Synergistic and chemosensitizing effects of bovine lactoferrin or muramyl dipeptide in Ehrlich solid tumor-bearing mice treated with cisplatin

Dalia S. Morsi, Mohamed L. Salem, Hany M. Ibrahim, Gamalat Y. Osman, Azza H. Mohamed and Amany E. Nofal
Welcome letter from Editor-in-Chief

Welcome to the Int J Cancer and Biomedical Research (IJCBR)!

It is with great pleasure that I write this editorial to welcome you to the IJCBR. This journal provides a platform for publication of original and reviews research articles, short communications, letter to editor, thesis abstract, conference report, and case studies. These types of publication are directed at the interface of the fields of cancer and biomedical research.

The IJCBR relies on a distinguished expert of the Advisory and Editorial Board Members from the top international league covering in depth the related topics. They timely review all manuscripts and maintain highest standards of quality and scientific methodology and ethical concepts. Meanwhile, we take all possible means to keep the time of the publication process as short as possible.

I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

Mohamed L. Salem,
Editor in Chief
Synergistic and chemosensitizing effects of bovine lactoferrin or muramyl dipeptide in Ehrlich solid tumor-bearing mice treated with cisplatin

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ABSTRACT

Background: Despite the effectiveness of anti-cancer chemotherapy, it is associated with serious adverse side effects and the development of drug-resistance mechanisms. Immune dysfunction is considered one of the most serious adverse effects of anti-cancer chemotherapy, which increases the susceptibility of the patients to infection. Co-administration of immunomodulatory agents as adjuvant therapy with chemotherapy will result in better anti-tumor responses with fewer side effects. Aim: This study was designed to evaluate the ameliorative effects of bovine lactoferrin (bLF) and muramyl dipeptide (MDP) against toxicity induced by cisplatin in tumor-bearing mice. Materials and Methods: In this study, MDP or bLF was co-treated with cisplatin in mice bearing Ehrlich solid tumor (EST). Results: Co-treatment of cisplatin with MDP or bLF enhanced the anti-tumor effects of cisplatin to induce a reduction of the tumor size, proliferative capabilities of tumor cells accompanied by an elevation in the apoptotic profile of tumor cells. Moreover, co-treatments of Cisplatin with bLF or MDP reversed the Cisplatin-induced immune suppression and partially restored splenocyte proliferation, immune organ indices, hematological profile, liver and kidney functions, and histological structure. Conclusion: Both bLF and MDP were able to act as adjuvant therapy with anti-cancer chemotherapy through their abilities to enhance the chemotherapy curative effects, modulate the immune response against tumor cells, and to some extent ameliorate the adverse toxic effects of the chemotherapy. Keywords: Cisplatin, Immunohistochemistry, Immunosuppression, Lactoferrin, Muramyl dipeptide.

INTRODUCTION

Despite several analyses and speedy developments that occurred throughout the previous decades, most cancers still a worldwide killer. Even though superior medical technology, chemotherapy with its severe negative effects nonetheless the cornerstone of the traditional therapies of various cancers (Aston et al., 2017). Moreover, chemotherapy alone in lots of instances can’t achieve passable therapeutic outcomes and, in addition to countless most cancers sorts can develop drug resistance (Park et al., 2009). Cisplatin is one of the most potent chemotherapeutic agents used for the treatment of a wide range of cancers such as ovarian, cervical, and head and neck cancers (Karadeniz et al., 2011 and Dkhil et al., 2013). Cisplatin, an anticancer drug, acts on cancer cell DNA forming adducts, causing cell cycle arrest, and thus triggering apoptosis (Eastman, 1999; Florea and Büsselberg, 2011). But cisplatin cannot distinguish cancer cells from naïve cells, lack tumor specificity, (El-Sayyad et al., 2009). It exhibits many adverse side effects including immunosuppression, myelosuppression, nephrotoxicity, hepatotoxicity, ototoxicity, neurotoxicity, and cardiotoxicity (Mir et al., 2015). Subsequently, the necessity to use adjuvant therapies to promote immune responses and attenuate the
chemotherapy toxicities has become fundamental to defeat these obstacles.

Immunomodulators are substances that elicit the immune functions to promote disease-fighting (Ibrahim et al., 2018; Abdel Ghaffar et al., 2019). The best immunomodulators come from natural sources like plants, animals, fungi, and bacteria (Mukherjee et al., 2014). Among these immunomodulators, bovine lactoferrin (bLF), is a glycosylated globular protein with a molecular weight of 78 kDa that nearly consists of 690 amino acid residues (Baker and Baker, 2005). LF is found in many mammalian animals like camels and goats but it is more prominent in bovine milk as well as in humans (Baker and Baker, 2005). It is found in human secretions such as breast milk (especially in the colostrum), seminal fluid, uterine secretions, tears, and saliva and synthesized by different cell populations, including neutrophils, macrophages, and glandular epithelial cells, and it is mainly secreted in response to inflammatory processes (Baker and Baker, 2005; Legrand et al., 2008; Actor et al., 2009; González-Chávez et al., 2009). bLF is also known for its anti-bacterial, anti-fungal, anti-viral, anti-oxidant, anti-inflammatory, anti-parasitic, anti-allergic, and most importantly anti-neoplastic properties (Yamauchi et al., 2006; Parhi et al., 2012, Ibrahim et al., 2020; Morsi et al., 2020). bLF exerts an immunomodulatory function on antigen-presenting cells by enhancing their activation, maturation, and migration to injured areas (Legrand et al., 2008; Actor et al., 2009; González-Chávez et al., 2009; Puddu et al., 2009; Ibrahim et al., 2019).

