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Effects of Cyclosporine and Azacitidine on Some Hematologic and Biochemical Parameters of Benzene-Induced Aplastic Anemia in Rats

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A B S T R A C T

Aplastic anemia, marked by deficiencies in hematopoietic stem cells, leads to peripheral blood pancytopenia and hypocellular bone marrow. This study aimed to evaluate the therapeutic efficacy of cyclosporine and azacitidine, administered either alone or in combination, in rats with benzene-induced aplastic anemia, focusing on restoring normal blood cell levels and preventing disease complications. Thirty adult female Wistar rats (Rattus norvegicus) were randomly divided into five groups: negative control (C-, untreated), positive control (C+, induced aplastic anemia with distilled water), cyclosporine-treated (CsA, 5.86 mg/kg), azacitidine-treated (Aza, 5.75 mg/kg), and combination-treated (CsA+Aza, 3.68 mg/kg each). Benzene (1940 mg/kg) was administered orally for fifteen days to induce aplastic anemia. Post a 30-day treatment period, evaluations included differential WBC and reticulocyte counts, serum IL-2 levels, and alkaline phosphatase (ALP) activity. Results showed significant improvements in WBC% and reticulocyte% in all treated groups compared to the C+ group, with the combination-treated group showing the highest enhancement. IL-2 levels in the combination group were significantly reduced compared to other treatment groups, aligning closely with the negative control. The ALP activity was significantly higher in both the cyclosporine and azacitidine-treated groups compared to the positive control, with the combination group showing a marked increase over the azacitidine group but no significant difference from the cyclosporine group and negative control. In conclusion, the study demonstrates the potential therapeutic benefits of cyclosporine and azacitidine in treating benzene-induced aplastic anemia in rats. The combination therapy, in particular, showed improved efficacy in all tested parameters, suggesting a potential strategy for dose reduction and toxicity mitigation.

Keywords: cyclosporine, azacytidine, bone marrow, aplastic anemia, rat

INTRODUCTION

Aplastic anemia is one type of acquired myeloid hematological system disease. It is characterized by immune-mediated primary bone marrow failure (BMF) leading to pancytopenia and hypocellular marrow (1, 2). Remarkably, reticulocytopenia, monocytopenia, neutropenia and thrombocytopenia are noteworthy because they can be extremely life-threatening due to the risk of infection and bleeding, which is exacerbated by severe anemia (3). In addition, acquired aplastic anemia was associated with a quantitative and qualitative hematopoietic stem progenitor cell deficiency that resulted in peripheral blood pancytopenia and hypocellular BM (4, 5). Moreover, IL-2 stimulates the synthesis of proinflammatory cytokines like IFN- and IL-4 as an

autocrine consequence of antigen-stimulated T cells, which are necessary for the proliferation and expansion of both antigen-specific clones of CD4+ and CD8+ T cells. (6). In this respect, previous studies have demonstrated that the peripheral blood IL-2 levels of cases with acquired aplastic anemia were significantly higher than those of healthy controls (4). Nevertheless, by preventing hematopoietic stem progenitor cells (HSPC) from proliferating and impairing the hematopoietic regulatory system, acquired aplastic anemia can be developed (6). A powerful immunosuppressive drug (calcineurin inhibitor) cyclosporine was first identified in 1971. It is an undecapeptide with a cyclic lipophilic structure of fungal origin called *Tolypocladium inflatum* (7). In numerous animal species for a variety of organs, aplastic anemia, allograft rejection, delayed hypersensitivity, and graft vs. host disease have all been found to be inhibited by cyclosporine, as well as certain humoral immunity and, to a greater extent, cell-mediated immune responses. (8).

Azacitidine (5-azacytidine), a pyrimidine nucleotide analog of cytidine, inhibits the activity of the DNA methyltransferase (DNMT) by altering the pyrimidine ring's fifth carbon. Depending on the dosing regimen, azacitidine exhibited two unique properties: cytotoxicity and DNA hypomethylation. Due to its incorporation into RNA and DNA, azacitidine enhances cytotoxicity at high doses while causing DNA hypomethylation effects at low doses (9). The medicine combination is most frequently using for treatment of the worst illnesses, such as cancer, AIDS, and aplastic anemia. The primary goals of combination medications are typically to reduce dosage and toxicity, generate synergistic therapeutic benefits, and prevent or postpone the development of drug resistance. The present study aimed to evaluate the role of cyclosporine and azacitidine in treating of benzene-induced aplastic anemia in rats.

