Role of cyclosporine and azacitidine on the treatment of induced aplastic anemia in female rats

Ghanem Taher Abdulrazzaq and Huda Falah Hasan

College of Veterinary Medicine, University of Baghdad, Baghdad - Iraq

Department of Physiology, Biochemistry and Pharmacology

Abstract…The aim of the research was to determine the therapeutic role of cyclosporine and azacitidine in the treatment of aplastic anemia induced by benzene in rats. The experiment was performed in 30 rats divided into five groups as C-: negative control group, C+: positive control group (benzene 1940 mg/kg), CsA: received cyclosporine at dose of 5.86 mg/kg, Aza: received azacitidine at dose of 5.75 mg/kg and combination-treated group: received combination of cyclosporine and azacitidine at dose 3.68 mg/kg for each drug. Then body weight, red cell indices, total BM cell count, total protein and histopathology for BM and hepatic tissues were measured. In conclusion, there are a good role of both cyclosporine and azacitidine alone and in combination therapy in the treatment of induced aplastic anemia.

Keywords--- Aplastic anemia, cyclosporine, azacitidine, drug combination.

Introduction

The potentially fatal hematopoietic disorder that can be classified as inherited or acquired is defined as aplastic anemia which caused by an immune response-mediated bone marrow failure [1, 2]. It can be clinically distinguished by a combination of peripheral pancytopenia and morphologically and histologically hypocellular bone marrow (BM), most often due to injury to the pluripotent stem cells; the damage to the pluripotent stem cells causes the reduction of normal levels of erythrocytes, neutrophils, and platelets, as well as evoke hypofunctional BM hematopoiesis [3].

It is noteworthy that deregulated polyclonal T-cell activation in acquired plastic anemia caused the death of HSCs either directly by inducing apoptosis or indirectly by producing excessive amounts of myelosuppressive cytokines like IL-2, IL-6, IL-8, IFN-gamma, TNF-alpha and others. Additionally, these cytokines contributed to the inhibition of hematopoiesis, which was correlated with the severity of the disease [4, 5]. Interestingly, BM-MSCs from aplastic anemia cases were more easily stimulated to develop into adipocytes than they were into osteoblasts, which may lead to enhanced adipogenesis and aberrant HSC microenvironment formation [6]. Although aplastic anemia typically idiopathic, it can also be

caused by cytotoxic medications such as chloramphenicol and gold, radiation, toxic chemicals such as benzene, infection with viruses such as Epstein-Barr virus infection and seronegative non-A-G hepatitis, immune-related conditions like graft vs. host disease, and other factors [7].

The first substance was benzene, which was discovered to be connected to aplastic anemia [8]. The chemical benzene is used extensively in medicines, dyes, explosives, rubber, and leather items, and prolonged exposure to benzene causes BM depression, which in turn leads to aplastic anemia in both humans and animals [9].

Furthermore, the immunosuppressive compound cyclosporine A, a well-known inhibitor of calcineurin with immunosuppressive properties, was derived from the *Tolypocladium inflatum* fungus and was first identified in 1971[10, 11]. Moreover, it has produced significant advancements in treating various blood disorders, including autoimmune illnesses and aplastic anemia [12]. In addition, the pyrimidine nucleoside analogue azacitidine has the ability to block DNA methyltransferase, focusing on epigenetic gene silencing, which was utilized by cancer cells to silence some genes in order to prevent the development of malignant phenotypes, as well as the FDA authorized it to treat a class of blood and BM diseases [13].

Materials and Methods

Experimental animals

This study was carried out at the animal house/ College of Veterinary Medicine/University of Baghdad. Thirty adult female rats (average weight 220g). Rats were reared in plastic cages in an air-conditioned area under appropriate environmental conditions of (22± 3°C) and relative humidity (60± 5%). The animals were given freshly prepared food and free use of the water supply.