Muramyl dipeptide (MDP) is peptidoglycan found in the bacterial cell wall as a thin layer in a gram-negative and thick layer in gram-positive bacteria (Vollmer et al., 2008). MDP is recognized by nucleotide-binding oligomerization (NOD) domain-2, which is found in several kinds of leukocytes, mainly monocytes and macrophages. MDP signaling via NOD2 activates the leucocytes, leading to increased production of tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, IL-12, and intercellular adhesion molecule (ICAM)-1, which in order activate leucocytes to attack tumor cells (Inohara et al., 2003 and Thundimadathil, 2012).

Souvannavong et al. (1990) reported that MDP has been shown to promote the effect of other immunostimulants like interferon (IFN)-γ and to synergize with cytokines to enhance lymphocytes differentiation and proliferation.

In this study, the Ehrlich tumor-bearing mouse model was used to investigate the ameliorative effects of either bLF or MDP against cisplatin-induced toxicity.

MATERIALS AND METHODS

Animals

Adult female Swiss CD1 albino mice (6-8 weeks, 28±2 g) were obtained from the National Research Centre "NRC", Giza, Egypt. Mice were housed in polypropylene cages under controlled conditions (temperature 25±2ºC and 12 hours dark/light cycle). This work was performed after the approval of the Institutional Animal Ethical Committee, Menoufia University (approval ID: MUF5/F/IM/1/16).

Cell line

Ehrlich ascites carcinoma cells (EACs) were obtained from the National Cancer Institute, Cairo University, Egypt. EACs were maintained in female mice, and then the cells were collected using a sterile syringe and diluted with normal saline. Then the cells were counted and their viability was examined using trypan blue dye exclusion method before transportation to naïve female CD1 mice for experimentation.

Chemicals

Cisplatin (Cis), bLF, and MDP were purchased from Sigma (Sigma-Aldrich company, CA, USA), and reconstituted in PBS in stock solutions and stored at -80 ºC until use.

Experimental Design

Forty-five female albino mice were divided into five groups (n=9/group). All animals were weighed at the beginning and the end of the experiment; the first group was given normal saline, serving as healthy control. Then, the remaining mice were challenged with intramuscular (i.m.) injection in the right thigh of the hind limb with 2.5×10^6 EACs on day 0 (Noaman et al, 2008) to form Ehrlich solid tumor (EST). The mice were then treated after 10 days as follows: group (II): given normal saline,
Serving as EST control. Group (III): injected intraperitoneally “i.p.” with cisplatin (2mg/kg) (Salem et al., 2016) three doses at days 11, 13, and 15. Group (IV): treated with cisplatin as a group (III) and then orally administrated bLF (100mg/kg) (Iigo et al., 1999) for successive ten days. Group (V): treated with cisplatin as a group (III) and then injected subcutaneously “s.c.” with MDP (0.5mg/kg) three times each other day from day 11 (Bloksma et al., 1984) (Figure 1).

Tumor size assessment

After thirteen days of tumor inoculation, dimensions of the right thigh of the lower limb were measured using two-end electronic digital caliber (Switzerland), each other day till the 21st day. Tumor size was then calculated according to Goto et al. (2000) using the formula: Tumor volume (mm$^3$) = Length × (width)$^2$/2

Sampling and cell preparation

On day 21, blood samples were collected from the orbital sinus. Each blood sample was divided into two tubes, one of them was mixed with EDTA anticoagulant and another was allowed to clot. Serum samples were separated by centrifugation at 4000 rpm for 20 minutes and stored at -80 °C until use (Alekseeva et al., 2017). The mice were then sacrificed by cervical dislocation to obtain spleens as well as liver, kidney, and right thigh muscles to be prepared for subsequent analysis.

Splenocytes single-cell suspension was prepared after RBCs lysing using ACK lysis buffer according to Ibrahim et al. (2010). Splenocytes count was determined using Trypan blue dye exclusion method. Spleen and thymus indices were assessed according to Zhao et al. (2009) using the following formula:

Organ index = organ weight (g)/ body weight (g).

Histopathological examination

Liver, kidney, and right thigh muscle tissue samples with or without tumor masses were carefully removed from all groups of the experiment and rinsed in an isotonic solution, then fixed in 10% neutral formalin. After 24h., tissue samples were dehydrated through ascending concentrations of ethanol and then embedded in paraplast paraffin wax. Paraffin blocks were then cut into 5µm sections and stained with haematoxylin and eosin for light microscope investigations (Suvarna et al., 2018). The sections were viewed on a light microscope (Olympus BX 41, Japan) and photographed using an Olympus digital camera.

Determination of tumor cell proliferation and apoptosis using immunohistochemistry

Tumor cell proliferation and apoptosis were determined by immunohistochemical staining of proliferating cell nuclear antigen (PCNA), anti-apoptotic protein Bcl-2, and tumor suppressor protein p53. Paraffin sections of skeletal muscles were stained using the avidin-biotin-peroxidase method, then counterstained by Mayer’s hematoxylin, cleared in xylene, and mounted in DPX, the immunohistochemical staining of each PCNA, Bcl-2, and p53 was carried out according to Arriazu et al., (2006). Slides were then examined and photographed by a light microscope (Olympus BX 41, Japan).

Figure 1. The applied treatment schedule
The represented photos were photographed using an Olympus digital camera. To quantitively the IHC, 1×10^3 cells from at least five separate tissue sections were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

**Splenocytes immunophenotyping by flow cytometry**

Splenocytes were stained for 30 minutes at 4°C in the dark through two panels with anti-mouse mAbs against CD4 (APC labeled, clone GK1.5), CD8 (PerCP, clone 53-6.7), CD3 (FITC labeled, clone 17A2), CD69 (PE.Cy7 labeled, clone H1.2F3), CD11b (APC labeled, clone M1/70) and Ly6G (FITC labeled, clone RB6-8C5). Then, splenocytes were washed with PBS twice and resuspended to 1×10^6 cells/50µl flow cytometry staining buffer (0.5% bovine serum albumen-PBS, 0.02% sodium azide in PBS). Surface marker expression was measured with BD FACS Canto II Flow Cytometry using BD FACS DIVA™ software (Abdel Salam et al., 2017).

