



Antianemic Effect of Cyclosporine Loaded with Chitosan Nanoparticles on Induced Aplastic Anemia in Adult Male Dogs

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Abstract | The study of aplastic anemia was induced in adult male dogs using benzene. the efficacy of cyclosporine-laden chitosan nanoparticles in treating induced aplastic anemia was evaluated and the effect of cyclosporine loaded with chitosan nanoparticles with their doses alone was compared. The investigation of possible side effects of treatment regimens used. This study aimed to prepare chitosan nanoparticle and cyclosporine loaded on nanochitosan and pure chitosan. Pure chitosan was prepared using the gelation method. The appropriate cross-linking ratio and cross-linking times of 2% (w/v) chitosan CNP_s/cyclosporine nanofibers and solution were prepared. It was dissolved in deionized water at a weight ratio of 2G CNP_s diluted with 100 ml DW, and mixtures were prepared by stirring at 80 Co for three and adding five drops of acetic acid. The physical properties and chemical composition were characterized using FE-SEM. Preparation of chitosan nanoparticles with sizes ranging from 20 – 50 nanometers. For succession. Bone marrow examination, counting myeloid cells to nucleated red blood cells revealed significant differences ($P < 0.05$) between the negative control group and the other groups, where the myeloid cells to nucleated red blood cells after induction of aplastic anemia in adult male dogs decreased from 2 on day 0 to 0.61 On day 15 for the positive control group, from 2.13 to 0.8 for the first group, and from 2.1 to 0.3 for the second group and from 2 to 1.07 for the third group and in the induction stage before giving it the treatment compared to the negative control group, respectively. In a hematological examination, the means of RBC count ($10^{12}/l$) did not reveal differences in the negative control group. They were 6.5, 7.1, and 6.99 in three consecutive periods, respectively, while the positive control group, there were significant differences in RBC count ($10^{12}/l$) between zero time, 15-day, and 45-day periods, as it decreased from 7.3 to 3.24, then to 2.48, respectively the results of the blood test after a month of treatment confirmed that there was a clear response to the treatment for the three groups compared to the positive and negative control groups.

Keywords | Chitosan nanoparticles, Hematological parameters, Bone marrow analysis, Benzene-induced aplastic anemia, Cyclosporine

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Aplastic anemia in dogs is a life-threatening disease resulting from the bone marrow's inability to replenish all three major cell lines in the peripheral blood supply and presence of hypocellular bone marrow (Brazzell and Weiss 2006). These cells include erythrocytes, responsible for carrying oxygen to the tissues in the body; leukocytes, responsible for protecting the body from infections; and thrombocytes, responsible for preventing bleeding through primary hemostasis. Aplastic anemia is also more appropriately named aplastic pancytopenia since "pan" meaning "all" of the cell lineages are affected, not just the erythroid lineage as the term "anemia" implies (Brazzell and Weiss 2006). The bone marrow spaces that lack these important progenitor cells are also replaced with adipose tissue (Weiss 2003). Patients that are clinically affected usually are young and show very non-specific signs such as lethargy, weight loss, and possibly vomiting. Bleeding tendencies, such as petechiae, are the most common clinical manifestations and are directly related to thrombocytopenia. Bone marrow aplasia is believed to result from destruction of or genetic defect in stem cells, an altered marrow microenvironment including vascular and stromal components, and/or dysregulation of cell production from abnormal humoral mediators or other cellular products (Brazzell and Weiss 2006).

During the 1970s, nanotechnology was seen as a potentially useful tool for creating special materials. One to one hundred nanometers was the range of their sizes (Youssef *et al.*, 2020). Additionally, it was involved in a variety of sectors, including drug delivery, biology, and agriculture (Mohammed Badawi *et al.*, 2022; Underwood and van Eps 2012). 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 (Youssef *et al.*, 2019); (Num and Useh 2013).

According to (McMillan *et al.*, 2011), applying nanostructured materials in veterinary medicine has demonstrated great effects against bacterial strains and multi-resistant pathogens. Because chitosan is less hazardous and more biodegradable, it is advised for use in a variety of applications. In comparison to other biological polymers, it is also regarded as one of the polymers that is employed the most widely. Many types of literature confirmed that the synthesis of chitosan nanoparticles is primarily which based on the ionic gelation method, which involves the interaction of opposite charged macromolecules; chitosan nanoparticles are unique carriers for less soluble and poorly absorbed materials at the field of drug delivery systems (Shanmugarithnam and Puratchikody 2014).

In terms of distribution, use, and availability, chitosan is a naturally occurring polysaccharide that comes in second to cellulose (Younus and Abdallaha 2023; Mincea *et al.*, 2012). According to (Mourya and Inamdar 2008), chitosan is chemically composed of residues of glucosamine and N-acetyl glucosamine. After chitin is N-deacetylated, a linear polyamine saccharide known as chitosan [(1, 4)-2-amino-2-deoxy-D-glucan] is produced. According to (Illum *et al.*, 2001), chitin is a structural element of the exoskeletons of shrimp, lobsters, and crabs. It is also found in the cell walls of yeast, fungi, as well as in the pens of squid. The antimicrobial, pain-relieving, hemostasis-promoting, microorganism-inhibiting, and epidermal cell-growth characteristics of chitosan (Balakrishnan *et al.*, 2005; Howling *et al.*, 2001) are a little different. Their prospective uses in pharmaceutical and medical fields are of great interest. Chitosan's positive qualities like its biocompatibility, capacity to hold certain organic molecules, capability for enzymatic hydrolysis, and intrinsic physiological activity with along its non-toxicities are the reasons for the surge in interest in the material (Wang *et al.*, 2003). 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 (Okay 2010).

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

METHODOLOGY

STUDY DESIGN

The study was applied to 25 healthy adult male dogs of a local breed. Every animal's health was regularly checked both before and during the study by clinical examination and all experimental animals were clinically examined and housed in the Animal House belongs to the Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Baghdad, where they experienced controlled environmental conditions characterized by moderate temperature and 12 hours light and 12 hours dark cycle. As a part of the acclimatization process, the animals were housed in cages (1×2×1 m³) for a duration of two weeks prior to the commencement of the experiment, in which they were provided with standard dog food, meat, and bread, offered twice daily (morning and evening), and had constant access to tap water throughout the experimental period. A prophylactic anthelmintic treatment (oral drontal

plus, prazquantal 50 mg/febantel 150mg /pyrantel 144 mg) and injected intramuscularly with a combined preparation of linco- spectin (lincomycin 50mg andspectinomycin 100mg) was provided before starting the experiment.

