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Enhancement of Cyclosporine Loaded with Chitosan Nanoparticles on Some Hematologic Parameters of Benzene-Induced Aplastic Anemia in Adult Male Dogs.

[1]Essa D Alhtheal, [2]Sufian Saleh Salman

[1]Nanotechnology and advanced material research Center, University of Technology-Iraq, Baghdad, Iraq
[2] College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

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Abstract : Aplastic anemia, characterized by a deficiency of hematopoietic stem cells, leads to pancytopenia in peripheral blood and bone marrow cells. The aims of this study to evaluate the therapeutic efficacy of cyclosporine alone, cyclosporine loaded with chitosan nanoparticles, and chitosan nanoparticles alone, with emphasing on restoring normal blood cell levels and preventing disease complications. Twenty five adult male dogs aged from 12 to 24 month were randomly divided into five groups: the first group was control negative (C-), given only distilled water, and the positive control (C+), which was untreated. The first treatment group (20 mg/kg) treated with chitosan nanoparticles, the second treatment group was with cyclosporin (5 mg/kg) alone, and the third treatment group was with cyclosporin loaded with chitosan nanoparticles (chitosan nano particles 5mg + cyclosporine 20mg/kg for each one). After a 60-day treatmentcourse. Assessments of adult male canines included serum IL2 levels and a complete blood count.When compared to group C+, all treated groups exhibited a signifiantly impr oved in RBCs, Hct, Hb, and RDWc. The group treated with cyclosporine loaded with chitosan nanoparticl es shown the strongest responseto treatment when compared to the other trea drop in IL2 levels, which tment groups, the cyclosporine group loaded with chitosan nanoparticles showed a considerable was in close agreement with the negative control. In conclusion, the research shows that cyclosporine loaded onto chitosan nanoparticles has the best therapeutic effect in treating benzene-induced aplastic anemia in adult male dogs. The treatment showed an improvement in all the factors tested, which indicating the nanomaterial role of the in delivering the treatment to the desired target.

Keywords: Chitosan Nanoparticles, Biomedical, Drug delivery male dogs , Benzene-induced aplastic anemia.

Corresponding author: (Email: 11645@uotechnology.edu.iq**)**

Introduction

Aplastic anemia in dogs is a life-threatening disease that results from the inability of the bone marrow to replenish all three essential cells in the peripheral blood supply and the presence of subcellular bone marrow (28) (6). These cells include red blood cells responsible for carrying oxygen to the body's tissues; White blood cells are responsible for protecting the body from injuries and infections. Platelets are responsible for preventing bleeding through primary hemostasis. Aplastic anemia is also called aplastic pancytopenia(37) (6). The bone marrow spaces that lack these important progenitor cells are also replaced with adipose tissue(38)(37). Those clinically affected are usually young and present

with non-specific signs such as weight loss and possibly vomiting and lethargy. The most frequent clinical signs and symptoms are bleeding tendencies, or petechiae, which are associated with thrombocytopenia. The degeneration of stem cells, a genetic abnormality in the marrow microenvironment, including vascular components, or dysregulation of the cells' production of aberrant humoral mediators or other cellular products are thought to be the causes of bone marrow aplasia (31)(6) .

During the 1970s, nanotechnology was considered seen as useful tool for creating special materials. One to one hundred nanometers was the range of their sizes (27); (39). Additionally, it was involved in a variety of sectors, including drug delivery, biology, and agriculture (19) (33) . The large surface area, higher potency, and bioactivity of nanoparticles, along with their controlled molecule size and excellent drug delivery, have been confirmed by numerous articles and references as reasons why they are more beneficial and effective than bulk materials (40) (20) These characteristics are helpful for biological applications such "tissues engineering, medication delivery and targeting, wound healing, and Nano biotechnology". "Chitosan" has gained popularity as drug delivery ingredient and delivering other macromolecules due to its biological and physicochemical qualities (21) (35). The synergistic effect of the combination treatment between cyclosporine and chitosan, cyclosporine make the immunosuppressant effect that reduce the inflammatory response and prevent occurrence of chemical mediators of inflammation and proinflammatory cytokine especially tumor necrotic factor alpha and IL 2, and this action of cyclosporine give or promotes the chitosan to be giving a good action during its work by act as antioxidant, anti-bacterial, anti-viral effect, and this agreement with (41), who conclude that Stanozolol combined with Cyclosporine is more effective than Stanozolol alone in treatment of aplastic anemia.