**Splenocytes proliferation assessment**

Splenocytes proliferative responses to mitogen Con-A were determined according to Ibrahim et al., 2013. Spleen cells were washed twice in PBS. 5×10^7 cells were re-suspended in PBS and stained with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Bio legend, San Diego, California, USA) for eight minutes at 37°C. Then 5 volumes of ice-cold RPMI 1640/10% FBS was used to stop the reaction. Cells were washed 3 times in PBS. The CFSE-labeled cells were in vitro cultured in six-well plates with or without the Con-A, for 3 days. Data were analyzed using a BD FACS Canto II flow cytometer and Flow Jo software (BD Biosciences). To estimate the Con-A induced spleen cells proliferation, the percentage of un-stimulated spleen cells in the absence of the ConA was subtracted from the percentage of ConA-stimulated cells.

**Biochemical and Hematological Analysis**

The serum concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine were examined using the available kits (Human, Max-Plank, Wiesbaden, Germany) according to the manufacturer instructions. Hematological measurements (red blood cells "RBCs" count, hemoglobin concentration, hematocrit value, mean corpuscular volume "MCV", mean corpuscular hemoglobin "MCH", mean corpuscular hemoglobin concentration "MCHC", white blood cell "WBCs" count, platelet count) were performed manually using blood samples mixed with EDTA previously mentioned according to (Dacie and Lewis, 1984).

**Statistical analysis**

The results of different groups were expressed as mean ± SD. The differences between groups were evaluated using a statistical package of social science (SPSS) software for Windows, version 22. One way analysis of variance (ANOVA) followed by least significant difference (LSD) for post hoc analysis was used for multiple comparisons. Statistical significance was considered when P<0.05.

**RESULTS**

**Effect of co-treatment on body weight and immune organ indices**

Mono-treatment with cisplatin significantly (P<0.05) decreased bodyweight, spleen, and thymus indices compared to control mice (Table 1). Co-treatments of cisplatin with bLF or MDP significantly increased both body weight and spleen index compared to the cisplatin-treated group. Cisplatin co-treatment with bLF or MDP increased thymus index without any significant difference compared to the cisplatin-treated group.

**Tumor Size**

Cisplatin induced a significant (P<0.05) reduction in tumor size compared to control mice. Co-treatments of cisplatin with bLF or MDP significantly (P<0.05) decrease the tumor size compared to control mice, without any significant differences when compared to cisplatin-treated animals (Figure 2).

**Histopathological observations**

**Liver:** Sections of naïve mice liver revealed that the polyhedral round hepatocytes with eosinophilic cytoplasm and round basophilic nucleus radially arranged in hepatic strands which are separated by irregular blood sinusoids. Examination of the liver of control mice displayed apparent signs of degenerative changes, such as cytoplasmic vaculation of the
Synergistic and chemosensitizing effects of bovine lactoferrin or muramyl dipeptide in Ehrlich solid tumor-bearing mice.

Table 1. Effect of Cisplatin co-treatment with bLF or MDP on body weight, spleen, and thymus indices in mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bodyweight (g)</th>
<th>Spleen Index (%)</th>
<th>Thymus Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>32.4±1.14</td>
<td>4.68 ±0.23</td>
<td>1.21±0.22</td>
</tr>
<tr>
<td>Control</td>
<td>30.8±0.83</td>
<td>6.04±0.38</td>
<td>3.15±0.36</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>18.9±1.3*</td>
<td>3.52±0.37</td>
<td>0.9±0.03*</td>
</tr>
<tr>
<td>Cisplatin + bLF</td>
<td>21.9±0.68*</td>
<td>5.56±0.35*</td>
<td>1.55±0.15*</td>
</tr>
<tr>
<td>Cisplatin + MDP</td>
<td>30.2±0.83*</td>
<td>5.31±0.41*</td>
<td>1.15±0.11*</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD, n = 5. *P < 0.05 indicates a significant difference compared to the Control group. *P<0.05 indicates a significant difference compared to Cisplatin-treated mice. bLF: bovine lactoferrin; MDP: muramyl dipeptide.

**Figure 2.** The effect of Cisplatin co-treatment with bLF or MDP on tumor size. Data were expressed as the mean ± SD, n=5. Statistical difference was calculated with an ANOVA and follow-up test (LSD). *P < 0.05 indicated a significant difference compared to the control group. *P < 0.05 indicated a significant difference compared to Cisplatin-treated mice.

hepatocytes with apoptotic nuclei and infiltration of a variable cell population of polygonal-shaped tumor cells with hyperchromatic nuclei beside an enlarged branch of the portal vein and bile ductule. Liver sections of EST-bearing mice treated with cisplatin were revealed marked cytoplasmic vacuolation of hepatocytes, number of binucleated cells, and congested portal veins, while co-treatments with bLF or MDP exhibited partially an improvement in liver structure (Figure 3).

**Kidney:** kidneys of naïve mice showed the normal structure of renal glomeruli with intact Bowman’s capsule and renal tubules which are lined with epithelial cells. Control mice exhibited degeneration, shrinkage, atrophy, and infiltration of inflammatory cells into the glomeruli, degeneration of epithelial cells of some renal tubules, and inflammatory leucocytic infiltration. Kidney sections of EST-bearing mice treated with cisplatin were exerted nephrotoxic effects, as edematous spaces, enlarged degenerated glomeruli with infiltrated inflammatory cells, and wider lumen of renal tubules with exfoliated degenerated epithelium. Fortunately, cisplatin co-treatment with bLF or MDP ameliorated the nephrotoxicity induced by cisplatin as they showed a nearly normal structure with several infiltrated inflammatory cells inside the glomeruli and between the renal tubules (Figure 4).