INDUCTION OF APLASTIC ANEMIA

The study was conducted on five groups of adult male dogs over six months. The first group was the negative control group and left without inducing aplastic anemia. The other four groups, which were from the second group to the fifth, respectively, these groups were exposed to aplastic anemia induced with 2ml/Kg of benzene for 15 dose (i.e. for 15 days), the dose and duration have been chosen according to our experience and literature survey (Elsayed 2017).

GROUPING OF THE ANIMALS

The dogs were divided into five in adult male dogs, and five were included in each group. The first group was a negative control group that was discharged without receiving any medication; the second group was a positive control group that was given aplastic anemia; the third group (T1) received oral chitosan nanoparticles (20 mg/kg) for 30 days following the occurrence of aplastic anemia; the fourth group (T2) received oral cyclosporine (5 mg/kg) for 30 days following the occurrence of aplastic anemia; and the fifth and final group (T3) received oral cyclosporine loaded with chitosan nanoparticles (5 mg cyclosporine/20 mg of chitosan nanoparticle).

PREPARATION OF CHITOSAN NANOPARTICLES

Three different concentrations were prepared from a solution of Chitosan provided by GLENTHM company according to the modulating method (H.Nm Mustafa 2021) (Mustafa 2021; Okay 2010), where the concentrations of 0.5 , 1 , 1.5, and 2 mg/ml of chitosan solutions were prepared by adding 25 , 50, 75 and 100 mg of chitosan powder to 50 ml deionized distilled water contains 1% acetic acid for each and left for 24 hours at room temperature, after that mixed by magnetic stirring on hotplate stirrer for 30 minutes at 900 rpm, leading to a semi- colloidal solution. The pH was adjusted at 4.6, and then the fluid was filtered using filter paper (400–800 nm) after being subjected to sonication for three minutes using a probe sonication(Pires *et al* 2014).

LOADING OF CYCLOSPORINE ON CHITOSAN NANOPARTICLES

According to previous studies (Ali *et al.*, 2018; Ibrahim *et al.*, 2015) 50 mg of To achieve a 1:4 loading ratio of cyclosporine with Chitosan, 1 ml of acetone was dissolved, and the mixture was then slowly distilled into 50 ml of a Chitosan solution (2 mg/ml) while being continuously stirred in a heated plate stirrer for 30 minutes at 900 rpm. To allow the cyclosporine molecules to adsorb on the surface of the

chitosan particles, 10 ml of TPP (0.25%) was added at a 5:1 ratio of solution by slow distillation, and the mixture was stirred continuously for 30 minutes. Next, the solution was subjected to sonication for a minute. Subsequently, a 1:4 (5:20) loading ratio of cyclosporine to Chitosan was obtained by slowly distilling 250 mg of cyclosporine in 1 ml of distilled water while “continuously stirring on a heated plate stirrer for 30 minutes at 900 rpm. After that, the mixture was subjected to sonication for a minute”.

In order allowing the cyclosporine to adsorb on the surface of the chitosan particles, the solution was put back to continuous stirring, 10 ml of TPP (0.25%) was added at a ratio of 5:1 of solution by gradual distillation, and stirring was left for 30 minutes (Saeed *et al.*, 2023).

HEMATOLOGICAL EXAMINATION

The blood samples were obtained from the cephalic vein in tubes supplemented with EDTA on days 0, 15, and 45 of the experiment. Then subjected to a complete blood examination by the vet. The automated hematology analyzer examined the following parameters: Blood samples from the cephalic vein were collected in tubes supplemented with EDTA on days 0, 15, and 45 of the trial. Then, he undergoes a complete blood test with a veterinarian. Automated hematology analyzer, where the following parameters were examined and include Red Blood Cell count, hemoglobin, percentage of Hematocrit (MCV, MCH, MCHC)

BONE MARROW EXAMINATION

The bone marrow of all dogs was examined before starting the experiment only to ensure that the dogs were free from any defect in bone marrow, e.g., depression or destruction, to exclude any bias that may occur. Bone marrow aspiration biopsy was performed according to (Blue 2002). After that, the following parameters were examined in the bone marrow smears: estimation of cellularity, M: E ratio, Maturation of cellular lineages (erythropoiesis), Presence of dysplastic changes, and Presence of parasites.

STATISTICAL ANALYSIS

Using the Statistics software version 8.1, the data was statistically analyzed using one-way analysis of variance (ANOVA) appropriate to design (CRD) totally and correlation coefficient. Least significant difference (LSD) was used to compare mean values at $P \leq 0.05$. and compared the results according to (Steel and Torrie 1980) by using the SPSS system.

RESULTS AND DISCUSSION

RESULTS OF PREPARING OF CHITOSAN NANOPARTICLES AND LOADING OF CYCLOSPORINE ON CHITOSAN NANOPARTICLES

Preparation of chitosan nanoparticles loaded with cyclo-

sporine revealed nanoparticles at measures ranging between 20 – 50 nm (see Figure 1 and Figure 2), which is considered a good size of nanoparticles, as the acceptable Nano scale of particles should be less than 100 nm (Mohammed and Rafeeq 2023; Saeed *et al.*, 2023).