The present study aims to determine the efficiency of cyclosporine loaded chitosan nanoparticales for treating the induced aplastic anemia , compare the effect of cyclosporine loaded with chitosan nanoparticles with their alone doses and investigate the possible side effects of the used treatment regimens.

Materials and Methods

Induction of Plastic Anemia

Male adult dogs 12-24 month were given an oral dose of 2 ml/kg of benzene for fifteen days to cause aplastic anemia (28)(8). After the end of the induction period, 20 adult male dogs were examined and a complete blood count was measured to confirm the induction of the disease and 5 adult male dogs act as negative group.

Animals of study

Twenty five clinically healthy local breed mongrel adult male dogs aged from 12 to 24 month. The study included participants with body weights between 9 and 20 kg (average: 15.63 ± 0.78 kg), acquired from a commercial provider in the province of Baghdad. During the period from November (2022) to April (2023). All dogs were subjected to detailed clinical examination daily according to with a special attention to body temperature, heart rate and respiratory rate and housed in the animal house of the College of Veterinary Medicine, University of Baghdad, where they experienced controlled environmental conditions, the animals were housed in cages Approximately two weeks before the experiment started, during which

time they were fed regular dog food—meat and bread—twice a day in the morning and evening. (36) (22).

The Department of Internal and Preventive Medicine/College of Veterinary Medicine, University of Baghdad, Committee of Research Ethics, approved the study (PG/ 314 on 13/2/2024).

Experimental design

The current study was designed into three main parts:

Part One: preparation of chitosan and cyclosporine and characterization of chitosan nanoparticles

Part Two: Inducing aplastic anemia in dogs using benzene experimentally.

Part Three: Evaluation of the activity of chitosan nanoparticles, cyclosporine and loading of cyclosporine on chitosan nanoparticles in treating of aplastic anemia.

G(1): Contral negative

In group 2.3.4.5 induce aplastic anemia by uses Benzene 2 ml / kg day for 15 days.

G(2): Contral positive (Benzene 2 ml/kg BW orally for 15 days)

G(3): T1 (20 m /kg Chitosain nanoparticle orally for 60 days)

G(4): T2 (5mg/kg cyclosporine orally for 60 days)

G(5): T3 (5mg/kg cyclosporine loaded with 20 Chitosain nanoparticle orally for 60 days).

Preparation of Nanoparticles and Loading

Chitosan Nanoparticle Preparation

The preparation of nanoparticles for loading materials was conducted at the Faculty of University of Technology, Baghdad, Iraq's Center for Nanotechnology and Advanced Materials Research. Using the modulating method of chitosan (Avonchem Ltd, UK, 99.99% purity, CAS: 9002-4, Batch No: AC00671K, 100MPa.s), four distinct concentrations were created (14). Chitosan nanoparticles 2g/1L (200 mg/100 ml) concentration of chitosan solution was made by mixing 200 mg of chitosan powder with 100 mL of deionized distilled water (DW) that included 1% v/v of acetic acid (CDH, India). The mixture was let to soak at room temperature for a full day. After that, a semi-colloidal solution formed after 30 minutes of continuous stirring with a magnetic bar in a hotplate stirrer at 900 rpm (11). After determining the need for NaOH (0.1 N, CDH, India) to lower the pH to 4.6 using a pH meter (Hanna Instruments SRL, Romania), the solution was sonicated for three minutes in a probe sonicator (29)(16) (13) (40)

Loading of Cyclosporine on Chitosan Nanoparticles

The loading of cyclosporine on chitosan was carried out using an ionic gelation method, using tripolyphosphate (TPP) solution as a linkage material following the procedures described by (13) (17) with some modifications. Initially, the TPP solution was prepared by using the method mentioned (34) which added 250 mg of sodium tripolyphosphate powder to 100 mL of deionized distilled water to obtain a ratio of 0.25% w/v. After that, 50 mg of cyclosporine was dissolved in 100 milliliteres of D.W. and then added dropwise at a 1:1 loading ratio of cyclosporine to chitosan in 100 milliliteres of chitosan solution. For 30 minutes at 900 rpm, the mixture was constantly swirled on a hot plate stirrer. After one minute of sonication, the mixture was put back to continuous stirring, and 10 milliliters of TPP at a 0.25% w/v concentration was added dropwise. Thirty more minutes were spent stirring the mixture. Cyclosporine was consequently loaded onto chitosan nanoparticles (29,10) .

Sampling

1- Blood sample collect.