**Skeletal muscle:** Histological examination of longitudinal sections of thigh muscles obtained from naïve mice showed the normal structure of muscle fibers arranged into bundles and separated by perimysium connective tissue. Each muscle fiber is polygonal with peripheral pale oval nuclei and surrounded by endomysium connective tissue. Longitudinal sections of thigh muscle of control mice showed that the remaining skeletal muscles were completely replaced and infiltrated by polygonal-shaped tumor cells with hyperchromatic nuclei and abundant eosinophilic cytoplasm, edema, irregular muscle fiber structure, pale staining degenerated myocytes with homogenized appearance without striations and wide gaps between them. The skeletal muscles of EST-bearing mice treated with cisplatin showed marked disruption and irregular fibers structure with nuclear clumps, edema, and most myofibers were replaced by fibro-adipose tissue as large fat vacuoles (macrosteatosis) or very small fat droplets (microsteatosis). The striations in the edematous area were partially disappeared and the myocytes had mild swellings with eosinophilically stained cytoplasm. Fortunately, cisplatin co-treatment with bLF or MDP showed an improvement in muscular strands with slight abnormalities (Figure 5).
Tumor cells proliferation and apoptosis

Table 2 shows that tumor cells in control mice exhibited the highest level (19.79±0.3%) of the proliferation marker PCNA expression. The current study revealed that cisplatin mono-treatment significantly (P<0.05) suppressed PCNA expression in tumor cells (6.98 ± 0.07%). Interestingly, cisplatin co-treatment with bLF or MDP significantly (P<0.05) reduced PCNA expression 5.59±0.1% and 5.74±0.1% respectively when compared to both control mice and those mono-treated with cisplatin (Figure 6). Furthermore, it was clear that tumor cells in control animals showed immunohistochemical overexpression of the anti-apoptotic Bcl-2 protein (19.45 ± 0.25%). Cisplatin mono-treatment and co-treatments with bLF or MDP significantly (P<0.05) succeeded to suppress the anti-apoptotic Bcl-2 protein expression 3.0±0.05%, 2.07±0.48%, and 2.3±0.2% respectively, compared to control mice and cisplatin-treated mice (Figure 7). Moreover, cisplatin mono-treatment significantly (P<0.05) elevated the pro-apoptotic p53 expression level (18.73 ± 0.25%) compared to control mice, also, cisplatin co-treatment with bLF or MDP significantly (P<0.05) increased the pro-apoptotic p53 expression 18.7±0.4% and 20.95±0.05%, respectively compared to both control mice and those mono-treated with cisplatin (Figure 8).

Effect of co-treatment on splenocytes proliferation

Cisplatin mono-treatment significantly (P<0.05) decreased the rate of splenocytes proliferation compared to control mice. Co-treatment with bLF or MDP significantly (P < 0.05) restored splenocytes proliferation rate compared to control and cisplatin-treated mice (Figure 9).

Effect of co-treatment on splenocytes immunophenotypic analysis

Cisplatin mono-treatment induced non-significant changes in the percentages of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD3⁺CD8⁺CD69⁺ compared to control mouse. On the other hand, it resulted in a significant (P<0.05) decrease in the percentages of CD3⁺CD4⁺CD69⁺ compared to control mice. Co-treatments of cisplatin with either bLF or MDP significantly (P<0.05) and partially improved the percentages of CD3⁺CD4⁺, CD3⁺CD4⁺CD69⁺, and CD3⁺CD8⁺CD69⁺ compared to control mice and cisplatin-treated mice. Moreover, those co-treatments minimally increased the percentage of CD3⁺CD8⁺ cells without any significant difference compared to control mice and those treated with cisplatin. Furthermore, co-treatments of cisplatin with bLF significantly (P<0.05) alleviated the percentage of CD11b⁺Ly6G⁺ spleen cells compared to control mice. Cisplatin co-treatment with MDP showed significant (P<0.05) improvement in CD11b⁺Ly6G⁺ spleen cells percentage when compared to cisplatin-treated mice. Ultimately, cisplatin co-treatment with bLF or MDP succeeded to partially restore the percentages of investigated immune cells when compared to cisplatin mono-treatment (Table 3).

Biochemical and Hematological changes

Tumor development was correlated with alterations in liver and kidney functions as approved by a marked rise in serum AST, ALT, urea, and creatinine concentrations (Table 4). Cisplatin mono-treatment induced significant (P<0.05) elevation in serum ALT, AST, urea, and creatinine levels as compared to control mice.

Co-treatments of cisplatin with bLF or MDP caused a significant (P < 0.05) decrease in the AST, ALT, urea, and creatinine as compared to control and cisplatin-treated animals. Fortunately, cisplatin co-treatment with bLF or MDP tend to attain significant (P<0.05) amelioration in serum urea and creatinine levels toward normal values as compared to the cisplatin-treated group.

Cisplatin mono-treatments significantly (P<0.05) reduced total RBCs, WBCs, platelets count, relative lymphocytes, hemoglobin concentration, and packed cell volume when compared with control mice. On the other side, cisplatin mono-treatment induced a significant (P<0.05) increase in relative granulocytes as compared to control mice (Table 5).

Cisplatin co-treatment with bLF resulted in significant (P<0.05) improvement in RBCs, WBCs, platelets count, relative lymphocytes, relative granulocytes, and hemoglobin concentration when compared to control mice.
Synergistic and chemosensitizing effects of bovine lactoferrin or muramyl dipeptide in Ehrlich solid tumor-bearing mice.