MATERIALS AND METHODS

Ethical Approval

All procedures used in this study were approved by the local Scientific Research Committee of the College of veterinary medicine, University of Baghdad in compliance with the ethical principles guidelines on the care and use of animals in research of animal welfare (Approval Number: 1254 P.G. dated 30/4/2023).

Induction of Plastic Anemia

Benzene was administered orally for fifteen days at a dose of 1940 mg/kg to induce this disease. After the end of the induction period, five female rats were randomly taken and euthanizing to study histopathological changes in BM and liver tissues, as well as measuring the complete blood picture to confirm the occurrence of disease.

Experimental Animals

Thirty adult female Wister rats, 3 months old, with an average body weight 220±12 g were obtained from the animal house of the College of Veterinary Medicine, University of Baghdad. The rats were accommodated in plastic cages (20×30×50 cm^3) with stainless-steel wire mesh lids in an air-conditioned room with proper climatic parameters of temperature (22±3 °C), relative humidity (60±5%), and 12 h dark/light cycle. Animals were allowed a two-week adaptation period prior to the beginning of the experiment. The animals received free access to fresh food and water (5).

Experimental Design

Thirty rats were divided equally into five groups, as follows: C-: was not treated with benzene and left without any treatment as a negative control. C+: induced aplastic anemia by oral administration of benzene for 15 days and treated with distilled water as a positive control. The CsA group was treated with cyclosporine (Novartis, Turkey) at a dose of 5.86 mg/kg. The Aza group: was treated with azacytidine (Celgene, Turkey) at a dose of 5.75 mg/kg and the CsA+Aza group received a combination of cyclosporine and azacytidine at a dose of 3.68 mg/kg for each drug. The doses of the drug alone and in combination were chosen based on previously published depended on Abdulrazzaq and Hasan (12). After the day 30th of the experiment, the animals were sacrificed after receiving all treatments orally through a stomach tube.

Blood samples

The animals were given chloroform (SDFL, India) inhalation anesthesia at the end of the study period. Blood samples were obtained from the heart using disposable syringes, gage 22 (5 mL capacity). The blood sample was split into two tubes, the first of which was filled with EDTA-anticoagulant, the other was centrifuged at 4000 rpm for five min to separate the serum, and the samples were then kept in the freezer at -18 °C until they were used for serum IL-2 and alkaline phosphatase tests.

Differential WBCs and Reticulocytes

Differential WBC count was measured by using an automated hematology analyzer (HEMAVET, Germany). In addition, the reticulocyte percentage was measured in accordance with (14). Methylene blue solution was added to EDTA-anticoagulated blood and incubated at 37 °C for 20–25 min. Blood smears were made for reticulocyte counting, showing a proportion of reticulocytes to the full number of RBCs.

Determination of serum IL-2

Serum IL-2 concentration was measured quantitatively by using Abcam's IL-2 rat ELISA kit (ab221834) (6).

Determination of Serum ALP Activity (ALP)

The rat serum ALP activity was determined by using a rat ALP ELISA kit (Kamiya Biomedical, USA). The microtiter plate provided in this kit is pre-coated with an antibody specific to ALP. Calibrators and samples were added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for ALP. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated for 15 - 25 min at 37 °C. Then the TMB substrate solution was added to each well. Only those wells that contained ALP, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm±2 nm. The concentration of ALP in the samples was then determined by comparing the optical density (OD) of the samples to the calibration curve.

Statistical Analysis

SAS (Statistical Analysis System, version 9.1) was utilized for analyzing the data. The ANOVA (one-way) with least significant differences (LSD) post hoc test was employed to assess significant differences among means (18). P≤0.05 was employed to determine statistical significance.

RESULTS

WBCs and Reticulocytes

The lymphocytes %, monocytes %, basophils %, neutrophils %, eosinophils %, and reticulocytes % in the positive group of control were significantly (P<0.05) decreased in compression with all treatment groups as well as negative control group. In addition, cyclosporine and azacitidine treated groups significantly (P<0.05) increased compared with the positive control group. There is a P≤0.05 decrease compared with negative control as well as combination treated groups. Furthermore, the combination treated group showed non-significant (P>0.05) difference in compression with the negative group of control for basophils, neutrophils and reticulocytes percent. As shown in (Table 1).

Serum IL-2

The IL-2 data are shown in Table 2. in compression with all treatment groups, the IL-2 in the positive group of control had a significantly (P<0.05) higher average value. Additionally, the mean values of IL-2 in the cyclosporine and azacitidine treatment groups were considerably (P<0.05) lower than those of the positive control group. Furthermore, the mean value of IL-2 in the combination-treated group was significantly (P<0.05) decreased when compared to all other treatment groups and the positive control group, with no marked change when compared to the negative control group.

