion to white pulps & thickening of spleenic arterioles (Fig. 1).

This results in accordance with Latha and Panikkar, (1999) who reported leucopenia in mice treated with CP. In addition, Holly, et al., (2003) recorded leucopenia, lymphopenia and neutropenia in female rats treated with CP for 30 days. Unni and Martinus (2002) reported significantly lymphopenia in rats treated with a single dose of the CP (250 mg/kg Bw), Zuluaga et al., (2006) who reported that I/P injection of female mice with 150 and 100 mg/kg of CP on days 1 and 4, respectively leading to leucopenia, lymphopenia, neutropenia and monocytopenia.
There was increase of (TLC) by LF in CP-treated mice group as well as increase of lymphocyte, neutrophil, eosinophil and monocyte blood cells comparing with CP group. Zimecki, et al., (2004) recorded increase bone marrow neutrophil lineage cell content following 24 h pretreatment mice with LF. In addition, oral LF treatment resulted in partial recovery and induced splenocyte proliferation, which confirmed with our histopathological result (Fig. 2). These agree with Jolanta et al., (2003) and Jolanta et al., (2004) who revealed that treatment of mice with LF induced a strong mobilization/recruitment of myelocytes and band forms in bone marrow. Also Rich, (1988) and Zimecki, et al., (1999) reported that LF can accelerate neutrophil recruitment in humans and animals.

Lymphocytes significantly increased in LF & CP treated group in comparing with CP group. The described activity of LF is in agreement with Sekine et al., (1997) who reported enhancement of natural killer (NK) activity and cytokine production by spleen cells in response to mitogen. Jolanta et al., (2003) concluded that oral LF treatment resulted in induced splenocyte proliferation, increased the cellularity of spleens, the content of peritoneal and alveolar macrophages and elevation of leukocytes by LF in CP-immunosuppressed mice.

In CP group showed significant decrease of total protein, albumin, alpha and beta globulin comparing with control group. This result may be due to decrease proteins synthesis as a result of liver damage as reported by Subramanian et al., (2006) and approved in our histopathological results by focal necrosis of hepatocytes and inflammatory cells infiltration (Fig. 3). Hypoproteinemia was reported in rats administered CP (150 mg/kg. BW) for two days Subramanian et al., (2006).

The present result showed significant decreases of γ-globulins in CP group in comparable with control one. The immunosuppressive effect of CP in rats was agree With Unni & Martinus (2002) and Holly et al., (2003) also, in mice with Artym (2003) and Zhang et al., (2007).

γ-globulins were significantly elevated in CP and LF treated group in comparing with CP group. Our result in hand with Zimecki, et al., (2007) who reported that LF accelerated reconstruction of the immune system function (cellular and humoral immune response) after administration of a sublethal dose of CP to mice. This conclusion was approved in our histopathological studies by mild depletion of white pulp in CP and LF treated group (Fig. 2)

TNF-α is amplify, propagate, and coordinate proinflammatory signals, resulting in the synchronized expression of effectors molecules that me-
mediate diverse aspects of innate immunity. TNF is capable of eliciting expression of chemokines and adhesion molecules and thus may be critical to the recruitment of neutrophils from the blood (Joseph et al., 2001). Our results show significantly increase TNF-α in CP group in compare with control one. The results are in accordance with Cruz-Chamorro et al., (2007) who reported elevated TNF in mice treated with CP. TNF-α was significantly decreased in CP plus LF treated group in compare with CP group. This attributed to immunomodulatory action of LF.

In conclusion CP has significant depression of blood cell production, as well as severe immunosuppressant. Oral treatment with LF can partially reconstitute humoral and cellular immune response in rats given a sub-lethal dose of CP.
Table (1): Some Hematological Parameters (Mean ± S.E), One Week Post Treatment with Lactoferrin in Immunosuppressed Rats with Cyclophosphamide.

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<tbody>
<tr>
<td>Control (A)</td>
<td>7.75c ± 0.45</td>
<td>2.74b ± 0.25</td>
<td>0.154b ± 0.041</td>
<td>0.016 ± 0.01</td>
<td>4.39c ± 0.31</td>
<td>0.47b ± 0.072</td>
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<tr>
<td>Cyclophosphamide (B)</td>
<td>3.07a ± 0.35</td>
<td>1.185a ± 0.19</td>
<td>0.015a ± 0.015</td>
<td>0.00</td>
<td>1.65a ± 0.34</td>
<td>0.22a ± 0.038</td>
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<tr>
<td>Lactoferrin &amp; Cyclophosphamide (C)</td>
<td>4.72b ± 0.46</td>
<td>1.34a ± 0.17</td>
<td>0.118b ± 0.031</td>
<td>0.00</td>
<td>3.02b ± 0.32</td>
<td>0.24a ± 0.040</td>
</tr>
<tr>
<td>Lactoferrin (D)</td>
<td>8.35c ± 0.59</td>
<td>2.52b ± 0.29</td>
<td>0.138b ± 0.039</td>
<td>0.017 ± 0.01</td>
<td>5.09c ± 0.36</td>
<td>0.56b ± 0.071</td>
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</table>

Means in the same column not followed by the same letter differ significantly (P<0.05).

