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Protective Role of Concanavalin-A and/or IL-27 Against Adverse Effects of Cyclophosphamide on Hematological and Biochemical Parameters in Tumor-Bearing Mice

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Abstract

Cyclophosphamide (CTX) is a chemotherapeutic drug used to treat mammary gland tumors. Despite its cytotoxic effect on cancer cells, it causes harm to hepatic and renal tissues, ranging from mild infiltration to necrosis and finally cytolysis, depending on the dosage and duration of treatment. It also suppresses bone marrow, resulting in leukopenia, anemia, and thrombocytopenia. This study aimed to evaluate the protective effects of concanavalin A (con-A) and IL-27 conditioned immune therapeutics on Ehrlich ascites carcinoma (EAC) tumor-bearing mice treated with CTX. To achieve this, naive splenocyte cells were activated in *vitro* for 3 days with the T cell mitogen concanavalin A (Con-A; 5 µg/mL) and/or IL-27 (10; ng/mL). After a single i/p injection of CTX (4 mg/mouse), they were adoptively transferred into (EAC) tumor-bearing mice. After tumor implantation, hematological analysis, liver function, and kidney function were evaluated. According to the findings, the CTX-treated group had significantly lower total leucocyte count, lymphocytes, neutrophils, hematocrit, and RBCs than the control group. Interestingly, Con-A + IL-27 normalized the total leucocytes' count, especially lymphocytes and neutrophils. When Con-A + IL-27 was compared to CTX, there was a significant increase in hematocrit and platelet count. AST, ALT, bilirubin, creatinine, and urea levels were not significantly different between normal and Con-A + IL-27, but CTX significantly increased AST compared to normal. According to the findings, adoptive transfer of Con-A + IL-27 conditioned splenocyte cells into lymphopenic hosts restored hematological and biochemical parameters and recovery from chemotherapy-induced lymphopenia.

Keywords:
Cyclophosphamide; Con-A, IL-27; Immunotherapy; Ehrlich Ascites Carcinoma

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1. Introduction

Mammary gland tumors are cancers that result from the tissue of the breast, most commonly the lining of the duct or the lobules that feed the duct (Rakha et al., 2010). Despite the development of traditional treatments to combat Mammary gland tumors, including chemotherapy, these treatments damage many tissues, increase resistance and lead to treatment failure. Therefore, to minimize these problems, it is important to find alternatives to cancer treatments where herbs are an important source of new bioactive compounds for the development of chemotherapeutic agents (Roy et al., 2018).

Immunotherapy is a modern approach to treating mammary gland tumors by stimulating the host's immune system to eradicate cancer cells (Qiu et al., 2021). The anti-tumor activity of some chemotherapeutic agents can be achieved by inducing immune cells such as type 1 T helpers (Th1) and cytotoxic T lymphocytes, in addition to their direct inhibitory effect on cancer cells. Type 1 (Th1) helper T cells secrete IL2, tumor necrosis factor (TNFβ), and interferon-gamma (IFNγ) that activate macrophages and are responsible for phagocyte-dependent protective responses and cell-mediated immunity (Romagnani 1996). Cyclophosphamide (CTX) is one of the alkylating agents widely used to treat cancer. Aside from its cytotoxic effects on tumor cells, it has a strong immune suppressive impact, as well as other harmful effects, including myelosuppression, hemorrhagic cystitis, alopecia, and gonadal damage (Ahmed and Hombal 1984).

CTX induces changes in liver function by regulating all liver enzymes (Davila et al., 1989). Since the liver is the most abundant source of both ALT) and AST enzymes, damage to liver cells increases both of these enzymes (Lutz and Bausbach 1992). In experimental animals, a single intraperitoneal dose of CTX (200 to 300 mg/kg) was found to cause severe damage to the kidneys, liver, and brain (El-Naggar et al., 2015; Zhai et al., 2018). Mice exposed to CTX developed several harmful consequences. It prevented weight gain, reduced spleen coefficient and leukocyte intensity, disrupted the oxidative and antioxidant balance of the liver, impaired kidney function, and disrupted liver and kidney amino acid metabolism (Akay et al., 2006; Zhang et al., 2021).