Table 2. Effect of Cisplatin co-treatment with bLF or MDP on PCNA, Bcl-2, and p53 immunohistochemical expression in tumor mass of different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>PCNA %</th>
<th>Bcl-2 %</th>
<th>PS3 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.79 ± 0.3</td>
<td>19.45 ± 0.25</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>6.98 ± 0.07</td>
<td>3.0 ± 0.05</td>
<td>18.73 ± 0.25</td>
</tr>
<tr>
<td>Cisplatin + bLF</td>
<td>5.59 ± 0.1</td>
<td>2.07 ± 0.48</td>
<td>18.7 ± 0.4</td>
</tr>
<tr>
<td>Cisplatin + MDP</td>
<td>5.74 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>20.95 ± 0.05</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD, n = 4. *P < 0.05 indicates a significant difference compared to the control group.

Table 3. Effect of Cisplatin co-treatment with bLF or MDP on splenocytes immuno-phenotyping in EST-bearing mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD3+CD4+ (%)</th>
<th>CD3+CD8+ (%)</th>
<th>CD3+CD8+CD69+ (%)</th>
<th>CD3+CD4+CD69+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>37.2 ± 0.56</td>
<td>14.6 ± 2.4</td>
<td>3.7 ± 1.24</td>
<td>0.077 ± 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>16.5 ± 2.1</td>
<td>8.32 ± 1.2</td>
<td>33.07 ± 2.8</td>
<td>16.3 ± 2.4</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>13.8 ± 2.3</td>
<td>7.4 ± 0.08</td>
<td>31.2 ± 4.4</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>Cisplatin + bLF</td>
<td>17.85 ± 2.4</td>
<td>8.42 ± 1.8</td>
<td>24 ± 3.11</td>
<td>2.0 ± 0.48</td>
</tr>
<tr>
<td>Cisplatin + MDP</td>
<td>17.15 ± 1.0</td>
<td>10.1 ± 2.9</td>
<td>4.4 ± 0.61</td>
<td>0.18 ± 0.06</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD, n = 5. *P < 0.05 indicates a significant difference compared to Cisplatin-treated mice. bLF: bovine lactoferrin; MDP: muramyl dipeptide.

Table 4. Biochemical changes of EST-bearing mice co-treated with Cisplatin and bLF or MDP.

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>Urea (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>63.28±8.3</td>
<td>31.3±0.81</td>
<td>42.6±3.72</td>
<td>0.82±0.26</td>
</tr>
<tr>
<td>Control</td>
<td>214.54±20.13</td>
<td>121.78±9.47</td>
<td>45.68±2.4</td>
<td>0.91±0.1</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>434.82±72.28</td>
<td>145.78±12.9</td>
<td>60.4±11.3</td>
<td>2.47±0.42</td>
</tr>
<tr>
<td>Cisplatin + bLF</td>
<td>380.8±20.9</td>
<td>183.3±11.78</td>
<td>37.2±4.59</td>
<td>1.38±0.33</td>
</tr>
<tr>
<td>Cisplatin + MDP</td>
<td>380.84±24.7</td>
<td>185.7±4.64</td>
<td>42.24±3.69</td>
<td>1.50±0.58</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD, n = 5. *P < 0.05 indicates a significant difference compared to Cisplatin-treated mice. bLF: bovine lactoferrin; MDP: muramyl dipeptide.

Table 5. Hematological changes of EST-bearing mice co-treated with cisplatin and bovine lactoferrin or muramyl dipeptide.

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Cisplatin</th>
<th>Cisplatin + bLF</th>
<th>Cisplatin + MDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^6/mm3)</td>
<td>4.58 ± 0.52</td>
<td>4.12 ± 0.25</td>
<td>3.38 ± 0.34*</td>
<td>4.1 ± 0.33*</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>12.38 ± 1.6</td>
<td>11.5 ± 0.8</td>
<td>9.32 ± 0.97</td>
<td>11.5 ± 0.93</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>38.6 ± 4.5</td>
<td>34.4 ± 2.4</td>
<td>28.6 ± 2.8*</td>
<td>35 ± 2.9*</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>31.24 ± 1.7</td>
<td>29.48 ± 0.91</td>
<td>30.7 ± 1.7</td>
<td>30.2 ± 0.26</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>27.01 ± 1.54</td>
<td>27.9 ± 1.66</td>
<td>27.61 ± 0.73</td>
<td>28.1 ± 0.77</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>32.04 ± 1.7</td>
<td>33.42 ± 0.37</td>
<td>32.6 ± 1.7</td>
<td>33 ± 0.0</td>
</tr>
<tr>
<td>Platelets (10^12/mm3)</td>
<td>395 ± 29.7</td>
<td>474 ± 56.05</td>
<td>297 ± 53.3*</td>
<td>361 ± 58.1*</td>
</tr>
<tr>
<td>WBCs (10^3/mm3)</td>
<td>7.06 ± 3.1</td>
<td>8.06 ± 3.0</td>
<td>4.65 ± 0.97*</td>
<td>10.8 ± 2.01*</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>35.3 ± 4.1</td>
<td>57 ± 2.6</td>
<td>75.6 ± 1.14*</td>
<td>52.2 ± 7.29*</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>60 ± 4.0</td>
<td>39 ± 1</td>
<td>19 ± 2.2*</td>
<td>42 ± 6.63*</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>5.3 ± 1.17</td>
<td>4 ± 0.1</td>
<td>5.4 ± 1.6</td>
<td>4.8 ± 1.7</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD, n = 5. *P < 0.05 indicates a significant difference compared to the control group. #P < 0.05 indicates a significant difference compared to Cisplatin-treated mice. bLF: bovine lactoferrin; MDP: muramyl dipeptide; RBCs: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBCs: white blood cells.
Figure 3. Light micrograph of liver sections Naïve mice showing normal hepatic structure; hepatocytes (H), central vein (CV), blood sinusoids (S), control mice showing infiltration of leukocytes mixed with tumor cells (thick arrows) around the enlarged branch of the portal vein (PV) and bile ductule (BD), cytoplasmic vaculation of hepatocytes (thin arrow); mice treated with cisplatin exhibiting marked cytoplasmic vacuolization of hepatocytes with several binucleated cells (arrows), and congestion of portal vein with eroded lining (PV); co- treatments (Cisplatin + bLF) and (Cisplatin + MDP), both showing an improvement in liver structure, (H&E, ×400).
Synergistic and chemosensitizing effects of bovine lactoferrin or muramyl dipeptide in Ehrlich solid tumor-bearing mice..