Experimental design

Aplastic anemia was induced by the administration of benzene at a dose of 1940 mg/kg for fifteen days [14, 15, and 16]. Following the end of the induction period, 30 rats were divided into five equal groups as follows: The first group was lifted without any treatment; the second group had induced aplastic anaemia and was treated with distilled water. The third group was given cyclosporine at a dose of 5.86 mg/kg. The fourth group was treated with azacitinde at a dose of 5.75 mg/kg, and the fifth group received a combination of cyclosporine and azacitinde at a dose of 3.68 mg/kg for each drug. All treatments were given orally by stomach tube, and the animals were sacrificed on day 30 at the end of the experiment.

Blood Sampling

After the termination of the experimental period, the animals were anaesthetized with chloroform inhalation, and then euthanized, blood samples were obtained from the heart using disposable syringes (5 mL capacity). In addition, the required amount of blood was collected

in two tubes, one of them contained EDTA-anticoagulant for hematological studies, and the second was a serum separator tube, which was centrifuged at 4000 rpm for ten minutes, and then isolated serum samples were stored in the freezer at -18 oC until use in biochemical studies.

Determination of total bone marrow cell count:
Total BM cells were counted according to method described by Lezama *et al* [17].

Determination of total protein concentration:
Total protein concentration in the serum was determined by using the Biuret method described by Zheng *et al* [18].

Detection of red blood cell Indices

Blood indices; MCV, MCH, MCHC and RDW% were calculated by using automated hematology analyzer [19].

Histopathological examination:
At the end of the treatment period, the rats were euthanized with chloroform. Samples from the liver and femur bone were cut out and then cleaned off any associated tissue and fat. These samples were then preserved in 10% formalin for fixation. In the following step, samples were dehydrated through an ascending series of ethyl alcohols (70%, 80%, 90%, and 100%), two changes each for 2 hours, then cleared with xylene for 1/2 hour. Samples infiltrated with paraffin wax (58–60 ^{o}C) were then embedded with new paraffin wax to obtain blocks of paraffin. Sections of 5-6 um thickness was obtained via rotary microtome, stained with haematoxylin and eosin stain, and then tested beneath a light microscope [20].

Statistical analysis
The statistical analysis of the data was performed using SAS (Statistical Analysis System, version 9.1). A one-way ANOVA and least significant differences (LSD) post hoc test were performed to assess significant differences among means. $P \leq 0.05$ was considered statistically significant.

Results
The results of body weight, total bone marrow cell count and total protein are shown in table (1). When compared to all treated groups as well as the negative control group, the positive control group had a significant ($P \leq 0.05$) decrease in body weight in the mean value (159.83±2.31). However, there was no significant ($P \geq 0.05$) difference in body weight between the cyclosporine and azacitidine treated groups, but they both had a significant ($P \leq 0.05$) increase in the mean value (204.03±1.80 and 202.20±2.25, respectively) when compared to the positive control group. Furthermore, when compared to all other treated groups, the combination treated group showed the highest significant ($P \leq 0.05$) increase in body weight in the mean value (220.40±4.62), with a significant ($P \leq 0.05$) decrease when

compared to the negative control group. The total BM cell count in the positive control group was significantly (P≤0.05) reduced in average value (390.00±47.25) when compared to all treated groups and the negative control group. In addition, total BM cell count in the cyclosporine and azacitidine treated groups was significantly (P≤0.05) elevated in mean values (580.70±53.22 and 480.00±37.85, respectively), as compared with the positive control group, with a significant (P≤0.05) reduction compared with the combination treated group as well as the negative control group. Furthermore, the total BM cell count in the combination treated group increased significantly (P≤0.05) in mean value (601.00±70.00) when compared to all other treated groups, with no significant (P≥0.05) difference when compared to the negative control group. When compared to all experimental groups, the total protein in the positive control group was significantly (P≤0.05) decreased in mean value (4.69±0.33). Additionally, when compared to the positive control group, the total protein in the cyclosporine and azacitidine treated groups was statistically (P≤0.05) higher in mean values (6.12±0.23 and 6.24±0.28, respectively). Furthermore, the total protein in the combination treated group increased dramatically (P≤0.05) in mean value (7.23±0.22) compared to all other treated groups as well as the positive control group, with significant (P≤0.05) reduction compared to the negative control group. The MCV and RDW% showed no significant difference in all experimental groups. Besides, MCH and MCHC were significantly (P≤0.05) reduced in the positive control group in mean values (16.15±1.57 and 23.00±2.12), respectively), compared with all other treated groups as well as the negative control group. In addition, when compared to the positive control group, MCH and MCHC in the cyclosporine and azacitidine treated groups were significantly (P≤0.05) higher in average values (18.71±1.13, 28.03±1.41 and 18.13±1.21, 27.70±0.96bc, respectively). Furthermore, MCH and MCHC in the combination treated group significantly (P≤0.05) increased in mean values (21.25±0.67 and 31.00±0.83), respectively) in comparison with all other treated groups as well as the positive control group, with no significant (P≥0.05) difference compared with the negative control group, as shown in the table (2).