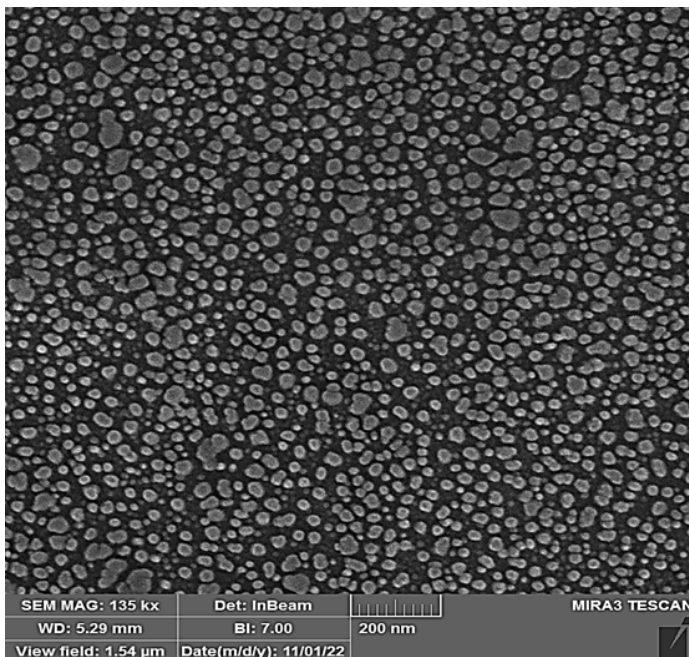


Figure 1: FE-SEM images of chitosan nanoparticles.

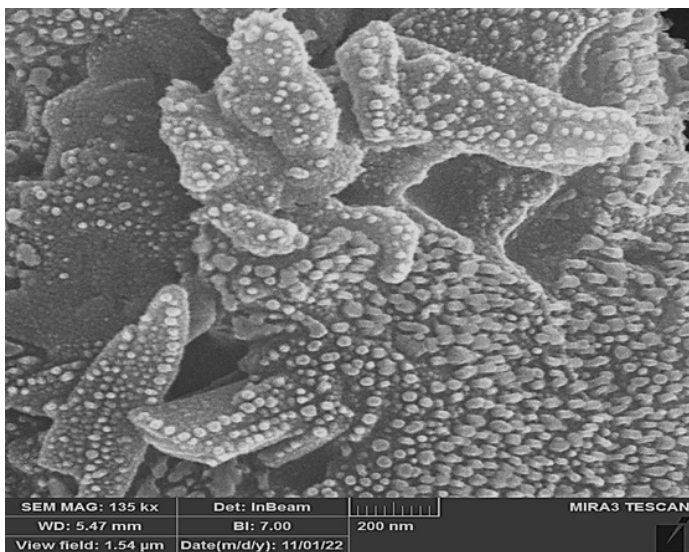


Figure 2: FE-SEM images of cyclosporine loaded chitosan nanoparticles.

RESULTS OF BONE MARROW EXAMINATION

Estimation of cellularity: In the negative control group, the cellularity remained within reference limits of dogs, which ranged between 25 – 75 % (Grondin *et al.*, 2010), throughout the experiment, and on the contrary, the cellularity decreased significantly in the other groups after exposure to benzene reaching to about 10 %, as shown in Figure 3.

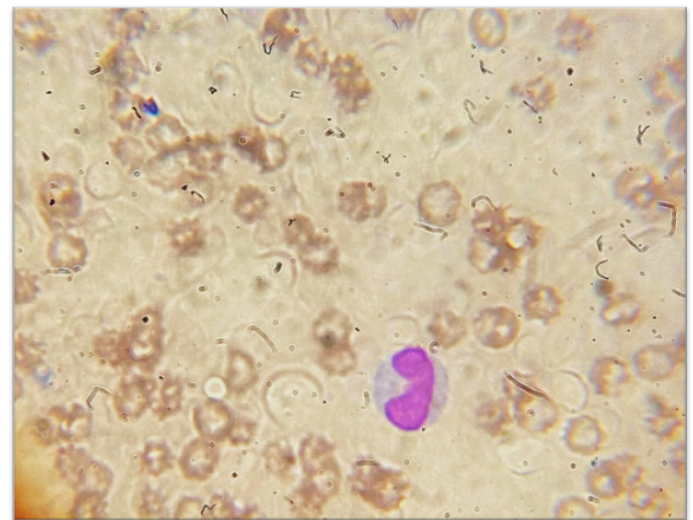


Figure 3: The cellularity of a bone marrow smear in PC group after induction of aplastic anemia by using of benzene.

In the T1 group, after administration of benzene, the cellularity was restored to about 20 %, but it remained under the reference limits.

In the T2 and T3 groups, the bone marrow restored near to its norm cellularity after 45 days, reaching about 25 %, as in adults, the normal cellularity is 25% (Weiss and Wardrop 2010), as shown in Figure 4.

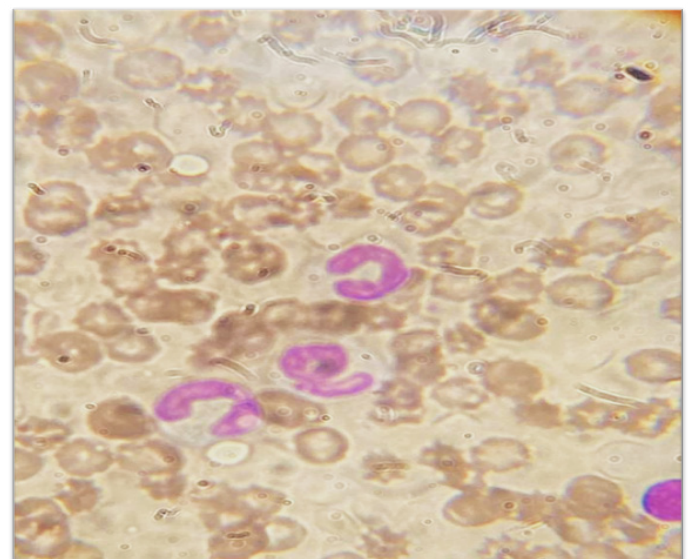


Figure 4: The cellularity of a bone marrow smear in T 3 group after treatment by cyclosporine - loaded chitosan nanoparticles, which estimated as 30 %.

MYELOID: Erythroid ratio: The calculation of M:E ratio reveal significant differences ($P < 0.05$) between negative control group and other groups , as shown in Table. 1. as after induction of aplastic anemia the Myeloid:Erythroid

ratio was decreased from 2 in day 0 to 0.62 in day 15 , from 2.13 to 0.8 , from 2.1 to 0.3 and from 2 to 1.06 in positive control , T 1 , T 2 and T 3 groups, respectively.