Figure 4. Light micrograph of kidney sections Naïve mice showing the basic normal structure; Bowman’s capsule with glomerulus (G) and renal tubules (RT); control mice showing degeneration of glomeruli (G) and epithelial cells of some renal tubules (D), and leucocytic infiltration (Li); mice treated with Cisplatin exhibiting edematous spaces (ES), enlarged fragmented glomeruli (G) and cytoplasmic vacuolation of the damaged renal tubule with pyknotic nuclei (arrows); co- treatments (Cisplatin + bLF) and (Cisplatin + MDP) both showing a nearly normal structure with several infiltrated inflammatory cells inside the glomeruli and between the renal tubules, (H&E, ×400).
Figure 5. Longitudinal sections in the skeletal muscle Naïve mice with the normal architecture of muscle fibers, peripheral nuclei (arrow) situated in the side of the myofiber (MF) by narrow intercellular endomysium (curved arrow); sections of control mice variable cell population consisting of polygonal-shaped tumor cells with hyperchromatic nuclei (TC), and irregular muscle fiber structure with pale staining degenerated myocytes (arrows); mice treated with Cisplatin exhibiting marked tumor area (TC) with irregular muscle fiber structure (arrow) with nuclear clumps (NC), and fatty vacuoles (FV); the co-treatment (Cisplatin + bLF) showing the reappearance of normal muscle structure with the remaining of tumor cells (TC); the co-treatment (Cisplatin + MDP) showing an improvement in muscle structure with slight leucocytic infiltration (Li), dilation of blood vessels (arrow), and few necrotic myocytes (N), (H&E, ×400).
Synergistic and chemosensitizing effects of bovine lactoferrin or muramyl dipeptide in Ehrlich solid tumor-bearing mice.

Figure 6. Representative light micrograph of the proliferation marker PCNA expression in tumor mass in EST-bearing mice of different groups” The brown stain referred to the positive reaction, the blue stain is hematoxylin counterstain (×400).

Figure 7. Representative light micrograph of the anti-apoptotic protein Bcl-2 expression in tumor mass in EST-bearing mice of different groups” The brown stain referred to the positive reaction, the blue stain is hematoxylin counterstain (×400).
Figure 8. Representative light micrograph of the pro-apoptotic protein p53 expression in tumor cells in EST-bearing mice of different groups. The brown stain referred to the positive reaction, the blue stain is hematoxylin counterstain (×400).

Figure 9. The effect of Cisplatin co-treatment with bLF or MDP on splenocytes proliferation rate. Data were expressed as the mean ± SD, n=5. Statistical difference was calculated with an ANOVA and follow-up test (LSD). *P < 0.05 indicated a significant difference compared to the control group. #P < 0.05 indicated a significant difference compared to Cisplatin-treated mice.
and those mono-treated with cisplatin, while an induced significant decrease in the hematocrit value compared to control mice. On the other hand, cisplatin co-treatment with MDP significantly (P < 0.05) increased RBCs, WBCs, platelets count, relative lymphocytes, hemoglobin concentration compared to cisplatin-treated mice, while caused a significant decrease (P<0.05) in the relative granulocytes compared to cisplatin-treated animals. Fortunately, cisplatin co-treatment with bLF or MDP partially succeeded to restore many hematological values compared to cisplatin mono-treatment.

**DISCUSSION**

The current study showed that co-treatments of cisplatin with bLF or MDP can be potent anti-tumor agents. These results are in the same line with Li et al. (2017) LF can inhibit the growth of colon cancer cell line (HT29) in nude mice when administered alone in dose (200mg/kg) or combination with the chemotherapeutic agent 5-FU. Consistently, Sun et al. (2012) demonstrated that oral administration of iron saturated -LF augmented tamoxifen therapy to delay the appearance of palpable tumors in breasts of female Balb/c mice, besides inhibiting their subsequent growth. Consistently, Varadhachary et al. (2004) found that oral rhLF inhibited the growth of squamous cell carcinoma (O12) tumors in T-cell immunocompromised nu/nu mice. Previous report used oral rhLF alone and in combination with cisplatin to treat HNSCCA in a syngeneic murine model and the authors found that mono-treatment with rhLF or Cisplatin caused 61% or 66% tumor growth inhibition over placebo respectively, while co-administration showed 79% tumor growth inhibition.

MDP has been shown to successfully reduce tumor size when co-administered with cisplatin. These results are consistent with Ma et al., (2011) who demonstrated that MDP conjugate with paclitaxel (MTC-220) was efficacious in inhibiting tumor growth in xenograft models using human ovarian (A2780, ES-2), breast (MDA-MB-231, MCF-7), and lung (H460, A549, H1975) tumor cell lines. Furthermore, MDP derivatives have been shown to have tumoricidal activity via up-regulating the expression and production of macrophage cytokines as IL-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor α (Worth et al., 1999).