Table (1): Effect of cyclosporine, azacitidine and their combination on body weight, total bone marrow cell count and total protein after 30 days in female rats

Parameters Groups	Body weight (gram)	Total BM cell count (cells/µl suspension)	Total protein (g/dl)
C-	231.66±3.62a	650.00±83.66a	8.91±0.28a
C+	159.83±2.31d	390.00±47.25d	4.69±0.33d
CsA	204.03±1.80c	580.70±53.22 b	6.12±0.23c
Aza	202.20±2.25c	480.00±37.85 c	6.24±0.28c
CsA+Aza	220.40±4.62b	601.00±70.00a	7.23±0.22b
LSD	9.04	100.60	0.79

*Means with a small letter in the same column are significantly different (P≤0.05).

*C-: control negative; C+: control positive; CsA: cyclosporine; Aza: azacitidine; CsA+Aza: combination treated grou

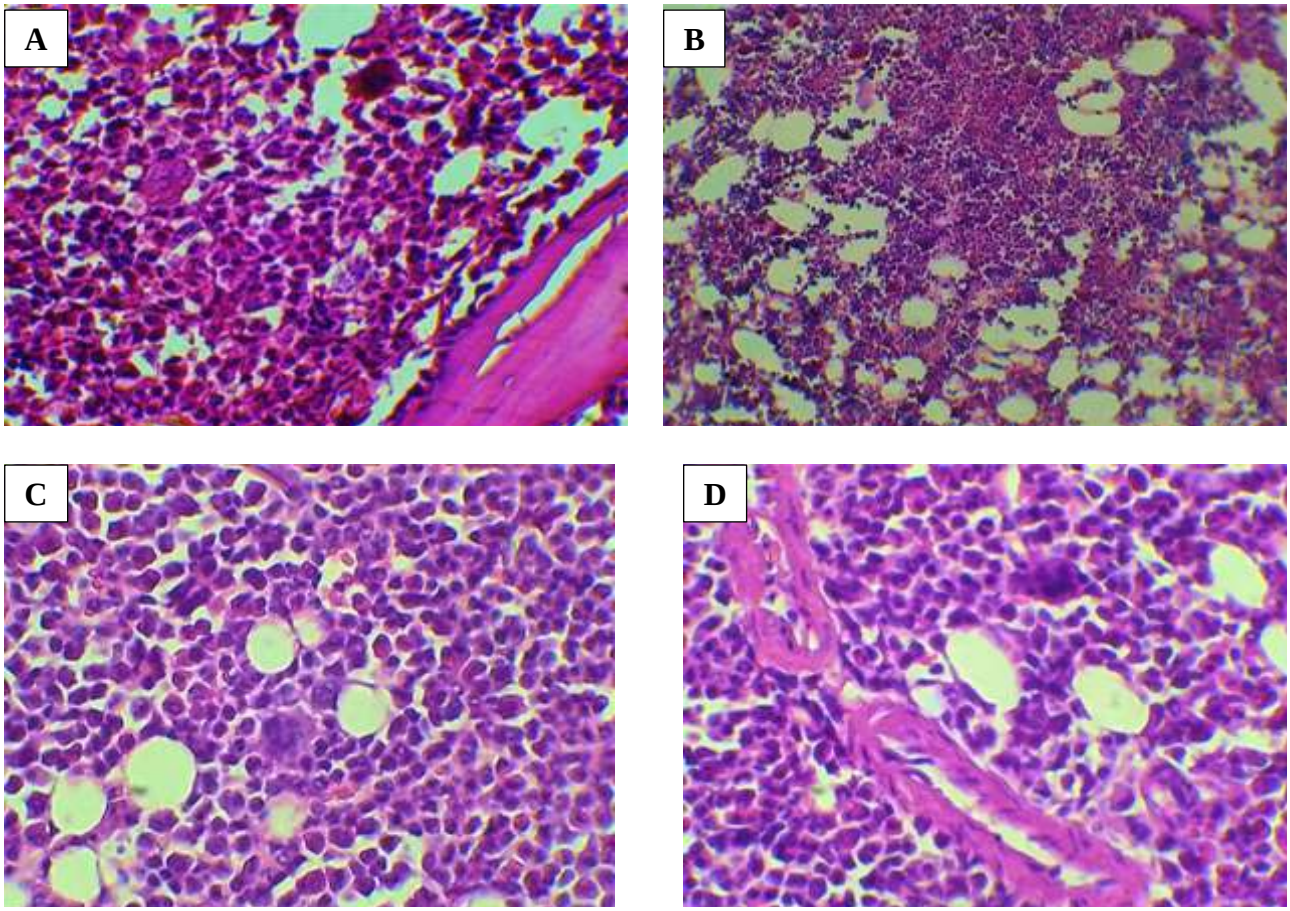
Table (2): Effect of cyclosporine, azacitidine and their combination on red cell indices after 30 days in female rats