Serum ALP

The ALP activity in the positive group of control was significantly (P<0.05) declined in compression with all treatment groups. In addition, the ALP activity in the cyclosporine and azacitidine treatment groups was significantly (P<0.05) increased compared with the positive control group. Furthermore, its activity in the group of the combination was significantly (P<0.05) increased compared with the positive control and azacitidine treated groups, but with no significant (P>0.05) variation in comparison with the cyclosporine-treated and negative control groups.

Table 1. Effects of cyclosporine, azacitidine, and their combination on the percent of differential white blood cells and reticulocytes of female rats

Parameter (%)	C-	C+	CsA	Aza	CsA+Aza	LSD
Lymphocytes	59.1 ± 2.96 ^{a}	28.8 ± 2.69 ^{d}	34.8 ± 2.07 ^{c}	34.1 ± 1.84 ^{c}	42.4 ± 2.09 ^{b}	6.11
Monocytes	3.14 ± 0.63 ^{a}	0.87 ± 0.24 ^{c}	0.99 ± 0.08 ^{c}	1.10 ± 0.17 ^{bc}	2.00 ± 0.11 ^{b}	0.93
Basophil	0.75 ± 0.06 ^{a}	0.21 ± 0.07 ^{c}	0.48 ± 0.07 ^{b}	0.46 ± 0.08 ^{b}	0.63 ± 0.08 ^{ab}	0.22
Neutrophils	38.4 ± 1.19 ^{a}	19.9 ± 2.36 ^{d}	29.1 ± 2.63 ^{c}	30.9 ± 0.90 ^{bc}	36.9 ± 3.22 ^{ab}	6.53
Eosinophils	3.50 ± 0.51 ^{a}	0.93 ± 0.11 ^{c}	1.80 ± 0.22 ^{b}	1.76 ± 0.21 ^{b}	2.62 ± 0.31 ^{b}	0.82
Reticulocytes	3.72±0.19 ^{a}	0.88 ± 0.09 ^{d}	1.90 ± 0.21 ^{c}	1.82 ± 0.24 ^{c}	2.95 ± 0.27 ^{a}	0.64

Values are Means ± SEM, n= 6. $^{a-d}$ Means with different superscripts in the similar row are statistically different (P≤0.05). C-: negative group of control; C+: positive group of control; CsA: cyclosporine-treated group; Aza: azacitidine-treated group; CsA+Aza: combination-treated group

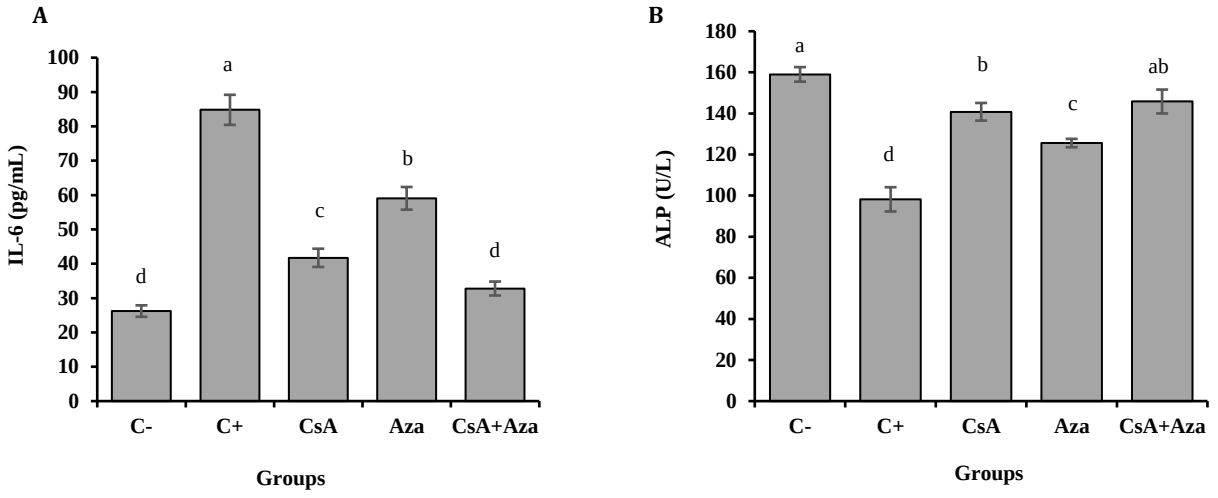

Figure 1. Effects of cyclosporine, azacitidine, and their combination on serum IL-2 level (pg/ml) **(A)** and serum alkaline phosphatase (ALP) levels (U/L) **(B)** of female rats. The bars represent the means (n=6), and the error bars indicate the standard error of the mean (SEM). [a-d] Bars without a common letter among treatment groups differ (P≤0.05). C-: negative group; C+: positive group; CsA: cyclosporine-treated group; Aza: azacitidine-treated group; CsA+Aza: combination-treated group)