Table 1: Myeloid:Erythroid ratio of all groups in the three periods.

Time	Negative control	Positive control	T1	T2	T3
0-day	1.88	2	2.13	2.1	2
15-day	1.55	0.62 aA	0.80 aA	0.30 aA	1.07 a
45-day	1.72 A	0.88 a	2.90 B	1.59 A	1.92 A

The small letter refer to significant differences ($P < 0.05$) in the rows. Different capital letters refer to significant differences ($P < 0.05$) in the columns.

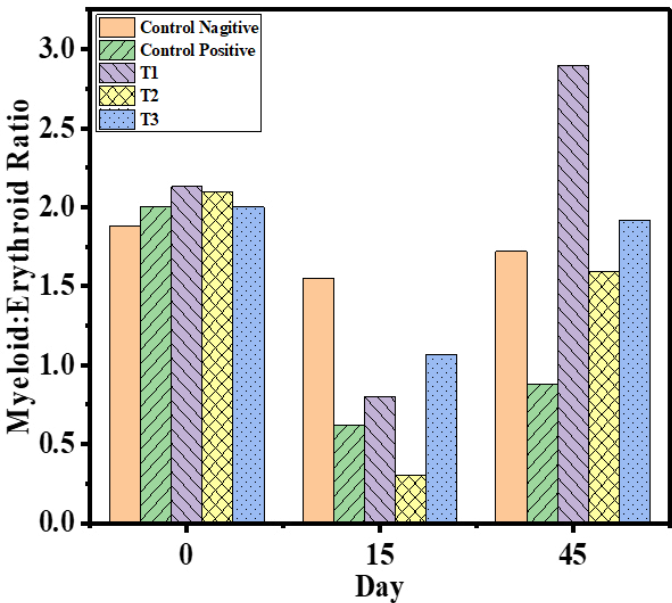


Figure 5: Result of Myeloid:Erythroid ratio.

MATURATION CELLULAR LINEAGE: Every step of elytroid maturation was observed in the current investigation, and as every stage was impacted by benzene and reacted uniformly to treatment, no variations in the effect of benzene on distinct maturation stages were noted.

DYSPLASTIC ALTERATIONS ARE PRESENT: In this investigation, dysplastic alterations were not seen.

THE EXISTENCE OF PARASITES: The bone marrow smears showed no signs of infection or parasite infestation. The hepatotoxicity effects of benzene, which harm hematopoietic stem cells (HSCs), are responsible for the bone marrow examination results. Its metabolites are a major factor in bone marrow (BM) depression, which is what led to the hypo cellularity seen in this study (Hamzah et al., 2019). Additionally, the bone marrow's HSCs with

varying differentiation stages (i.e., distinct lineages) are susceptible to hematological damage caused by benzene. A body of research indicates that benzene and its metabolites cause bone marrow cells to die, severely damaging the bone marrow and resulting in cellular aplasia (Atsdr 2020). Studies have shown that benzene and its metabolites induce various adverse effects in HSCs, such as chromosomal aberration, apoptosis, oxidative stress, inhibition of the cell cycle, and DNA damage (Wang et al., 2012). As a result, benzene affected all lineages equally and there were no differences observed in the effect of benzene on different maturation stages in the present study. This induced toxicity toward HSCs includes direct short- and long-term damage to HSC progenitors as well as effects on the bone marrow hematopoietic microenvironment.

The current study's significant decrease in the M:E ratio after benzene administration can be explained by the pathological analysis of a previous study, which found that benzene exposure promoted elytroid hyperplasia and inhibited granular hyperplasia in bone marrow (Sun et al., 2014). The reference limits of the M: E ratio in dogs are 1.8 ± 0.61 (Grondinet al., 2010). However, after treatment with cyclosporine-loaded chitosan nanoparticles at day 45, the ratio returned to near normal, indicating the therapeutic effect of the nanoparticles.

These results confirm that cyclosporine can be an efficacious drug for patients with refractory aplastic anemia, and that the long term treatment with a relatively low dose of cyclosporine may be essential for obtaining a high response rate (Al-Ghazaly et al., 2005) and reinforce the belief that immunosuppressive therapy was markedly effective for pancytopenia (Perkins et al., 2001) , as mainly the aplastic anemia resulted from T- cells mediated immune destruction of bone marrow cells, therefore aplastic anemia can be effectively treated by stem cells transplantation and immunosuppressive drugs such as cyclosporine , as it block T- Cells function , therefore this researcher with his colleagues found that approximately half of patients with severe aplastic anemia treated with anti-thymocyte globulin and cyclosporine have durable recoverable and excellent long-term survival (Bacigalupo et al., 1995; Rosenfeld et al., 2003), especially when loaded on nanoparticles, as they are potential carriers for drug delivery, imaging molecules, and even genes (Badawi et al., 2022) .

HEMATOLOGICAL EXAMINATION

As shown in Tables 2, 3, and 4, the means of RBC count ($10^{12}/l$) did not reveal differences in the negative control group. They were 6.5, 7.1, and 6.99 in the three consecutive periods, respectively, while in the positive control group, there was a significant difference in RBC count ($10^{12}/l$)

Table 2: Results of hematological examination in 0-day.

T3 group	T2 group	T1 group	Positive control group	Negative control group	Parameters
7.87	6.37	5.95	7.3	6.5	RBC ($10^{12}/l$)
16.7	14.8	14.64	15.5	16.3	Hemoglobin (g/l)
45.95	43.4	38.8	39.75	41.65	Percentage of Hematocrit (Hct)(%)
72	74	75	74	64	MCV (fL)
25.2	23.2	25.8	22.5	23.5	MCH (pg.)
35.4	33.2	38.2	36.7	36	MCHC (g/l)

Table 3: Results of hematological examination in 15-day.