Parameters / Groups	MCV (fl)	MCH (pg/cell)	MCHC (g/dl)	RDW (%)
C-	63.60±2.04	22.80±1.98a	35.20±2.41a	17.20±1.51
C+	63.80±1.81	16.15±1.57c	23.00±2.12c	17.60±0.86
CsA	62.95±1.59	18.71±1.13b	28.03±1.41b	16.93±0.69
Aza	63.20±1.57	18.13±1.21b	27.70±0.96bc	17.00±0.74
CsA+Aza	62.33±1.51	21.25±0.67a	31.00±0.83ab	16.56±0.47
LSD	5.00 NS	2.05	4.87	2.70 NS

*Means with a small letter in the same column are significantly different (P≤0.05).

*C-: control negative; C+: control positive; CsA: cyclosporine; Aza: azacitidine; CsA+Aza: combination treated group. * NS; Non significant.

Histopathological study of bone marrow



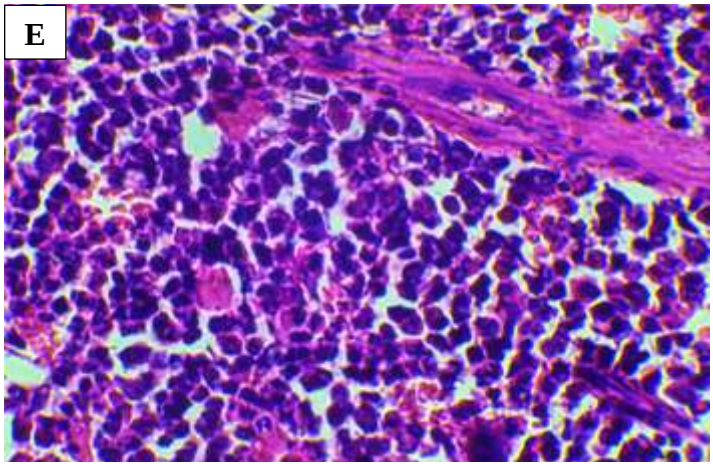


Figure (1): Histopathological section in the BM, A: the negative control group showed normal cellularity and equal proportion of erythyroid and myeloid cells, B: the positive control group showed hypocellular marrow and marrow spaces consisting primarily of adipose tissue and vascular sinuses, C: the cyclosporine-treated group demonstrated severe BM cellularity associated with treatment. There is a dramatic increase in cell density affecting erythyroid and myeloid cells, as well as megakaryocytes in the BM, D: the azacitidine-treated group's BM demonstrating increased BM cellularity associated with treatment. The marrow is dominated by immature and mature myeloid cells, E: the combination treated group revealed erythryoid hyperplasia and an increase in erythropoiesis which is typically characterised by normal synchrony of erythyroid cell maturation. H&E stain 40X.