Blood samples were obtained sterilely from the cephalic vein of each dog treated and apparently healthy groups , and a tube containing EDTA as an anticoagulant was used to perform blood tests at day zero and after 15 and 75 days. The following hematological parameters were performed using the automated hematology analyzer;(Red Blood Cell count (RBC) (10^{12}/l), Hemoglobin (g/l), Percentage of Hematocrit (Hct %) , Red blood cells Distribution width concentration (RDWc).

The blood samples were collected aseptically from the cephalic vein of each dog in a tube without anticoagulant to obtain the serum for biochemical tests to estimate the IL-2 in dogs.

2- Bone marrow aspiration examination

The bone marrow of all adult male dogs were examined before starting the experiment to ensure that the dogs free from any defect in the bone marrow.

Method for aspirating bone marrow from the sternum. Clean gloves, sterile gauze, a 10 mL syringe, and a 16-gauge needle are among the supplies. Pharmaceutical sedation was typically not necessary due to the short process, which could take one to two minutes. Only aggressive or agitated dogs required a small amount of xylazine administered intramuscularly (i.m.). 10–15 minutes study design.

The smears were fixed by wrapping them with absolute methanol for not less than 3 minutes and then stained by Giemsa Stain for 20 – 30 minutes, the slides were air dried and then examined under immersion oil objective. After that the bone marrow smears were evaluated according to (Blue 2002) and (6) depending on the following criteria: Myeloid : Erythroid (M:E) ratio by counting 500 cells in the smears where the normal M:E ratio in dogs is1:1 to 2:1 (Myeloid cells 25-55% Erythroid cells 8-14%)

Statistical Analysis.

The Statistical Analysis System-SAS (2018) program was used to detect the effect of different factors (group and time) in study parameters. The least significant difference-LSD was used to significant compare between means (ANOVA/ Two- way) on this study.

Results and Discussion

Loading of cyclosporine on Chitosan Nanoparticles

The nanoparticles were obtained from chitosan nanoparticles using an ultrasonic technique (Figure:1). shows the morphology of chitosan. The obtained chitosan solution contains almost spherical particles with a narrow size distribution. The average size of the chitosan particles was approximately 30 nm, as shown in (Figure:1). The Field emission scanning electron microscopy (FE-SEM images) indicate individual spheres with agglomeration and porosity. the homogeneous composition and distribution of CS nanoparticles are due to the high level of association between ultrasound and the chitosan nanoparticle molecule (38)(1)(3) (41) (18).

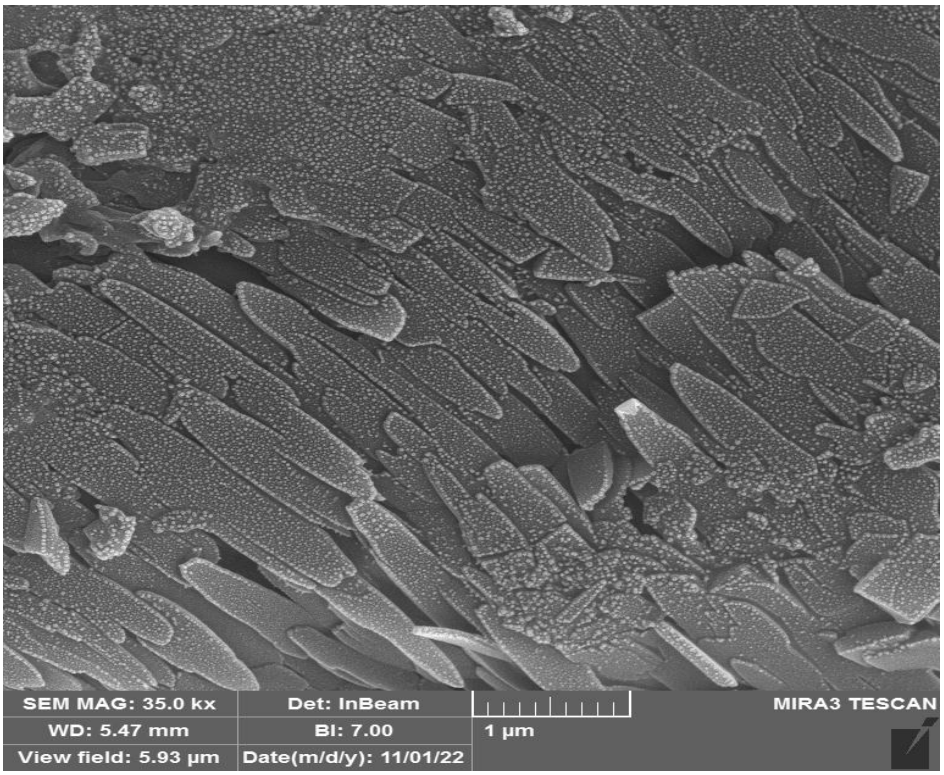

Figure (1): Loading of cyclosporine on Chitosan Nanoparticles

Complete blood count and level of IL-2.