T3 group	T2 group	T1 group	Positive control group	Negative control group	Parameters
3.19 a	3.68 a	2.74 a	3.24 a	7.1	RBC ($10^{12}/l$)
7.8 a	6.3 a	6.5 a	7.7 a	15.9	Hemoglobin (g/l)
22.52 a	18.44 a	19.08 a	22.74 a	43	Percentage of Hematocrit(Hct %)
68	70	67	70	68	MCV (fL)
24.4	24.2	23.9	23.6	27.7	MCH (pg.)
34.5	33.7	34.3	33.7	38.7	MCHC (g/l)

The small letter refers to significant differences ($P < 0.05$) in the rows.

Table 4: Results of hematological examination in 45-day.

T3 group	T2 group	T1 group	Positive control group	Negative control group	Parameters
4.95 a	4.89 a	4.69 a	2.48 a	6.99	RBC ($10^{12}/l$)
12.6 b	11 b	10.2 b	6.3 a	16.2	Hemoglobin (g/l)
30.98 b	28.94 b	28.22 b	18.44 a	45.02	Percentage of Hematocrit (Hct)(%)
71	74	70.3	62.1	74	MCV (fL)
27.5	28.1	27.9	28	26.7	MCH (pg)
40.5	37.8	37.8	37.7	36.2	MCHC (g/l)

The different small letter refers to significant differences ($P < 0.05$) in the rows.

between zero time, 15-day, and 45-day periods, as it decreased from 7.3 to 3.24, then to 2.48, respectively.

On the other hand, in the T 1, T 2, and T 3 groups, the RBC count ($10^{12}/l$) were 7.87, 6.37, and 5.95 and decreased to 3.19, 3.68, and 2.74, respectively, after induced aplastic anemia 15 days after the occurrence of aplastic anemia. In comparison, they return near reference limits after 45 days of treatment by chitosan to 4.69, cyclosporine to 4.89, and cyclosporine-loaded chitosan nanoparticles to 4.95, but without significant differences ($P < 0.05$).

The means of Hemoglobin (g/l) did not reveal significant differences in the negative control group, and they were 16.3, 15.9, and 16.2 in the three consecutive periods, respectively. On the other hand, in the positive control group, there was a significant difference in Hemoglobin (g/l) between the zero time, 15 days, and 45 days after the occurrence of aplastic anemia, as it decreased from 15.5 to 7.7

and 6.3, respectively. On the other hand, in the T 1, T 2, and T 3 groups, the hemoglobin values (g/l) were 14.64, 14.8, and 16.7 on day 0 and decreased to 6.5, 6.3, and 7.8 after induced aplastic anemia on day 15. However, they increased and returned to normal reference limits after 45 days of treatment with chitosan 10.2 and cyclosporine 11 and 12.6 by cyclosporine-loaded chitosan nanoparticles.

The means of Hematocrit (Hct) did not reveal significant differences in the negative control group, as they were 41.65, 43, and 45.02 in the three consecutive periods, respectively. On the other hand, in the positive control group, there were significant differences in the Percentage of Hct between zero time, 15 days, and 45 days periods, as it decreased from 39.75% to 22.74 % and then to 18.44%, respectively. The results of Hct in T 1, T 2, and T 3 groups were 38.8%, 43.4%, and 45.95% on day 0, while 15 days after the occurrence of the aplastic anemia, they decreased to 19.08%, 18.44%, and 22.52%, respectively.

Table 5: Comprehensive discussion of comparisons with existing literature.

Reference	Time tretment	RBC	Hemoglobin	Method	Nps	No.
(Jia <i>et al</i> 2020)	15 days	1-6.1 (Cn) 2-3.30(AA+Nacl) 3-4.27(AA+Epo) 4-4.57(AA+ GF-NPs)	1-12.4(Cn) 2-6.70(AA+Nacl) 3-8.68 (AA+Epo) 4-9.30(AA+ GF-NPs)	1-water-soluble nanoparticles (GFNPs) 2- a simple sol-id-liquid reaction to modify gad-ofullerene	Gadofullerene nanoparticles	1
(Rodri-guez-Cobos and Atencia 2023)	24 days	7.92	1-15.9	drug	Eeltrombopag	2
(Zangeneh <i>et al</i> 2019)	Non 24	1-6.1 (Cn) 2- 4.57(Nacl) 3-5.90 (Mentha piperita)	1-15 (Cn) 2- 8 (Nacl) 3-12.3 (Mentha piperita)	New health-pro-moting products (nutraceuticals, cosmetics, and pharmaceutical products)	Mentha piperita	3
(Rezk, Mo-hamed, and Ammar 2018)	7 days	1-5.40 (Cn) 2-3.98 (Thorium)	1-9.03 (Cn) 2-7.55 (Thorium)	a naturally-occur-ring radioactive meta	Thorium	4
(Mahmoud 2022)	two week (5days/week)	1-4.74 (cn) 2- 2.78 treted of CeONPs	1-13.66 (cn) 2- 9.23 treted of CeONPs	CeONPs	CeONPs	5
Current Work	30 days	1-6.99(Cn) 2-2.48 (AA+Nacl) 3-4.95(AA+ Chi-tosan nanoparticles &cyclosporine).	1-16.2(Cn) 2-6.30(AA+Nacl) 3-12.6(AA+ Chi-tosan nanoparticles &cyclosporine)	Gelation methoeds	Chitosan nano-particles &cyclo-sporine	6

Table 5 shows a more comprehensive discussion of comparisons with existing literature.

In the third period (45 days after the occurrence of the aplastic anemia), the Hct increased after treatment by chitosan to 28.22% and by cyclosporine to 28.94% and to 30.9 by cyclosporine-loaded chitosan nanoparticles. As shown in **Tables 2, 3, and 4**, the means of MCV revealed no significant differences, in the negative control group, and they were 64, 68, and 74 fl in the three consecutive periods, respectively.