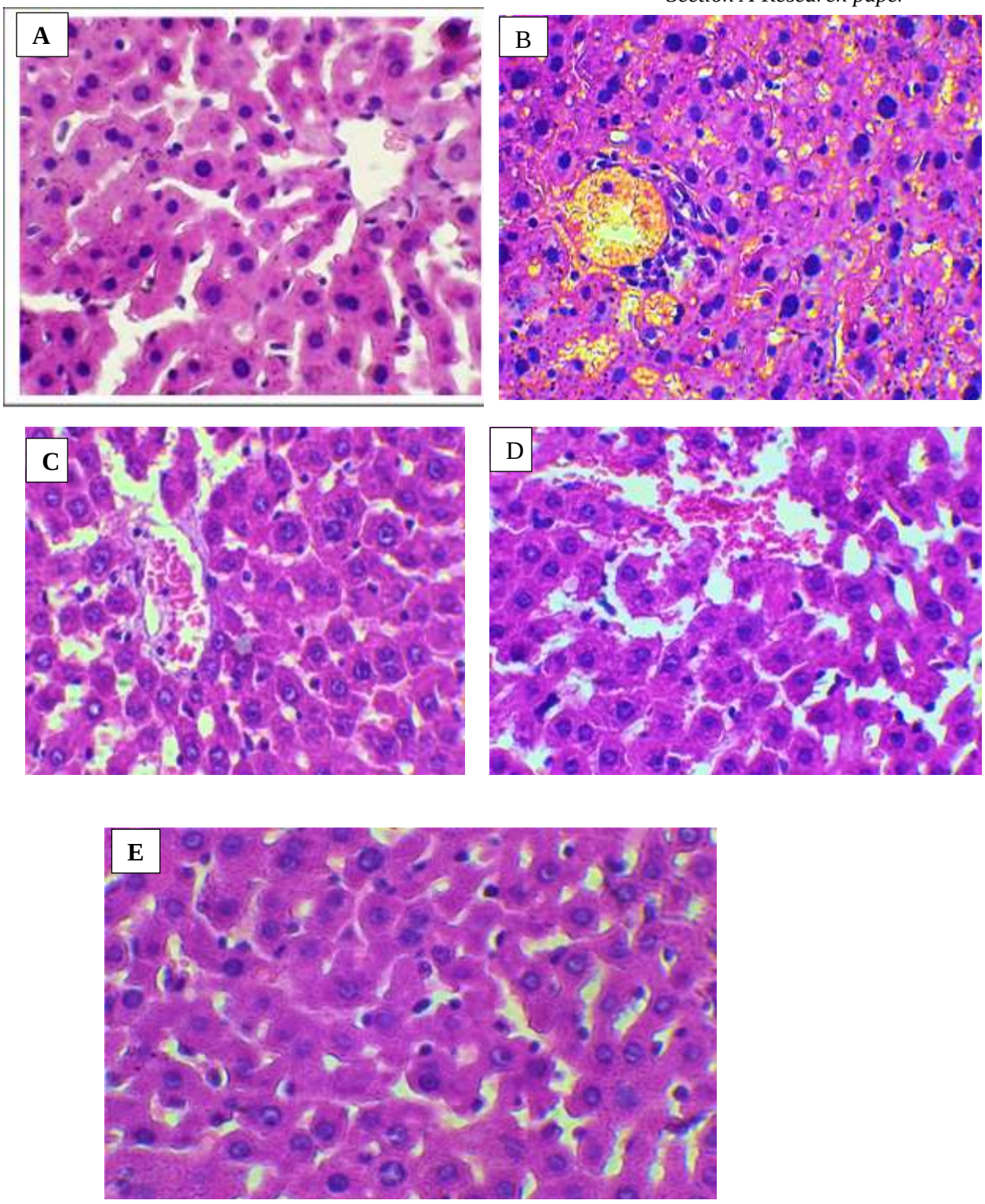


Figure (2): Histopathological section in the liver, A: the negative control group showed normal tissue with no pathological lesion ,B: the positive control group showed aggregation of mononuclear cells around the central vein and necrosis of hepatocytes with mitotic divisors