In this study, cisplatin-induced hepatotoxic and nephrotoxic effects represented in elevated levels of serum ALT, AST, urea, and creatinine besides histopathological alterations in liver, as the appearance of many abnormal bi-nucleated cells and marked cytoplasmic vacuolization of hepatocytes, besides histopathological aspects in the kidney as the occurrence of edema, enlarged fragmented glomeruli and renal tubule damage with cytoplasmic vacuolization. These results are inconsistent with Ma et al. (2015) and Osman et al. (2015) who reported that cisplatin induced renal toxicity by elevated blood urea nitrogen and serum creatinine levels compared to their levels in normal mice, also caused histopathological changes such as tubular necrosis, desquamation in the renal cortex, tubular congestion and swelling, loss of brush border, appearance of pyknotic nuclei and congestion of renal blood vessels. Moreover, previous studies recorded that cisplatin leads to pathological changes in the liver presented as hepatocytes necrosis, central vein congestion, bile duct proliferation, cytoplasmic vacuolization accompanied by disturbances in liver functions presented as elevation in serum levels of ALT and AST (Park et al., 2009; Gong et al., 2015; Niu et al., 2017). The obtained data showed that co-treatment of cisplatin with bLF or MDP succeeded to partially modulate serum levels of ALT, AST, urea, and creatinine, also induced partial improvement in the histological structure of both liver and kidney.

In accordance with the current results, previous studies recorded that LF improved liver functions and histological structure, reduced oxidative stress, and fibrosis in rats with liver fibrosis (Hessin et al., 2015). Moreover, the ameliorative effect of LF against nephrotoxicity induced by cisplatin was previously reported by Kimoto et al. (2013) and Hegazy et al. (2016), through modulating the levels of urea and creatinine, reducing renal tubule damage. The hepatoprotective effect of MDP was previously reported by Cursio et al. (1998) as they recorded that MDP treatment improved liver status after normothermic liver ischemia in rats by reducing...
liver injury, retrieving serum levels of ALT and AST.

The current results proved muscular destructions in the skeletal muscles of the right thigh due to intramuscular injection with EACs. These pathological alterations may be owed to the pro-inflammatory conditions induced by EACs which may, in turn, lead to a catabolic state causing such pathological alterations (Der-Torossian et al., 2013). These observations were in harmony with Areida et al., (2015) who observed that muscle fibers of thigh muscle of Ehrlich solid tumor-bearing mice were invaded by deeply stained tumor cells, with a large area of necrosis. Consistently, Aldubayan et al. (2019) reported that Ehrlich tumor cells under the light microscope were manifest as sheets of small, highly chromatophilic tumor cells with inconsistent morphology.

Deregulated proliferation and inhibition of apoptosis lie at the heart of all tumor development and they present obvious targets for therapeutic interventions in all cancers (Evan and Vosden, 2001). In the present study, tumor cells in control mice exhibited a high proliferation rate represented in elevated expression of PCNA and the anti-apoptotic protein Bcl-2 while very weak expression of the pro-apoptotic protein p53. In accordance, previous studies recorded that Ehrlich tumor cells exhibited a high proliferation rate and low apoptosis percentage (Ahmed and Ahmed, 2015; Aldubayan et al., 2019; Ghoneum et al., 2019).

Interestingly, the presented results showed that cisplatin co-treatment with bLF or MDP was more effective than cisplatin mono-treatment on tumor cell proliferation and apoptosis. As shown, cisplatin co-treatment with bLF or MDP significantly decreased proliferation rate while induced apoptosis by inhibiting anti-apoptotic Bcl-2 expression and increasing the expression of pro-apoptotic p53. In harmony with the current results, Guedes et al. (2018) reported that bLF inhibited proliferation, induced apoptosis in vitro of prostate cancer PC-3 and osteosarcoma Mg-63 cells. Consistently, bLF has been reported by Duarte et al., (2011) to induce apoptosis and inhibit proliferation in T47D and HS578D human breast cancer cell lines. Similarly, Xu et al., (2010) also reported that bLF induced apoptosis by reducing the levels of intrinsic protein Bcl-2 in stomach cancer cells. Furthermore, Gibbons et al., (2015) recorded that bLF both apo- or Fe- bLF markedly suppressed proliferation and inducing apoptosis in MDA-MB-231 and MCF-7 human breast cancer cell lines.

Moreover, MDP markedly suppressed tumor cell proliferation, whilst initiated tumor cell apoptosis by reducing levels of anti-apoptotic Bcl-2 and increasing the pro-apoptotic p53 expression. The observed anti-proliferative and apoptotic effects of MDP came following Yoon et al. (2016) who recorded that MDP can inhibit proliferation and induce apoptosis of oral squamous cell carcinoma by nucleotide oligomerization domain (NOD)-2 stimulation. Upon binding of NOD2 with its ligand, undergoes conformational changes, and various pathways could lead to nuclear factor-kappa B (NF-kB) activation which resulted in apoptosis and autophagy (Inohara et al., 2000; Yamaguchi et al., 2017). On another side, MDP is a peptidoglycan constituent of both gram-positive and gram-negative bacteria, it is composed of N-acetylmuramic acid (glycan moiety) linked by its lactic acid moiety to the dipeptide L-alanine-D-isoglutamin (Ellouz et al., 1974). The N-acetylmuramic (MurNAC) acid is a monosaccharide derivative of N-acetyl glucosamine (GlcNAC) (Bhagavan, 2002). N-acetyl muramic acid is probable to exert the apoptotic effect in the current examined tumor model. Liang et al. (2018) recorded that GlcNAC improved the effect of TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by activating death receptor (DR)-5 accumulations and clustering, which in turn recruited the apoptosis-initiating protease caspase-8 to form death-inducing signaling complex (DISC) and initiated apoptosis in human non-small cell lung cancer cells.