Table (2): Some Hematological Parameters (Mean ± S.E), Three Weeks Post Treatment with Lactoferrin in Immunosuppressed Rats with Cyclophosphamide.

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<tbody>
<tr>
<td>Control (A)</td>
<td>8.35b ± 0.51</td>
<td>2.53b ± 0.28</td>
<td>0.165b ± 0.044</td>
<td>0.016 ± 0.01</td>
<td>5.18c ± 0.36</td>
<td>0.46b ± 0.051</td>
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<tr>
<td>Cyclophosphamide (B)</td>
<td>3.95a ± 0.24</td>
<td>1.71a ± 0.19</td>
<td>0.022a ± 0.016</td>
<td>0.00</td>
<td>1.79a ± 0.29</td>
<td>0.42b ± 0.058</td>
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<tr>
<td>Lactoferrin &amp; Cyclophosphamide (C)</td>
<td>6.55c ± 0.38</td>
<td>2.51b ± 0.27</td>
<td>0.131b ± 0.029</td>
<td>0.00</td>
<td>3.53b ± 0.39</td>
<td>0.39b ± 0.061</td>
</tr>
<tr>
<td>Lactoferrin (D)</td>
<td>8.91b ± 0.57</td>
<td>2.98bb ± 0.31</td>
<td>0.178b ± 0.034</td>
<td>0.089 ± 0.09</td>
<td>5.29c ± 0.34</td>
<td>0.37b ± 0.065</td>
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</table>

Means in the same column not followed by the same letter differ significantly (P<0.05).
Table (3): Some Immunological Parameters One Week Post Treatment with Lactoferrin
(Mean ± S.E.) in immunosuppressed Rats with *E. coli* and Cyclophosphamide.

<table>
<thead>
<tr>
<th>Group</th>
<th>T. Protein gm/dl</th>
<th>Albumin gm/dl</th>
<th>α- globulin gm/dl</th>
<th>β- globulin gm/dl</th>
<th>γ- globulin gm/dl</th>
<th>TNF- α Pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (A)</td>
<td>7.45b ± 0.41</td>
<td>3.40b ± 0.25</td>
<td>1.41b ± 0.18</td>
<td>1.35b ± 0.17</td>
<td>1.29b ± 0.12</td>
<td>21.2 ± 2.45</td>
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<tr>
<td>Cycloph. (B)</td>
<td>5.59a ± 0.45</td>
<td>2.71a ± 0.21</td>
<td>1.01a ± 0.09</td>
<td>0.92a ± 0.10</td>
<td>0.95a ± 0.07</td>
<td>42.8 ± 4.15</td>
</tr>
<tr>
<td>Lactoferrin &amp; Cycloph.(C)</td>
<td>5.84a ± 0.42</td>
<td>2.81a ± 0.19</td>
<td>1.08a ± 0.08</td>
<td>0.96a ± 0.11</td>
<td>0.99a ± 0.05</td>
<td>39.8 ± 3.75</td>
</tr>
<tr>
<td>Lactoferrin (D)</td>
<td>7.52b ± 0.62</td>
<td>3.38b ± 0.46</td>
<td>1.46b ± 0.19</td>
<td>1.32b ± 0.16</td>
<td>1.36b ± 0.13</td>
<td>20.4 ± 2.01</td>
</tr>
</tbody>
</table>

Means in the same column not followed by the same letter differ significantly (P<0.05).

Table (4): Some Immunological Parameters Three weeks Post Treatment with Lactoferrin
(Mean ± S.E.) in immunosuppressed Rats with Cyclophosphamide.

<table>
<thead>
<tr>
<th>Group</th>
<th>T. Protein gm/dl</th>
<th>Albumin gm/dl</th>
<th>α- globulin gm/dl</th>
<th>β- globulin gm/dl</th>
<th>γ- globulin gm/dl</th>
<th>TNF- α Pg/ml</th>
</tr>
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<tbody>
<tr>
<td>Control (A)</td>
<td>7.48b ± 0.48</td>
<td>3.46b ± 0.23</td>
<td>1.39 ± 0.16</td>
<td>1.31 ± 0.17</td>
<td>1.32b ± 0.14</td>
<td>18.9 ± 2.08</td>
</tr>
<tr>
<td>Cycloph. (B)</td>
<td>5.93b ± 0.46</td>
<td>2.89a ± 0.20</td>
<td>1.09 ± 0.10</td>
<td>0.96 ± 0.11</td>
<td>0.99a ± 0.06</td>
<td>31.9 ± 3.05</td>
</tr>
<tr>
<td>Lactoferrin &amp; Cycloph.(C)</td>
<td>6.17a ± 0.35</td>
<td>2.88a ± 0.22</td>
<td>1.12 ± 0.10</td>
<td>0.99 ± 0.10</td>
<td>1.18a ± 0.08</td>
<td>25.2 ± 3.01</td>
</tr>
<tr>
<td>Lactoferrin (D)</td>
<td>7.52b ± 0.49</td>
<td>3.38b ± 0.32</td>
<td>1.34 ± 0.16</td>
<td>1.32 ± 0.18</td>
<td>1.48b ± 0.15</td>
<td>19.4 ± 1.95</td>
</tr>
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</table>