of nuclei ,C: the cyclosporine-treated group showed moderate congestion of the central vein with dilated blood sinusoids,D: the azacitidine-treated group revealed mild hemorrhage ,E: the combination-treated group revealed mild dilated blood sinusoids. H&E stain 40X.

Discussion

The results of the present study suggested that the systemic effects of this disorder, which have been linked to the production of free radicals could be responsible for the reduction in body weight in the positive control group. Thus, excessive levels of ROS could harm cellular macromolecules like proteins, lipids, and nucleic acids, potentially causing DNA damage and leading to the breakdown of cell tissue [21, 22]. It's worth noting that losing appetite may be related to aplastic anemia, which eventually led to weight loss [23]. While the group receiving cyclosporine treatment showed improvement in body weight, this improvement could be attributable to the drug's immunosuppressive effect, which can be seen in its modulation of numerous pathological responses by inhibiting transcription of T-cell cytokines, which in turn reduced many of the secondary systemic responses to cytokines in various body cell types [24]. In fact, cyclosporine, a cyclophilin ligand, and its coupling to cyclophilin D produced cytoprotective effects by inhibiting mPTP, which was mediated by cyclophilin D blocking [25]. Likewise, the increase in body weight in the azacitidine-treated group was regulated by epigenetic mechanisms in several related genes, which may have been primarily driven by energy homeostasis, particularly the genes affecting growth factors or regulators of genes controlling growth and energy metabolism [26]. Recent research discovered that the anti-oxidative and anti-inflammatory properties of azacitidine may have beneficial effects on the body tissues and it was hypothesized that therapy with azacitidine might reduce the degree of methylation and restore the production of genes that fight free radicals, thereby attenuating the systemic effects of aplastic anemia [28].

The reduction of total BM cell count in the positive control group was clearly associated with a profound deficit of hematopoietic stem and progenitor cells and BM hypocellularity, as well as enhanced adipogenesis, leading to the characteristic phenomenon of a fatty BM [5, 6]. Furthermore, cytotoxic T cell-induced apoptosis of CD34+ primary hematopoietic precursors, as well as Th1 inhibitory cytokines such as IL-2, TNF-α, and IFN-γ reduced the number of BM cells, as well as reduced levels of osteonectin+ cells might cause damage to stromal cells and, consequently, a BM defect by affecting tissue repair and regeneration [29]. Of note, MSCs in aplastic anemia may also showed aberrant morphology, decreased proliferation, increased apoptosis, and a propensity for adipogenic differentiation [30].

In another study, scientists found that adiponectin and FABP4, which closely related to adipogenesis, were significantly increased at the mRNA and protein levels, which have a negative regulatory effect on HSCs in the BM microenvironment and inhibit the proliferation of HSCs [31]. Correspondingly, the total BM cell number significantly increased in a group of rats given cyclosporine, confirming the drug's mechanism of action, which specifically

inhibited mammalian T cells by preventing activation of transcription factors involved in cytokine gene expression [32, 33]. It also suppressed BM-MSCs' adipogenetic differentiation and secretion of IL-6, which also exerted a positive effect by regulating the microenvironment of BM, which could be another mechanism responsible for aplastic anemia treatment [34, 35]. Meanwhile, the total BM cells in a group of rats treated with azacitidine substantially increased due to the drug's reduction of adipocyte proliferation and differentiation via the down regulation of peroxisome proliferator-activated receptor gamma and fatty acid-binding protein 4 and the up regulation of GATA-binding factor 2 [36]. Moreover, azacitidine inhibited T-cell proliferation and activation while decreasing the production of pro-inflammatory cytokines, including IL-2, thereby; preventing T-cell-mediated HSCs apoptosis and destruction and thus the total BM cells elevated [37].