Concanavalin A (Con-A) is a plant lectin extracted from *Canavalia ensiformis* (Jack bean) seeds that bind to various glycolipid proteins and D-mannose residues of the glycolipid (Sharon 2007).

Lectins can specifically differentiate cancer cells from normal ones and therefore act as a valuable tool in cancer research (Poiroux et al., 2017). Con-A has been shown to inhibit cancer cell growth *in vitro* and *in vivo*. Con-A induced cytotoxicity by triggering an apoptotic response in human leukemic (MOLT-4 and HL-60), glioblastoma (U87), and breast cancer (MCF-7) cells with no damage to normal cells (Faheina-Martins et al., 2012; Pratt et al., 2012; Shi et al., 2014).

Interleukin-27 (IL-27) stimulates lymphocyte proliferation, increases adhesion molecules' expression, and releases

leukocytes from various inflammatory mediators such as chemotactic factors, prostaglandins, proteases, and procoagulants. It is a heterodimeric cytokine that induces inflammation (Yoshida and Hunter 2015). IL-27 has a potent stimulus on the expansion and survival of lymphocytes, and there are reports addressing the effects of IL-27 on the hematopoiesis process (Imamichi et al., 2021) and proliferation (Charlot-Rabiega et al., 2011). IL-27 contributes to the development of natural killer cells (NK) and cytotoxic T lymphocytes (CTL), exerts a major immunomodulatory effect, and has potential anti-angiogenic and anti-metastatic activity and local anti-tumor. Strengthens anti-tumor immunity by exerting its effect (Murugaiyan and Saha 2013).

This study aimed to evaluate the protective effect of Con-A and IL-27 on tumor-bearing EAC carcinoma to overcome the side effect of CTX *in vivo*, thorough investigation of the hematological parameters, liver enzymatic activities (ALT and AST), total bilirubin concentration as well as kidney function tests (urea and creatinine).

2. Materials and methods

2.1. Ethics Statement

The study was approved in response to the "NIH Guide for the Care and Use of Laboratory Animals" by the Faculty of Veterinary Medicine Ethics Committee of the Damanhour University, Egypt.

2.2. Mice

All experiments were performed on adult female BALB/C mice of 20 grams aged 12 - 18 weeks (*n*= 90). The mice were purchased from the Holding Company for Biological Products & Vaccines (VACSERA) (Cairo, Egypt). Mice were acclimatized at least two weeks before experimentation and randomly divided into six experimental groups, 15 mice each. Mice were maintained at regular light and dark cycles and provided standard food and water.

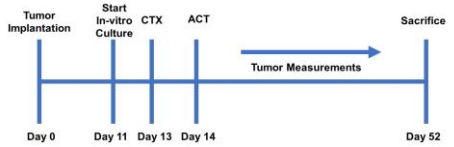
2.3. Tumor Cells

All experiments in this study were performed using spontaneous murine mammary adenocarcinoma [Ehrlich ascites carcinoma (EAC)]. This tumor grows aggressively as its ascites. The parent cell line was purchased from The National Cancer Institute, Cairo University, Egypt.

2.4. Experimental design

Mice were allocated into the following groups (*n*= 15 per group) (**Scheme 1**), including the healthy control group as follows:

1) **Group 1:** Control Group: Mice left without any treatment
2) **Group 2:** (EAC gp): Mice were injected with 2.5 $\times 10^5$ EAC cells subcutaneous (S.C.) at day zero (D0) without further treatment.
3) **Group 3:** (CTX): Mice were injected with 2.5 $\times 10^5$ EAC cells S.C. at D0 and treated with 4 mg/mouse (200 mg/kg) CTX at D13.
4) **Group 4:** (IL-27): Mice were injected with 2.5 $\times 10^5$ EAC cells S.C. at D0 and treated with 4 mg/mouse CTX at D13, and (5×10^6 cells/mouse) activated splenocytes with IL-27 at D14.
5) **Group 5:** (Con-A): Mice were injected with 2.5 $\times 10^5$ EAC cells S.C. at D0 and treated with 4 mg/mouse CTX at D13, and (5×10^6 cells/ mouse) activated splenocytes with Con-A at D14.
6) **Group 6:** (Con-A + IL-27): Mice were injected with 2.5 $\times 10^5$ EAC cells S.C. at D0 and treated with 4 mg/mouse CTX at D13 and (5×10^6 cells/mouse) activated splenocytes with Con-A+IL-27 at D14.



Scheme 1. Timeline of *in vitro* and in *vivo* experiments during this study

2.5. Cyclophosphamide (CTX)

CTX (ENDOXAN 200 mg) (C7H15Cl2N2O2P) was obtained from Baxter Oncology GmbH (D-33790 Halle, Germany), diluted to 4 mg/150 µl with PBS, and administered IP with 200 mg/kg (Zhang et al., 2021).

2.6. Cytokines

Recombinant Mouse IL-27 (Catalog # 2799-ML-010/CF) was purchased from research and diagnosis (R & D) Systems (Minneapolis, Minnesota, USA), and was used according to recommendation manufacturers.

2.7. Concanavalin A (Con-A)

It was purchased from Megazyme Ltd. (Chicago, Illinois, USA) and stored at -20°C. Con-A (Catalog # L-Con-A-1000MG), was in powder form and reconstituted in PBS to 5 µg/ml.

2.8. In vivo tumor implantation (passage)

Seven days after intraperitoneal (IP) implantation of 2.5×10^5 EAC cells, 3 mice were sacrificed by cervical dislocation, and ascetic fluid (EAC cells) was collected from the peritoneal cavity. A trypan blue dye exclusion assay determined the total number of EAC cells. The harvested cells were adjusted to 2.5×10^5 EAC cells in 150 µL for subcutaneous (S.C) injection into the normal BALB/c mice of the experimental groups (Zidan et al., 2018).

2.9. In vitro programming of BALB/c splenocytes cells

Splenocytes cells harvested from the BALB/C spleen of the control group were used for *in vitro* culture. Harvested BALB/c splenocytes cells were adjusted to 2×10^6 cells/mL and activated with Concanavalin A (Con-A) in the presence or absence of IL-27. Three days after activation, cells were harvested for the adoptive cell transfer (ACT) (Díaz-Montero et al., 2013).

2.10. Chemotherapy of recipient mice

One day before the adoptive cell transfer (ACT), BALB/c mice bearing 13-day-old EAC tumors were conditioned by a single I.P. injection with 4 mg/mouse (200 mg/kg) CTX (Diaz-Montero et al., 2013; Pelaez et al., 2001; Salem et al., 2007).

2.11. Adoptive cell transfer (ACT) by intravenous route

The harvested cells that had been primed were adjusted to $5x10^6$ cells and administered 24 hours after the conditioning of recipient mice by intravenous injection.

2.12. Blood sampling

Blood samples were collected from the submandibular vein on day 52 for hematological and biochemical analysis. Whole blood with EDITA for CBC analysis and serum without anticoagulant for analyses of liver and kidney function were collected. The collected blood was centrifuged for 2000 RPM to separate the serum and then stored at -20 ºc

2.13. Evaluation of Hematological Parameters

An automated hematology analyzer analyzed blood samples with anticoagulant EDTA were analyzed for hematological parameters by using an automated hematology analyzer.