The levels of RBC, Hct, Hb, and (RDWc) in the control positive group were ($P<0.05$) decreased in comparsion with all treatment groups and the negative control group. Furthermore, at 75 days, the cyclosporin loaded with chitosan nanoparticles group had a significantly ($P<0.05$) higher value in comparsion with all groups . According to (Tables 1, 2, 3, and 4). In (Figure:2) the outcomes of IL-2 were displayed. The positive control group's IL-2 was either considerably higher ($P < 0.05$) or had an intermediate value across all treatment groups. When cyclosporine was loaded onto chitosan nanoparticles, the treatment groups' mean IL-2 values were notably ($P<0.05$) lower than those of the positive control group. Additionally, the mean IL-2 level in the comparing the compound-treated group to the positive control group and all other treatment groups revealed a substantial ($P<0.05$) drop, whereas there was no significant change observed when comparing to the negative control group. The calculation of the Myeloid:Erythroid ratio shown in (Table:5) and (Figure:3). reveal significant differences ($P < 0.05$) between negative control group and other groups , as after induction of aplastic anemia the Myeloid:Erythroid ratio was decreased from 2 in day 0 to 0.61 in day 15 , from 2.13 to 0.76 , from 2.1 to 0.31 and from 1.98 to 0.72 in positive control , G3 , G4 and G5 groups, respectively (26) (12) (4) (23).

Table (1): Means ± SD of Red Blood Cells count(10^{10}/l)

Group	Mean ±SE of RBC (x10^6/ml)		
	0 Day	15 Days	75 Days
G1	6.50 ±0.26 AB a	6.86 ±0.43 A a	6.74 ±0.34 A a
G2	7.19 ±0.11 A a	3.27 ±0.35 B b	2.30 ±0.19 C b
G3	5.95 ±0.19 B a	2.91 ±0.34 B b	5.33 ±0.13 B a
G4	6.37 ±0.19 AB a	2.85 ±0.18 B c	5.21 ±0.26 B b
G5	7.25 ±0.40 A a	2.98 ±0.16 B c	7.63 ±0.19 A a
L.S.D. value = 1.082 * . Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).			

These results indicated that the decrease in erythrocyte count occurred in all groups in which aplastic anemia was induced on 15 day and in the positive control group compared to zero time and are consistent with (28,8); Durrani and Maciejewski (7) and (25) whom concluded that due to benzene poisoning, hematological indicators in the peripheral blood and bone marrow which affected as follows: A decrease in the number of red and white blood cells, platelets, and hemoglobin. The percentage of lymphocytes in the blood decreased and the percentage of neutrophils increased in all cases of poisoning groups, a decrease in bone marrow due to benzene, which led to a decrease in bone marrow cellularity and its slowdown cell maturation rate. While it was clearly observed in the three treatment groups and the time period at 75 days. Although there were some statistically significant differences between the red blood cell values, they all reached close to the normal reference limits in adult male dogs and this agreed with (32) and (9) who mentioned that the decrease in red blood cells, white blood cells, platelets, and hemoglobin and the percentage of lymphocytes in the blood decreased and the percentage of neutrophils increased in all cases of poisoning groups.

Table (2): Means ± SD of Hemoglobin concentration(g/l)

Group	Mean ±SE of Hb (g/dl)		
	0 Day	15 Days	75 Days
G1	16.30 ±0.54 A a	14.04 ±0.75 A b	14.87 ±0.39 A ab
G2	15.50 ±0.26 A a	6.21 ±0.46 B c	5.55 ±0.46 C c
G3	14.90 ±0.65 A a	6.15 ±0.49 B c	8.09 ±0.78 B b
G4	14.96 ±0.34 A a	6.58 ±0.58 B b	8.20 ±0.42 B b
G5	16.31 ±0.43 A a	5.73 ±0.31 B d	14.20 ±0.45 A b
L.S.D. value = 1.665 * . Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).			