DISCUSSION

Evaluation of the differential WBC percent and reticulocyte percent is imperative in the setting of aplastic anemia and a vital investigation for this disease; thus, a low differential WBC count and reticulocyte count are suggestive of aplastic anemia (19). As such, the reduction in the differential WBC and reticulocyte counts of the group of positive control was linked to the disease status, which is linked to the damaging effects of hematopoiesis, leading to the destruction of hematopoietic stem cells (HSCs) resulting from the inability of bone marrow to create blood cells. As a result, the differential WBC and reticulocyte were reduced below normal levels, the similar results reported by (20, 21).

In addition, deregulation of the T cell response and its activation mediated suppression through increasing expression of the first apoptosis signal (Fas) receptor and secretion of hematopoietic suppressing cytokines like IFN-γTNF-α and IL-2, resulting in HSC immune-mediated destruction and induced BM inhibition, as reported by (20). Cyclosporine has a direct positive effect on HSPC signal transduction pathways via interaction with elements in this pathway, resulting in the stimulation of hematopoiesis (23, 24). Likewise, the improvement in the differential WBC and reticulocyte % count of the azacitidine-treated group was mediated via the epigenetic regulation of the HSPCs, which enhanced normal hematopoiesis, and also via their ability to modulate the bone marrow microenvironment, specifically the mesenchymal stem cell, this finding supported by (5, 22). Azacitidine has been shown to increase the percentage of reticulocytes containing hemoglobin as well as the proportion of hemoglobin in BM cells (22, 25). Importantly, the combination of cyclosporine and azacytidine could significantly improve the differential WBCs and reticulocyte % count in rats, which might be accomplished primarily through synergistic co-treatment, dose reduction, and toxicity reduction (5).

IL-2, a type of interleukin, is also a potent signaling molecule in the signaling cascade of the immune-mediated activation of T Lymphocytes, leading to the destruction of hematopoietic stem cells (HSC) which is the basis of acquired aplastic anemia (8). The elevation in IL-2 serum levels in the positive control group was due to IL-2 acting as an autocrine and paracrine molecule, which is produced mainly by activated CD4+ T-cells, naive CD8+ T-cells, and dendritic cells (26). Furthermore, as a T-cell growth factor, serum levels of IL-2 increased drastically in rats with induced aplastic anemia (4- 12). While the IL-2 levels of the cyclosporine-treated group declined. It is worth noting that cyclosporine is a powerful immunosuppressive drug that inhibits the production of IL-2 and other pro-inflammatory cytokines such as TNF-α and IFN-γ via calcineurin inhibition (23, 24). As well as altering the fundamental function of multiple proteins (e.g., NFAT, AP3, and NFLB), all are included in the regulation of the IL-2 gene transcription, this discussion is supported by (27-28). The group that received azacitidine was also found to have significantly lower levels of IL-2, it inhibited proliferation and activation of T cell, thus reducing the pro-inflammatory cytokines release, of which IL-2 (29). It was also attributed to epigenetic modifications that have been shown to tightly control FOXP3, the signature transcription factor of regulatory T cells, the same explaining reported by (30). Notably, the group that received a cyclosporine and azacitidine combination had a significantly lower decrease

in IL-2 levels than either drug alone; these findings suggested that the two drugs might have a synergistic immunosuppressive effect (12).

ALP is considered a reliable marker for bone metabolism, and changes in its level in the blood could indicate issues relating to the liver or bones (31). The reduction of serum alkaline phosphatase level in the positive control group was likely due to defective osteoplastic cells' activity, which regulated the HSCs' microenvironment in the BM (32, 33). Specifically, ALP levels and bone metabolism are linked to the development and maturation of blood corpuscles (33, 34). Additionally, under anemic conditions, the activity of BM should be increased to compensate for hemoglobin insufficiency. However, if the osteoblasts cannot be activated by anemia, a lower ALP level may result (36, 37). Moreover, ALP is correlated with the production of HSCs, and lower levels of this enzyme may therefore be associated with anemia by signifying lower production of red blood cells, suggesting a lack of hyper function of the BM to compensate for anemia (38, 39). In comparison, the increase in alkaline phosphatase levels in the cyclosporine-treated group was clearly associated with its ability to compensate for anemia and hemoglobin reduction, as well as its immunosuppressive effect; TNF-mRNA gene transcription was discovered to be inhibited by cyclosporine, which could indirectly result in ALP elevation (40, 41).