2.14. Serum Biochemical Analysis

The activities of alanine aminotransferase (ALT) (Catalog # AL 10 31 (45)), aspartate aminotransferase (AST) (Catalog # AS 10 61 (45)), and total bilirubin (Catalog # BR 1111) kits were purchased from Biodiagnostic (Dokki, Giza, Egypt), according to the manufacturers' guidelines (Mullon and Langer 1987; Reitman and Frankel 1957)

The levels of urea (Catalog # UR 21 10) and creatinine (Catalog # CR 12 50) in the serum were estimated using commercial kits purchased from Biodiagnostic (29 Tahreer St., Dokki, Giza, Egypt). They were used according to manufacturers' guidelines (Fawcett and Scott 1960; Schirmeister et al., 1964).

2.15. Statistical analysis

Statistical analyses were performed using the one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (Tukey HSD) as a post hoc test. Using GraphPad Prism (GraphPad Software, Inc. San Diego, CA, USA) was used to graph and analyze the survival data. Every experiment was repeated three independent times under the same conditions, and all P values were two-sided, with $p < 0.05$ considered significant (*) **(**Overholser and Sowinski 2008**)**.

3. Results

3.1. Alterations in the tested hematological parameters

EAC carcinoma cells implantation resulted in a sharp decline in the total leukocyte count in circulation. In the treated CTX group, the reduction of leukocytes persisted and there was no significant change from the untreated group. Both Con-A activated splenocytes with CTX and IL-27 activated splenocytes with CTX treated groups induced a slight but significant upregulation of leukocyte count compared to treatment with CTX alone. Interestingly, Con-A + IL-27 combination with CTX resumed leukocytes level elevation, and there was no significant variation compared with the healthy group (as shown in **Figure 1a**).

Regarding leukocytic subpopulations, Neutrophils exhibited a sharp decline among untreated, CTX, Con-A, and IL-27 treated groups compared with the control group. However, Con-A + IL-27 showed a slight decrease in neutrophils compared with the control group and showed a significant increase compared with CTX treated mice, as shown in **Figure 1b**. Furthermore, there was a significant decrease in lymphocyte count in untreated, CTX, Con-A treated mice as compared with the normal control group, while there was no significant difference between Con-A + IL-27 and IL-27 with the normal group (**Figure 1c**). In addition, there was no significant difference in monocytes and basophils between all treated groups and the normal healthy one. Notably, monocytes were nearly similar in the IL-27 treated group to the control group and slightly higher in the Con-A + IL-27 group than in the normal control. There was no significant difference in eosinophils between normal, CTX, Con-A, and Con-A+IL-27, but a significant decrease in untreated IL-27 compared with normal (**Figure 1d, 1e, and 1f**). The tested parameters for an erythrocytic picture in all treated groups showed slight changes compared with the normal healthy group. Notably, RBCs, hemoglobin, and hematocrit values in the Con-A + IL-27 treated group (Means ± SD, 4.88 ± 0.27, 11.28 ± 0.33, and 34.71 ± 0.63) were slightly higher than in the CTX group (Means ± SD, 3.36 ± 0.5, 10.73 ± 0.24 and 32.34 ± 0.68) and were close to normal values in the healthy group (Means ± SD, 4.54 ± 0.48, 11.37 ± 0.76, and 35.16 ± 2.01), respectively (**Figure 2a, b, and c**). Treatment of mice with CTX alone resulted in a non-significant reduction in platelet count. Compared with the untreated, CTX, Con-A group, the Con-A + IL-27 group significantly increased platelet count (**Figure 2d**).

3.2. Alterations in Liver function analyzed parameters

When AST activities were compared to the normal group (Means ± SD, 26.8 ± 0.96) there was no significant difference between normal and Con-A + IL-27(Means ± SD, 30.25 ± 1.89). Still, there was a significant increase in AST activities in the untreated CTX, Con-A, and IL-27 (52.5 ± 3.87, 42.75 ± 1.71,39.82 ± 0.99, and 34.75 ± 0.96), respectively as shown in **Figure 3a**.