All results were in agreement with (28)(8) (7) (9) and (25) that mentioned aplastic anemia causes a low number of blood cells which occurred in red blood cells, white blood cells, platelets, and hemoglobin and the percentages of cells decreased. Levels of blood lymphocytes and neutrophils increased in all intoxicated groups, with a decrease in bone marrow benzene, which resulted in a decrease in bone marrow cells and a slow rate of cell maturation.

Table (3): Means ± SD of Hematocrit test (%)

Group	Mean ±SE of HCT		
	0 Day	15 Days	75 Days
G1	41.74 ±1.04 A a	43.00 ±1.30 A a	43.40 ±0.92 A a
G2	40.61 ±1.38 A a	21.08 ±1.43 B bc	19.70 ±1.53 C c
G3	40.49 ±1.29 A a	18.08 ±1.44 B c	27.78 ±1.31 B b
G4	41.95 ±0.86 A a	19.34 ±1.73 B c	28.80 ±1.39 B b
G5	42.82 ±1.12 A a	17.43 ±0.73 B c	40.00 ±0.89 A a
L.S.D. value = 4.317 * . Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).			

The result agreed with (32)(5) (30) (24).Evaluation of RBCs, Hct, Hb, and (RDWc) is essential in identifying aplastic anemia and performing a bioassay for this disease; Therefore, low differential RBC counts and Hct, Hb, and RDWc ratios indicate aplastic anemia. As such, the lower RBC counts, Hct, Hb, and (RDWc) of the positive control group were linked to the disease state, which is associated with adverse hematopoietic effects. Resulting from the inability of the bone marrow to form blood cells. As a result, red blood cells were reduced to below normal levels, similar results reported by the study agreed with (2) (31) (28) (30).

Table (4): Means ± SD of Red blood cells Distribution width concentration (RDWc).

Group	Mean ±SE of RDWc		
	0 Day	15 Days	75 Days
G1	16.36 ±1.34 A a	16.33 ±1.34 A a	16.80 ±1.07 A a
G2	17.64 ±0.94 A a	13.60 ±2.90 B b	13.40 ±1.78 C b
G3	16.34 ±A1.05 A a	13.20 ±1.46 B b	15.80 ±0.97 B a
G4	16.66 ±0.72 A a	12.15 ±1.47 BC c	15.20 ±0.58 B ab
G5	17.34 ±1.48 A a	11.32 ±2.61 C c	16.85 ±1.73 A a
L.S.D. value = 2.263 * . Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).			

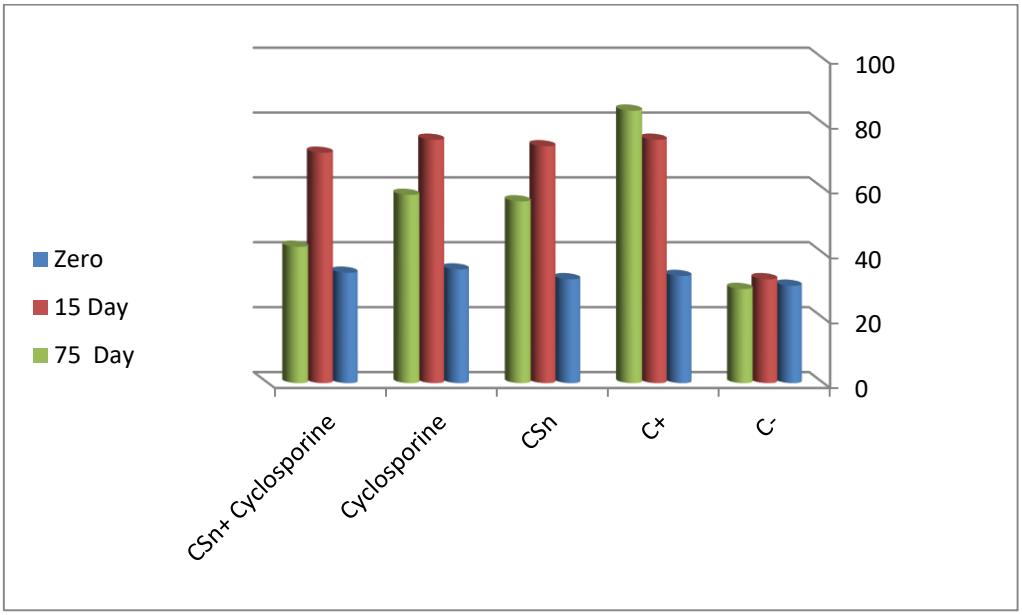

Figure (2): Effects of treated group compare negative and positive control group on serum IL-2 level (pg/ml)

The results for RBC count, PCV, hemoglobin, MCV, MCH, MCHC and RDWc indicated the following: The RBC count in the positive control group decreased significantly ($P < 0.05$) below the normal values in dogs, 5.7-8.5 (10^{12}/liter) and therefore the number of red blood cells can be considered normal even from a statistical standpoint there were significantly low values, and the results agreed with (36) (28) and (8).