Tumors often establish an almost symbiotic relationship with their hosts by suppressing excessive inflammation and antitumor immune response (Goldszmid et al., 2014). In the present study, EST significantly decreased the percentage of the effector T-lymphocytes (CD3+CD4+ and CD3+CD8+), while significantly increased the expression of CD69 antigen “a
negative regulator of the anti-tumor immune response on T-lymphocytes (CD3+CD4+CD69+ and CD3+CD8+CD69+) besides increasing the percentage of CD11b+Ly6G+ myeloid derived suppressor cells (MDSCs). These data are consistent with Murdoch et al., (2008) who reported that tumor-bearing mice have a markedly elevated number of MDSCs (CD11b+Gr1+) in their peripheral blood, bone marrow, and spleen compared with non-tumorized mice. Moreover, the present results agree with Wen et al., (2018) who reported that large numbers of MDSCs accumulated in the tumor-bearing mice to be ~7-folds that of normal mice. Herein, the administered dose of the chemotherapeutic agent cisplatin "2 mg/kg, body weight" randomly decreased the percentages of the investigated effector immune cells CD3+CD8+, CD3+CD4+CD69+, CD3+CD8+CD69+ and CD11b+Ly6G+.

The presented results are consistent with Shruthi et al. (2018) who reported the immunosuppressive action of cisplatin. On the other hand, co-treatments of cisplatin with bLF or MDP relieved the immunosuppressive impact of the administered dose of cisplatin through retrieving the percentages of CD3+CD4+, CD3+CD8+, CD3+CD4+ CD69+, CD3+CD8+CD69+, and CD11b+Ly6G+ cells toward the normal values. These results are inconsistent with Artym et al. (2003a) who reported that oral administration of LF notably reconstitutes the splenocytes cellularity and enriches both CD3+ and CD4+ cells in cyclophosphamide-immunosuppressed mice. In addition, a previous study demonstrated similar effects of LF on T lymphocytes by increasing expression of the CD4 marker in Jurkat T cells (Dhennin-Duthille et al., 2000). Similarly, the immunomodulatory role of LF was reported by Tomita et al. (2009) who found that LF bound to receptors on enterocytes, dendritic cells, lymphocytes inducing the release of cytokines and increasing the number of NK, CD4+ and CD8+ cells.

In harmony with the current results, Heinzelmann et al. (2000) and Uehori et al. (2005) reported that antitumor efficacy of MDP and its derivatives may be via stimulating the immune response of mononuclear macrophage to release some cytokines and express more co-stimulatory molecules that are necessary for generation and differentiation of effector T-lymphocytes. Furthermore, a previous study revealed that MDP could induce dendritic cells maturity which in turn promoted the antitumor effects of T lymphocytes (Wang et al., 2011).

The health and immune status of the host is mostly relative to the immune organ indices (Zhao et al., 2009). In the present study, mice mono-treated with cisplatin showed a decrease in splenocytes proliferation, spleen, and thymus indices. On the other hand, mice co-treated with cisplatin and bLF or MDP restored splenocyte proliferation, spleen, and thymus indices compared to those treated with cisplatin. These results are in accordance with Shruthi et al., (2018) who recorded that both cisplatin and cyclophosphamide markedly decreased the thymus index in mice. Moreover, Artym et al. (2003b) reported that oral administration of LF could partially reconstitute the humoral immune response associated with elevation in CD3+ and CD4+ T lymphocytes and B cells and enhancement of spleen cells proliferation. Furthermore, the stimulatory effect of MDP on the proliferative capabilities of splenocytes was previously reported by Christiana et al. (2016). Likewise, Iribe and Koga (1984) reported that MDP significantly augmented the proliferative response of thymic T-lymphocytes to phytohaemagglutinin.

To estimate the ameliorative effect of certain therapy on the host, hematological and biochemical changes should be recorded during tumor therapies (Ganger and Koul, 2010). In the present study, mono-treatment with cisplatin leads to a sharp decrease in RBCs count, Hb concentration, hematocrit volume, PLT count, WBCs count, relative lymphocyte count, inducing anemia which may be attributed to its known myelosuppressive effects reported previously (Song et al., 2017), while causing an increase in relative granulocyte count which might be due to the acute inflammatory response. In accordance, Khynriam and Prasad (2001) proved that cisplatin induced hematoxic features as decreased RBCs count, Hb content, WBCs count, lymphopenia, neutropenia besides the development of erythrocyte morphological anomalies (microcytes, macrocytes, echinocytes and acanthocytes) in Dalton...
lymphoma bearing mice. On the other side, co-treatments of cisplatin with bLF or MDP mostly enhanced all hematological anomalies induced by cisplatin mono-treatment. These results are matched with Kanwar et al. (2008) who proved that bLF restored both peripheral RBCs and WBCs counts depleted by chemotherapy in preclinical studies, indicating the ability of bLF in treating anemia. Furthermore, Moastafa et al. (2014) reported that bLF could alleviate RBCs and WBCs counts, hemoglobin content in colorectal cancer patients who received bLF for three months.

Finally, in this study, Cisplatin mono- and co-treatments with bLF or MDP, fortunately, exhibited atrophy in the tumor mass accompanied by improvement in muscular strands with slight abnormalities.

CONCLUSION

Adjuvant therapy of bLF and MDP with cisplatin has potent anti-tumor properties. Co-treatment of bLF or MDP with cisplatin enhances the chemotherapy curative effects, regulate the proliferation and apoptosis of the tumor cells, modulate the immune response against the examined tumor, and to some extent ameliorate the adverse toxic effects of the cisplatin.

CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

FUINDING

There is no financial support for this study.

REFERENCES


Egyptian Association for Cancer Research (EACR)
http://eacr.tanta.edu.eg/

EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

EACR Chairman,
Prof. Mohamed Labib Salem, PhD
Professor of Immunology
Faculty of Science, Tanta University, Egypt
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