Investigation of ALT revealed that the tumor (untreated) group had a significantly higher ALT activities than the normal group (48.50 ± 18.34 and 25.25 ± 4.65), correspondingly. Furthermore, there was no significant difference between normal and CTX, Con-A, IL-27, and Con-A + IL-27, as shown by (Means ± SD, 25.25 ± 4.65, 45.75 ± 0.96, 42.14 ± 13.03, 38.25 ± 4.35, 36.25 ± 7.81), respectively (**Figure 3b**).

Bilirubin (mg/dL) analysis revealed a substantial increase in the IL-27 group compared to the control group (Mean ± SD 1.03 ± 0.28, 0.46 ± 0.096) respectively. While there was no statistically significant difference between normal and untreated mice, CTX, Con-A, and Con-A + IL-27 mice demonstrated ((Means ± SD, 0.46 ± 0.1, 0.68 ± 0.36, 0.7 ± 0.14, 0.64 ± 0.31, and 0.73 ± 0.22) respectively, as shown in **Figure 3c**.

3.3. Alterations in kidney function analyzed parameters

As demonstrated in **Figures 3d and 3e**, creatinine and urea investigations revealed a slight elevation in all treated groups compared to the healthy control, but it was insignificant. Indeed, there was no significant difference in creatinine level (mg/dL) between normal and untreated, CTX, Con-A, IL-27, and Con-A + IL-27, which indicated (Means ± SD, 0.79 ± 0.09, 1.05 ± 0.51, 0.88 ± 0.09, 0.82 ± 0.1, 1.08 ± 0.13, and 0.83 ± 0.13), respectively. There was no significant difference in urea level (mg/dL) between normal and untreated, CTX, Con-A, IL-27, and Con-A + IL-27, which indicated (Means ± SD, 25 ± 9.42, 36.25 ± 12.76, 34.5 ± 3.32, 33.95 ± 3.49, 40.25 ± 8.92, and 32 ± 6.78) correspondingly.

4. Discussion

Cyclophosphamide is widely used to treat a variety of malignant and nonmalignant disorders. Although it has some tumor selectivity, it also has a wide range of side effects, including immune suppression, liver disease, and urinary problems. Many previous studies have shown that a single dose of the alkylating agent cyclophosphamide can be harmful (Akay et al., 2006; El-Naggar et al., 2015; Lavin and Koss 1971; Zhai et al., 2018; Zhang et al., 2021). The goal of the study was to see how con-A and IL-27 affected tumor-bearing EAC carcinoma mice' hematological analysis, liver enzymatic activities ((ALT and AST), and total bilirubin concentration), and kidney function tests (urea and creatinine) after chemotherapy (CTX).

According to our findings, the mice given CTX had a significant decrease in total leukocytes, primarily neutrophils and lymphocytes, but no significant decrease in monocytes, eosinophils, or basophils. It is well known that intraperitoneally administered cyclophosphamide kills hematopoietic stem cells and incapacitates the rest of the cells, thereby preventing proliferation and differentiation (Hellman and Grate 1971; Stackowicz et al., 2019). Similarly, (Huyan et al., 2011) reported the Immunosuppressive effect of CTX on white blood cells and lymphocyte subpopulations from peripheral blood of BALB/c mice. In contrast, (Moore 1991) showed that human white blood cell counts began to decline on day 6, reached their lowest level between days 9 and 12, and recovered 15 days after a single intravenous dose of CTX. Our data are consistent with previously reported data on the adverse effects of CTX on neutrophils and monocytes.

In this study, the Con-A-treated group helped to increase the number of differential leukocytic cells. ConA also helped patients with advanced cancer regain lymphocytes in their peripheral blood after they were given cyclophosphamide (Berd et al., 1984). Based on our findings, CTX's inducible myelosuppressive effect is impaired by con-A and/or IL-27 conditioned immune treatments. The combination of Con-A and IL-27 in combination with CTX had the best results in restoring the differential leukocytic counts to a healthy level. Treatment with cytokines is thought to enhance the immune system's ability in patients to fight cancer (Conlon et al., 2019). IL-27 receptor is widely expressed in the immune system, and the functional effects of IL-27R signaling have been observed in mast cells, B cells, natural killer (NK) cells, dendritic cells (DCs), macrophages, and neutrophils (Batten and Ghilardi 2007).