Table (5): Means ± SD of Myeloid:Erythroid ratio

Group	Mean ±SE of M:E Ratio		
	0 Day	15 Days	75 Days
G1	1.88 A a	1.73 A a	1.93 A a
G2	2 A a	0.61 B b	0.72 B b
G3	2.13 A a	0.76 B c	1.66 A b
G4	2.1 A a	0.31 B c	1.72 A b
G5	1.98 A a	0.72 B b	2.05 A a
L.S.D. value = 0.817 * . Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).			

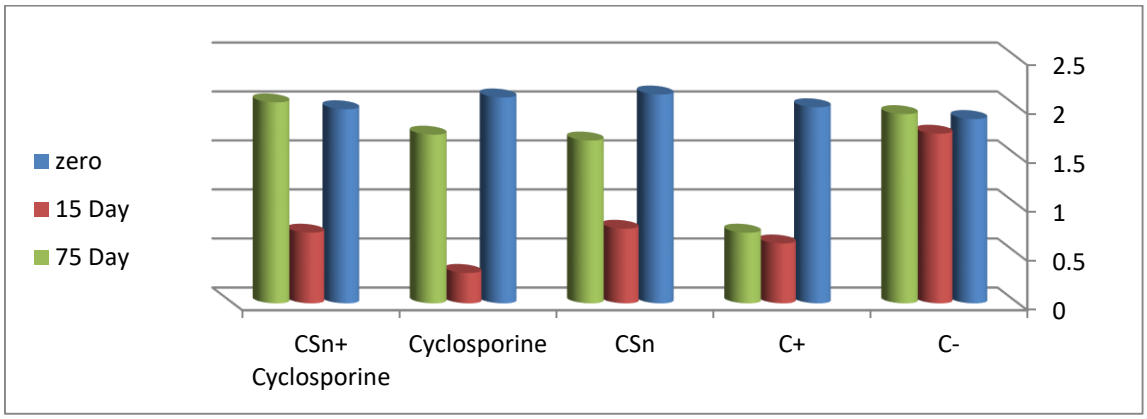

Figure (3): Effects of treated group compare negative and positive control groupf on M:E Ratio

These criteria are in agreement with (36) and indicated that there were no defects in the bone marrow in the dogs subjected to this study and that there was no bias that might occur when the bone marrow was damaged or defective. the results were consistent with (8) and (32), The rate of maturation of erythroid precursor cells also became slower by benzene intoxication, the percentages of cells were shifted toward the immature cells to yield a case of hypocellularity . Proerythroblasts showed significant decrease by benzene treatment in all croups compared to control ($P<0.01$), The bone marrow is an ordered environment with the hemopoietic stem cell being in close proximity to protective stromal cells. Ordered maturation of myeloid progenitors can be seen in relation to the normal hemopoietic stem cells and one of the diagnostic features of myelodysplasia is abnormal location of these immature precursor cells. In addition to these myeloid components, mature B and T cells are present which may exert significant effects on the stem cell compartment. Thus, hemopoietic stem cells are found in a relatively protected environment within the bone marrow. Oxygen and toxins are delivered by the vascular system, and the differentiating myeloid precursors, which are rich in myeloperoxidase, provide an environment which easily generates oxidative stress.

The combination of cyclosporine and chitosan nanoparticles considerably reduced the drop in IL-2 levels in the treated group compared to the single treatment; these results suggested that the two medications may have a synergistic immunosuppressive impact. (5) (15).

Conclusions

From the results of the current study, it can be concluded that benzene has side effects during the period of disease development. It was concluded that treating of cyclosporine loaded onto chitosan nanoparticles very important in treated aplastic anemia. Cyclosporin has a less therapeutic effect than the mixture in some cases of aplastic anemia in dogs. Still the best results were achieved by loading cyclosporine onto chitosan nanoparticles from all aspects and blood tests. , which indicates this. The nanoparticles had a clear effect in providing treatment in a faster period and a rapid recovery response.

Future research applications could be applied to humans and other important and essential animals. Nanotherapy is considered one of the most modern technologies. It reduces the doses used and quickly delivers the medicine to the target to achieve early recovery and reduce treatment periods.

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