Moreover, treatment of rats with azacitidine led to a significant improvement of alkaline phosphatase activity, which may be linked to epigenetic regulation via modulating the differentiation potentials in MSCs of osteoblasts. (42). Furthermore, hypomethylation of genomic DNA was observed in association with facilitated osteogenic development, demonstrating that epigenetic control via DNA demethylation occurred during osteogenic differentiation (43).

Cyclosporine and azacitidine have a potential therapeutic effect on Aplastic anemia induced by benzene. As well, the combination of cyclosporine and azacitidine revealed an improvement in blood picture, IL-2 and ALP activity which achieved via dose and toxicity reduction.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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تأثير السيكلوسبورين والأزاسيتيدين على بعض المعلمات الدموية والكيميائية الحيوية لفقر الدم اللاتنسجي المستحث في الجرذان

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الخلاصة

فقر الدم اللاتنسجي ، المرتبط بنقص في الخلايا الجذعية المكونة للدم على المستوى الكمي والنوعي ، يظهر في شكل قلة الكريات الشاملة في الدم المحيطي وكذلك انخفاض في خلايا النخاع العظمي. وهكذا ، كان الهدف من هذه الدراسة هو تقييم دور السيكلوسبورين والأزاسيتيدين في علاج فقر الدم اللاتنسجي المستحث في الجرذان. تم إحداث فقر الدم اللاتنسجي بواسطة البنزين بجرعة ١٩٤٠ مجم / كجم لمدة خمسة عشر يومًا. تم تقسيم الجرذان البالغة عددها ث ثلاثون أنثى إلى خمس مجموعات متساوية. المجموعة الأولى رفعت دون أي علاج كعنصر تحكم سلبي ، المجموعة الثانية تم استحداث في فقر الدم اللاتنسجي وتلقى الماء المقطر كعنصر تحكم إيجابي ؛ المجموعة الثالثة تلقت السيكلوسبورين بجرعة ٥,٨٦ مغ / كغ ؛ تلقت المجموعة الرابعة الأزاسيتيدين بجرعة ٥,٧٥ مجم / كجم ؛ والمجموعة الخامسة تلقت مزيجًا من السيكلوسبورين والأزاسيتيدي بجرعة ٣,٦٨ مجم / كجم لكل عقار. انخفض عدد خلايا الدم البيضاء التفاضلية٪ ونسبة الخلايا الشبكية في مجموعة التحكم الإيجابية بشكل معنوي P≤٠,٠٥ عند مقارنتها مع جميع المجموعات المعالجة فضلاً عن مجموعة التحكم السلبية. وزيادةً على ذلك ، في المجموعات المعالجة بالسيكلوسبورين والأزاسيتيدين ، زادت هذه المعلمات إحصائيًا P≤٠,٠٥ مقارنة بمجموعة التحكم الإيجابية ، مع انخفاض جوهري P≤٠,٠٥ مقارنة بمجموعة التحكم السلبية وكذلك المجموعات المعالجة المركبة. وبعد هذا كله ، انخفض متوسط قيمة IL-٢ في المجموعة المعالجة المركبة بشكل كبير عند مقارنتها بجميع مجموعات العلاج الأخرى ومجموعة التحكم الإيجابية ، مع عدم وجود تغيير ملحوظ عند مقارنتها بالمجموعة السلبية. انخفض نشاط الفوسفاتيز القلوي في مجموعة التحكم الإيجابية إلى حد كبير P≤٠,٠٥ مقارنة مع جميع المجموعات التجريبية. في الختام ، الأدوية المستخدمة في الدراسة الحالية لها تأثير علاجي محتمل على فقر الدم اللاتنسجي المستحدث في الجرذ ، وقد أظهر المزيج بين السيكلوسبورين والأزاسيتيدين تحسنًا في جميع معاملات هذه الدراسة. تم تحقيقه بشكل عام عن طريق تقليل الجرعة والسمية.

الكلمات المفاحية: السيكلوسبورين، أزاسيتيدين، نخاع العظم، فقر الدم الاتنسجي، إناث الجرذان