In the positive control group, there was a significant difference in MCV between day 0, day 15, and day 45; it decreased from 74 to 70, then to 62.1 fl., respectively. On the other hand, in the T 1, T 2, and T 3 groups, the MCV were 75, 74, and 72 fl on day 0 and decreased to 67, 70, and 68 FL after induction of aplastic anemia. At the same time, they returned to reference intervals after treatment to 70.3, 74, and 71 fl. The results of RBC count, Hct, and Hemoglobin (in addition to bone marrow findings mentioned above) reflect the occurrence of aplastic anemia, as the average reference interval of these parameters in dogs are 5.5 – 8.5 ($\times 10^{12}/l$), 12.0 – 18.0 g/dl and 37 – 55 %, respectively,

as there were many types of research considered benzene as one of the causes of anemia, especially the aplastic anemia (Shah and Naik 2006; Aksoy 1985) due to change of osmotic fragility of erythrocyte and shortening of erythrocyte survival time (Aksoy 1985) leading to aplastic anemia which characterized by reduction of all cellular elements in the peripheral blood and bone marrow, leading to fibrosis which is the irreversible replacement of bone marrow (Qasim and Badawi 2023). These results of MCV, although there was some drop in values of the mean of MCV after induction of aplastic anemia and increased after treatment, all still into the reference intervals in dogs, which ranged between 60 – 77 FL (Badawi and Yousif 2020; Grondin *et al.*, 2010), this may be attributed to the fact that there are vast differences between breeds of dogs in addition to effects of the ages on the reference intervals. Therefore, these intervals have several differences in the different scientific references. The means of MCH and MCHC revealed no significant differences ($P < 0.05$) in the negative control group in the three consecutive periods, and the same result was observed in the positive control group and also in T 1, T 2, and T 3 groups, as there were no significant differences in MCH between the three consecutive periods. This result reflects that in aplastic anemia,

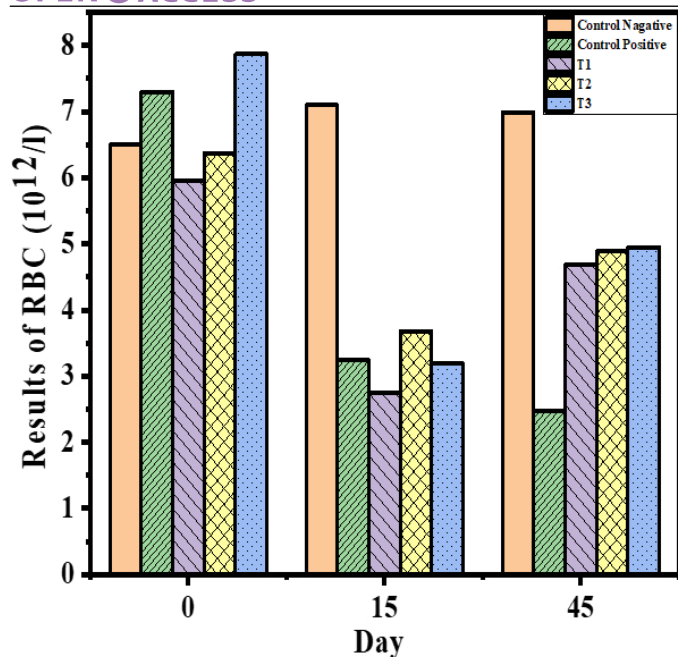


Figure 6: Result of RBC ($10^{12}/l$).

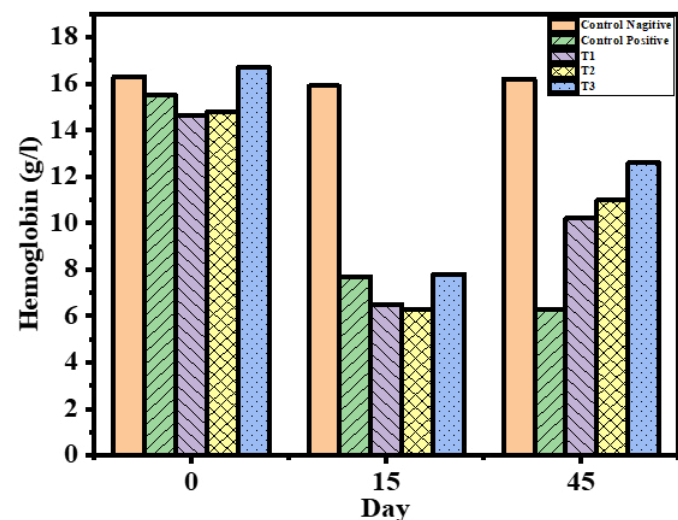


Figure 7: Result of Hemoglobin.

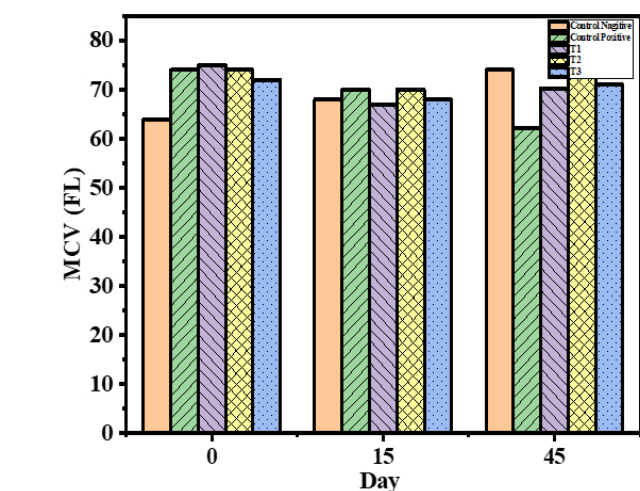


Figure 8: Result of MCV.

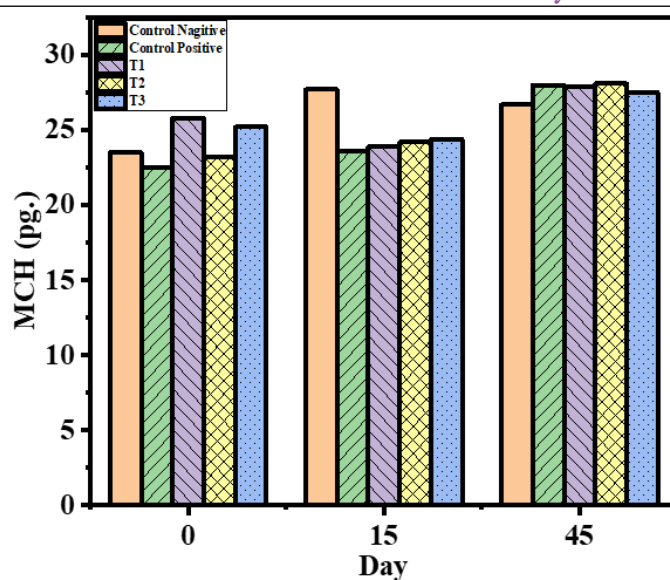


Figure 9: Result of MCH.