Figure 1. Hematological parameters analyzed in in the blood of EAC bearing mice treated with CTX, Con-A or/and IL-27; (a) White Blood Cells (WBCs) count, (b) Neutrophil Abs, (c) lymphocyte Abs. (d) Monocyte Abs, (e) Eosinophil Abs, (f) Basophil Abs. Data are presented as mean ± SD, n=15 mice per group, ns (non-significant) $P \geq 0.05$, $^{*}P$ <0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001, and $^{****}P$ < 0.0001.

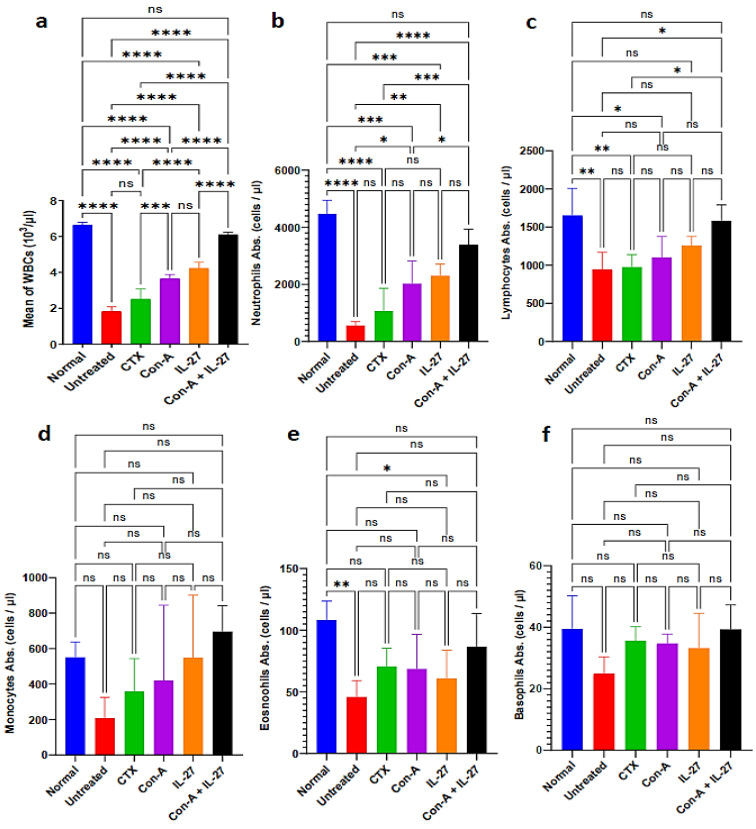
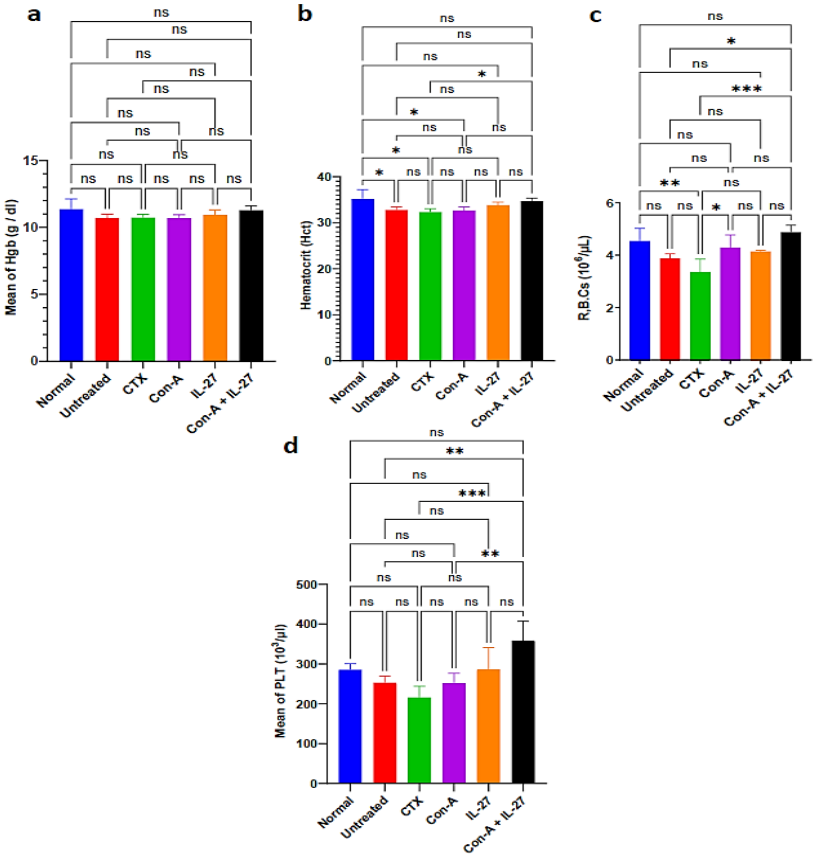