Means in the same column not followed by the same letter differ significantly (P<0.05).
Fig. (1): *Section in spleen of group (B)*, treated with CP one week post treatment showing severe lymphoid depletion to white pulps & thickening of splenic arterioles. H&E, X 300.

Fig. (2): *Section in spleen of group (C)*, treated with CP and LF three weeks post treatment showing mild depletion of white pulp. H&E, X 300.

Fig. (3): *Section in liver of group (B)* treated with CP one week post treatment showing focal necrosis with inflammatory cells infiltration. H&E, X 300.

Fig. (4): *Section in liver of group (C)*, treated with CP and LF three weeks post treatment showing normal hepatic cells with portal lymphocytic aggregation were common. H&E, X 300.
REFERENCES


Unni, C.N. and Martinus, L. (2002): Blood and spleen lymphocytes as targets for immunotoxic effects in the rat—a comparison. Toxicology. 174(3): 153-161


الملخص العربي

نظراً للإحتياج الكبير لمنشطات المناعة فقد وجد أن مادة الاكتوفيرين هي من أهم المواد المنشطة للمناعة المكتشفة حديثاً من أصل غذائي. كما أن له تأثير منشط للخلايا الليمفاوية كما أن له تأثير مضاد للسرطان ومضاد للحساسية بالإضافة للتآثر المسكن للألم.

ثمانون من ذكور الفئران متوسط وزنها حوالي 0.53 جرام تم تقسيمها عشوائياً إلى أربع مجموعات تحتوى كل منها على عشر فئران وقد تم معالجتها كما يلي: المجموعة الأولى (أ) تم حقنها بمحلول ملحي فسيولوجي (مجموعة ضابطة) والثانية (ب) تم إعطاءها (0.50 مجم/كج من وزن الفأر) من مادة السيكلوفوسفاميد جرعة واحدة بالحقن في غشاء المساريقا وال مجموعة الثالثة (ج) تم معالجتها بمادة السيكلوفوسفاميد بالإضافة إلى مادة الاكتوفيرين في مياه الشرب بعمر الفأر (60 %) و المجموعة الرابعة (د) تم إعطاءها مادة الاكتوفيرين فقط في مياه الشرب كما سابق. استمر إعطاء الاكتوفيرين في مياه الشرب طول فترة التجربة لمدة 02 يوم. تم تجميع عينات الدم من خمسة فئران من كل مجموعة في نهاية الأسبوع الأول والثاني وذلك لعمل اختبارات الدم واختبارات المناعة.

وكذلك أظهرت النتائج انخفاضاً معنوي في العدد الكلي لكرات الدم البيضاء والخلايا متعادلة الصبغة، الخلايا الليمفاوية والخلايا الأكولية وجميع الخلايا المناعية المختبرة في المجموعة (ب) التي تم حقنها بالسيكلوفوسفاميد بالمقارنة بمجموعة الضابطة ولكن المجموعة التي عولجت بالاكتوفيرين مع السيكلوفوسفاميد وجدت بها ارتفاع معنوي بالنسبة للمجموعة (ب) التي عولجت بالسيكلوفوسفاميد.

دراسة مستوى البروتين في الدم وجد انخفاض معنوي في مستوى الألبومين والبروتين الكلي في الدم في المجموعات (ب، ج) بالمقارنة بمجموعة الضابطة طوال فترة التجربة. أما مستويات الألفا والبيتا جلوبولين في الدم فقد شهد انخفاض معنوي في مجموعات (ب، ج) بالمقارنة بالمجموعة الضابطة.

وعند قياس مستويات مراقباً التأكسد (معامل التيمر نيكروزيس) في الدم وجد ارتفاع معنوي في المجموعات (ب، ج) في الأسبوعين الأول والثاني بالمقارنة بمجموعة الضابطة والمعالجة بالاكتوفيرين لم تشهد أي تغير معنوي في جميع الاختبارات السابقة طوال فترة التجربة.

ومن النتائج السابقة نستخلص أن مادة الاكتوفيرين يمكن استخدامها بأمان تام وبدون آثار جانبية لتنشيط المناعة بعد تثبيطها من قبل العقاقير المثبطة للمناعة مثل السيكلوفوسفاميد.