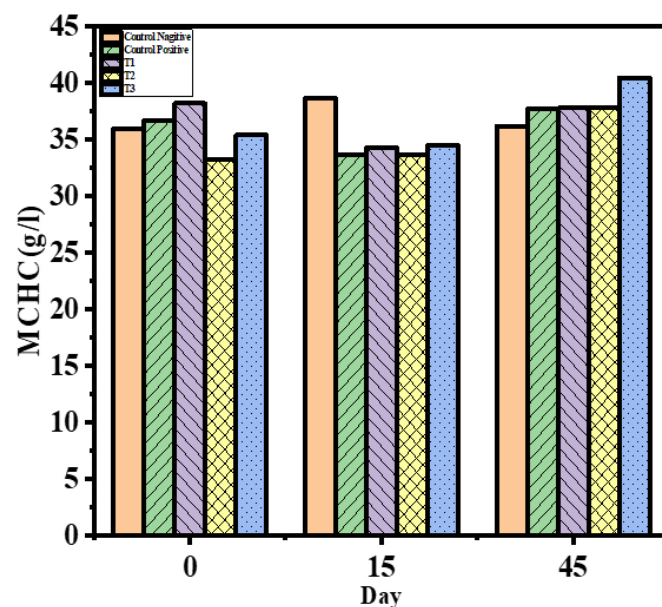


Figure 10: Result of MCHC.

the anemic dogs lose the regenerative ability of bone marrow to compensate for damaged RBC and its hemoglobin content. Thus, RBC count, hematocrit, and Hemoglobin are all decreased, leading to normochromic anemia, as tabled in Tables 2, 3, 4, and shown in Figure. 5, 6, 7, 8, 9,10.

CONCLUSIONS

From the results of the current study, it can be concluded that benzene has side effects during the disease development period. Chitosan nanoparticles are beneficial in a significant cure for aplastic anemia, and cyclosporine has a noticeable therapeutic effect on some cases of aplastic anemia in dogs, but the best results were by loading cyclosporine on chitosan nanoparticles from all clinical aspects

and blood tests, which indicates that the nanoparticles has a clear effect in delivering treatment in a faster period and a quick recovery response.

Future research applications can be applied to humans and other important and necessary animals. Nanotherapy is considered one of the modern and important technologies, as it reduces the doses used and quickly delivers the drug to the target to achieve early recovery and reduce treatment periods. This is useful in terms of reducing the toxicity of some treatments that are used for long periods.

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NOVELTY STATEMENT

What is new in this study is the focus on the protective effects of cyclosporine loaded with chitosan nanoparticles against benzene-induced aplastic anemia and the histological and hematological changes in adult male dogs with benzene-induced aplastic anemia.

AUTHOR'S CONTRIBUTIONS

All authors were contributed equally.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

- Aksoy, Muzaffer (1985). Benzene as a leukemogenic and carcinogenic agent. *Americ. J. Indust. Med.*, 8 (1): 9–20. <https://doi.org/10.1002/ajim.4700080103>.
- Al-Ghazaly, Jameel, Al-Dubai W, Al-Jahafi AK, Abdullah M, Al-Hashdi A (2005). Cyclosporine monotherapy for severe aplastic anemia: A developing country experience. *Annals of Saudi Medicine*, 25 (5): 375–79. <https://doi.org/10.5144/0256-4947.2005.375>.
- Ali, Abdelfattah ME, Aboelfadl MMS, Selim AM, Khalil HF, Elkady GM (2018). Chitosan nanoparticles extracted from shrimp shells, Application for removal of Fe(II) and Mn(II) from aqueous phases. *Separation Science and Technology (Philadelphia)*, 53 (18): 2870–81. <https://doi.org/10.1080/01496395.2018.1489845>.
- Atsdr (2020). 9 Chromium 2. Relevance to public health 2.1 Background and environmental exposures to chromium in the United States, no. Iii: 187–96.
- Bacigalupo A, Broccia G, Corda G, Arcese W, Carotenuto M, Gallamini A, Locatelli F, *et al* (1995). Antilymphocyte globulin, Cyclosporin, and Granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): A pilot study of the ebmt saa working party. *Blood* 85 (5): 1348–53. <https://doi.org/10.1182/blood.v85.5.1348.bloodjournal8551348>.
- Badawi, Mohammed N, Yousif AA (2020). Estimation of some hematological and biochemical references values of clinically healthy dogs in Baghdad Province, Iraq. *Biochemical and Cellular Archives* 20 (2): 4931–37.
- Balakrishnan, Biji, Mohanty M, Umashankar PR, Jayakrishnan A (2005). Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. *Biomaterials* 26 (32): 6335–42. <https://doi.org/10.1016/j.biomaterials.2005.04.012>.
- Blue JT (2002). Book Review: Atlas of veterinary hematology: Blood and bone marrow of domestic animals. *Veterinary Pathology* 39 (1): 165–165. <https://doi.org/10.1354/vp.39-1-165>.
- Brazzell, Jennifer L, Douglas J. Weiss (2006). A retrospective study of aplastic pancytopenia in the dog: 9 Cases (1996–2003). *Veterinary Clinical Pathology* 35 (4): 413–17. <https://doi.org/10.1111/j.1939-165X.2006.tb00157.x>.
- Elsayed, A (2017). Volume-7 , Issue-1 , Jan-Mar-2016 Coden IJABFP-CAS-USA Ata Sedik Ibrahim Elsayed Ph . D,” no. February.
- Ettinger SJ, Feldman EC, Côté E, Elsevier, Louis St, Missouri (1997). Book Review / Compte Rendu de Livre. *Contemporary Accounting Research* 14 (2): 203–13. <https://doi.org/10.1111/j.1911-3846.1997.tb00533.x>.
- Mustafa HN, Al -Ogaidi I (2021). Efficacy of zinc sulfide-chitosan nanoparticles agains bacterial diabetic wound infection”, 54 (1): 1–17.
- Grondin, T. M., Dewitt, S. F., Weiss, D. J., and Wardrop, K. J. (2010). *Schalm's Veterinary Hematology*.
- Hamzah, Jasim K, Mahmood AK, Hamzah KJ, Saad MH (2019). Comparative study of blood pressure between local breed and different breed of dogs in Iraq. *Biochemical and Cellular Archives* 19 (2): 2853–56. <https://doi.org/10.35124/bca.2019.19.2.2853>.
- Howling, Graeme I, Dettmar PW , Goddard PA, Hampson FC, Dornish M, Wood EJ (2001). The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro. *Biomaterials* 22 (22): 2959–66. [https://doi.org/10.1016/S0142-9612\(01\)00042-4](https://doi.org/10.1016/S0142-9612(01)00042-4).
- Ibrahim, Hassan M, El-Bisi MK, Taha GM, El-Alfy EA (2015). Chitosan nanoparticles loaded antibiotics as drug delivery biomaterial. *J. Appl. Pharma. Sci.*, 5 (10): 85–90. <https://doi.org/10.7324/JAPS.2015.501015>.
- Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS (2001). Chitosan as a novel nasal delivery system for vaccines. *Advanced Drug Delivery Reviews*, 51 (1–3): 81–96. [https://doi.org/10.1016/S0169-409X\(01\)00171-5](https://doi.org/10.1016/S0169-409X(01)00171-5).
- McMillan, Joellyn, Batrakova E, Gendelman HE (2011). Cell delivery of therapeutic nanoparticles. *Progress in molecular biology and translational science*, Vol. 104. <https://doi.org/10.1016/B978-0-12-416020-0.00014-0>.
- Mincea M, Negrulescu A, Ostafe V (2012). Preparation, modification, and applications of chitin nanowhiskers: A review. *Reviews on Advanced Materials Science*, 30 (3): 225–42.
- Badawi MN, Qasim MM, Al-Graibawi MA, Khalaf JM, Yousif AA (2022). First molecular detection and phylogenetic analysis of ehrlichia canis in dogs from Baghdad, Iraq. *Archives of Razi Institute*, 77 (6): 2431–37. <https://doi.org/10.1016/j.arsci.2022.04.001>.