Figure 2. Hematological parameters analyzed in the blood of EAC bearing mice treated with CTX, Con-A or/and IL-27; (a) Mean of Hgb, (b) Hematocrit, (c) Red blood cells, (d) Platelets count. Data are presented as mean ± SD, n=15 mice per group, ns (non-significant) $P \geq 0.05$, $^{*}P$ <0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001, and $^{****}P$ < 0.0001.



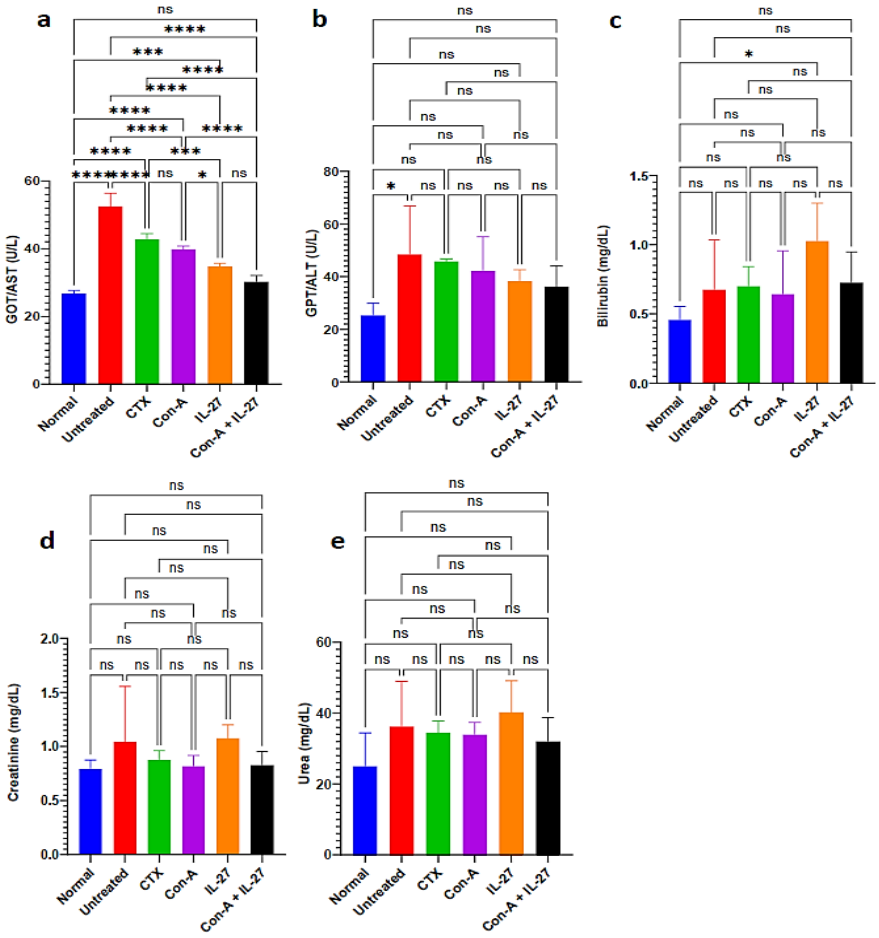


Figure 3. Biochemical parameters analyzed in the blood of EAC bearing mice treated with CTX, Con-A or/and IL-27; (a) aspartate aminotransferase (AST), (b) Alanine aminotransferase (ALT), (c) Bilirubin level. (d) creatinine level, (e) Urea level. Data are presented as mean ± SD, *n*=15 mice per group, ns (non-significant) $P \geq 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

An anemic event is usually accompanied by chemotherapy with CTX (Alenzi et al., 2010; Kirshner et al., 2004). Thus, the hematocrit values and RBCs count of CTX-treated mice were significantly lower. CTX-treated mice, on the other hand, experienced a rare decrease in Hb levels. Compared with CTX, the treated groups with Con-A + IL-27 and the Con-A showed a significant rise in hematocrit values and RBCs. Therefore, a combined regimen could reverse the anemia caused by a single CTX treatment. Since CTX is cytotoxic to the mature hematopoietic progenitor cells (Emadi et al., 2009) thrombocytopenia is constant with cyclophosphamide (Kuter 2015). Platelet counts are normally disordered in the Con-A and IL-27 treatment groups, with Con-A + IL-27 being the highest, suggesting that IL-27 consistently acts directly on hematopoietic stem cells (Orii et al., 2018). Increased exposure to CTX metabolites causes symptoms of liver dysfunction associated with sinusoidal obstruction syndrome with increased levels of hepatotoxicity, aminotransferases, and bilirubin (McDonald et al., 2003).