Moreover, The decrease in total protein concentration in the positive control group could be attributed to hepatic dysfunction caused by activation of circulating cytotoxic T cells, which tend to accumulate in the liver, and a large number of T cell infiltration from liver parenchyma [38, 39]. Furthermore, CD8 cells residing in the BM during aplastic anemia produced a high level of INF-γ, and cells derived from the BM residing in the liver may have activated these cytotoxic T cells, causing intrahepatic accumulation and severely damaging hepatocytes [40]. Moreover, it has also shown in several studies that an increased level of soluble IL-2 receptors considered the major reason for non-specific inflammation because IL-2 is known to activate immune effector cells, cell-mediated effects of Kupffer cells considered a potential mechanism of IL-2-mediated hepatic dysfunction [41, 42]. In addition, IL-2 significantly increased intrahepatic TNF-mRNA expression, and both IL-2 and TNF-α, a potent inflammatory cytokines that play important roles in the early development of inflammation and hepatocyte damage [43]. While the cyclosporine-treated group revealed improvement in total protein concentration due to the drug significantly down-regulated the expression of both IL-2 and TNF-α, thus ameliorating histological alterations in the liver and inhibiting the production of pro-inflammatory cytokines, resulting in optimization of hepatic function and subsequent elevation of total protein concentration [43]. Of interest, it was well known that cyclosporine exerted an anti-inflammatory effect besides its immunosuppressive activity and could prevent apoptotic effects by binding to cyclophilin-1 inside the cells to form the cyclosporine-cyclophilin complex. Subsequently, this complex inhibited calcineurin, which stopped the activation and dephosphorylation of NFAT, responsible for causing inflammatory reactions [44]. Similarly, the group that was treated with azacitidine showed an elevation in total protein concentration. This implied that azacitidine has not only immunomodulatory properties but also anti-inflammatory and anti-oxidant effects might provide protective benefits [45]. It was speculated that treatment with azacitidine could decrease the methylation level and restore the expression of anti-oxidative genes [28]. Recent studies found that inhibition of DNMTs by azacitidine suppressed the expression of pro-inflammatory cytokines and might result in enhanced expression of anti-inflammatory genes [45, 46].

In another hand, both MCV and RDW% appeared normal in the positive control group, which has been clearly attributed to the well-documented data that aplastic anemia considered one of the causes of normocytic anemia because most normocytic anemia appeared to be the outcome of impaired production of RBCs [47, 48]. Besides, MCH and MCHC were markedly depleted because aplastic anemia considered a hypoproliferative disorder, was recognized by an inappropriately low reticulocyte count, in which cellular depletion and a reduced production of blood cells occurred, as well as a low MCHC showed that red blood cells did not have enough hemoglobin [49]. Whereas, the group that was received cyclosporine exhibited significant elevations in MCH and MCHC values; revealed the presence of a significant increase of RBCs and Hb, which may be a result of an increase in hematopoietic tissue due to cyclosporine's stimulation of hematopoiesis and these findings supported cyclosporine's direct positive effect on signal transduction pathways in HSPC, thus indirectly increased MCH and MCHC [50]. Furthermore, MCV and RDW%, however, showed typical values. It was demonstrated that at therapeutic doses, cyclosporine was bound in the cytoplasm of erythrocytes and had no effect on the cell membrane or its capacity to deform. These findings could aid in improving our comprehension of how cyclosporine interacts with cells [51]. Meanwhile, the MCH and MCHC showed improved values in group that treated with azacitidine, which is a pyrimidine nucleoside analog, inhibited DNMT, thus resulting in a reduction of DNA methylation and altered gene expression that might, in turn, help to restore normal hematopoiesis [52]. As well, the combined direct effects of the drug on both the erythroid precursor and progenitor compartments resulted in an increase in HbF synthesis, consequently MCH and MCHC values improved [53]. However, MCV and RDW%, revealed typical values.

The deleterious histopathology changes in the BM of the positive control group attributed to the disease condition in which over-activated T-cell that related to hematopoietic failure, leading to hypocellularity and enhanced adipogenic differentiation of MSCs in BM, consequently, the over-activated CD8+ T cell driven adipogenesis could be the fundamental factor for the fatty BM [54]. Interestingly, CTLs played a critical role in BM destruction during aplastic anemia by releasing several deregulated cytokines and chemokine’s as a result of T-cell subset expansion or as a direct cause of immune response polarization and, ultimately, BM growth inhibition [55]. Furthermore, IL-2 and IFN-gamma and TNF-α levels in the BM increased, which reduced BM growth because of increased apoptosis through induction of Fas expression on HSPCs, and by inducing nitric oxide synthase and production of nitric oxide [28]. On the other hand, disruption of the HSC microenvironment, as well as decrease stromal cell growth and function in the BM, may lead to the progression of BM aplasia [56]. Obviously, the formation of free radicals has been suggested to be causally related to aplastic anemia and may be reflective of the dynamic ability of ROS to cause BMF or at least perturbations of its functions [57, 58]. Meanwhile, the histopathology for hepatic tissue in the positive control group could be due to the activation of circulating cytotoxic T cells, which tend to accumulate in the liver and intrahepatic accumulation of cytotoxic T cells

strongly causing the damage to hepatocytes [40, 59]. Moreover a study revealed that IL-2 significantly induced intrahepatic TNF-α mRNA expression and both IL-2 and TNF-α play critical role in the early development of inflammation and hepatocyte damage [43]. Of note, data suggested that IL-2 would induce secondary cytokines though Kupffer cell activation and would thereby alter leukocyte-endothelial interactions and ultimately obstructing hepatic blood flow, as well as activated Kupffer cells produced a variety of mediators such as active oxygen radicals, TNF-α, IL-l, IL-6 and several eicosanoids that are potentially important in mediating hepatic toxicities [60, 61]. Whereas, the cyclosporine-treated group demonstrated severe BM cellularity as a result of drug immunosuppressive properties, that implicated in selectively inhibiting calcineurin, impairing the transcription of interleukin-2 and other cytokines in T lymphocytes and hence interfere with T cell activation, proliferation, and differentiation, so that it shown to reduce the infiltration of T cells into BM, thus ameliorated BM defects and maintained functional hematopoietic microenvironment via inhibiting a key transcription factor of adipogenesis [44]. In addition to BM, cyclosporine ameliorated the histological alterations in the liver and inhibited the production of pro-inflammatory cytokines like IL-2, IL-1a and 1b, IL-6, IFN-γ, and TNF-α and other lymphokines [43, 62]. Promptly, the azacitidine-treated group's BM demonstrated increased in cellularity, which implied by a number of studies have shown that azacitidine via epigenetic modulation inhibited T-cell proliferation and activation, thereby decreasing the production of pro-inflammatory cytokines [63]. In another words, azacitidine has the ability in declining the proliferation and differentiation of adipocyte, via the down regulation of PPAR-γ and FABP4 in BM and the up regulation of GATA2 (an anti-adipogenic gene). As such, the reconstruction of BM microenvironment and repopulating capacity of HSCs ultimately enhanced [36]. Moreover, the protective benefits of azacitidine on hepatic tissue associated with its immunomodulatory, anti-inflammatory and anti-oxidative effects via increased level of GSH with a concomitant decreased of MDA level, suggesting that the oxidative stress was attenuated and liver injury alleviated [45]. It is interesting to note that the combination therapy showed better attenuation of histopathologic changes in the BM and hepatic tissues, which may have been achieved by reducing intra-BM and intra-hepatic T cell infiltration as well as by suppressing BM adipogenesis. This restoration of immune balance was then necessary to maintain a functional tissue microenvironment and ultimately prevent deleterious changes overall via synergistic co-treatment.

Conclusion

The combination of cyclosporine and azacitidine treatment exerted strong therapeutic effects on the improvement of body weight, total BM cells, total protein as well as histopathological changes of the BM and hepatic tissues. Chiefly through dose and toxicity reduction.

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