It also has a toxic effect on the kidneys and causes an increase in creatinine levels (Zhang et al., 2021). In line with our findings, when CTX-treated tumor-bearing mice were compared to healthy mice, they showed an increase in AST, ALT, bilirubin, creatinine, and urea. The herbal compound, Con-A, in a combination therapeutic regime reduced the previous biochemical changes to control levels, but the results were not statistically significant. This is consistent with the protective effect of thymoquinone, with significantly reduced activity of liver enzymes, bilirubin, urea, creatinine, triglycerides, and cholesterol in CTX-treated rats (Alenzi et al., 2010). In mice injected with CTX/propolis, the levels of ALT, AST, urea, creatinine, WBCs, and platelets all improved too (El-Naggar et al., 2015). The AST activity was significantly reduced in IL-27 conditioned splenocytes with CTX treatment, but bilirubin and kidney function parameters were negatively affected. Compared with CTX alone, only a combination therapy using Con-A + IL-27 conditioned splenocytes with CTX dramatically reduced ALT, AST, urea, and creatinine, but not bilirubin. Con-A + IL-27 conditioned splenocytes may protect tumor-bearing mice from the overall toxicity of CTX. In addition, some studies have shown that CTX causes oxidative stress, as high production of oxidative-promoting molecules is associated with a significant reduction in antioxidant enzyme activity (Premkumar et al., 2001).

5. Conclusion

Even though CTX is a widely used drug for treating both malignant and nonmalignant tumors, the clinical outcomes of these treatments are severely limited, owing to its toxicity to normal tissues. CTX's main side effects are bone marrow suppression and oxidative stress in hepatocytes. As a result, developing adjuvant therapy that can be used in conjunction with CTX to improve the treatment's efficacy or reduce the treatment's undesirable side effects is critical. During the chemotherapy course, Con-A + IL-27 is recommended as a good and effective therapy for the treatment of mammary gland tumors. According to the findings, con-A and IL-27 successfully normalized the hematological and biochemical parameters.

Compliance with Ethical Standards

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Conflict of Interest

The author(s) confirm that this article content has no conflict of interest.

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