- org/10.22092/ARI.2022.358868.2321.
- Rawnaq MR, RA Rafeeq (2023). Evaluation of the shear bond strength of chitosan nanoparticles-containing orthodontic primer: An in vitro study. *Inter. J. Dent.*, (2023). <https://doi.org/10.1155/2023/9246297>.
- Mourya VK, NN Inamdar (2008). Chitosan-modifications and applications: Opportunities Galore. *Reactive and Functional Polymers*, 68 (6): 1013–51. <https://doi.org/10.1016/j.reactfunctpolym.2008.03.002>.
- Num SM, Useh NM (2013). Nanotechnology applications in veterinary diagnostics and therapeutics. *Sokoto J. Vet. Sci.*, 11 (2). <https://doi.org/10.4314/sokjvs.v11i2.2>.
- Okay, Okorafor Ekpe (2010). *No 主観的健康感を中心とした在宅高齢者における健康関連指標に関する共分散構造分析Title*. *Inter. J. Dev. Manag. Rev.*, Vol. 5. <http://publications.lib.chalmers.se/records/fulltext/245180/245180.pdf%0Ahttps://hdl.handle.net/20.500.12380/245180%0Ahttp://dx.doi.org/10.1016/j.jsames.2011.03.003%0Ahttps://doi.org/10.1016/j.gr.2017.08.001%0Ahttp://dx.doi.org/10.1016/j.precamres.2014.12>.
- MayurP, Shah T, Amin A (2007). Therapeutic opportunities in colon-specific drug-delivery systems. *Critical Reviews in Therapeutic Drug Carrier Systems*, 24 (2): 147–202. <https://doi.org/10.1615/CritRevTherDrugCarrierSyst.v24.i2.20>.
- Perkins JL, Neglia JP, Ramsay NKC, Davies SM (2001). Successful bone marrow transplantation for severe aplastic anemia following orthotopic liver transplantation: Long-term follow-up and outcome. *Bone Marrow Transplantation*, 28 (5): 523–526. <https://doi.org/10.1038/sj.bmt.1703177>.
- Pires, Cléo TGVMT, Joice AP, Vilela, Airoidi C (2014). The effect of chitin alkaline deacetylation at different condition on particle properties. *Procedia Chemistry*, 9: 220–25. <https://doi.org/10.1016/j.proche.2014.05.026>.
- Qasim, Al-kareem NA, Badawi NM (2023). The haematological values and serum iron profile in dogs with some pathological and physiological conditions” 10: 737–45.
- Rosenfeld, Stephen, Follmann D, Nunez O, Young NS (2003). Antithymocyte globulin and cyclosporine for severe aplastic anemia: Association between hematologic response and long-term outcome. *Jama*, 289 (9): 1130–35. <https://doi.org/10.1001/jama.289.9.1130>.
- Saeed, Ruwaidah S., Attiya HG, Obead KA (2023). Synthesis and characterization of grafted chitosan blending with polyvinyl alcohol / nanocomposite and study biological activity. *Baghd. Sci. J.*, 20 (5): 1692–1700. <https://doi.org/10.21123/bsj.2023.7574>.
- Norman S (2007). Nanoscience in veterinary medicine. *Veterinary Research Communications*, 31 Suppl 1 (September): 139–44. <https://doi.org/10.1007/s11259-007-0083-7>.
- Ata S, Elsayed I (2015). Hematotoxicity and oxidative stress caused by benzene. *Pyrex J. Biomed. Res.*, 1 (6): 74–080. www.researchgate.net/publication/313938368%0Ahttp://www.pyrexjournals.org/pjbr.
- Shah, Kiran C, Naik PK (2006). Aplastic anemia due to exposure to benzene in diamond workers in Surat (India). *Blood* 108 (11): 3763–3763. <https://doi.org/10.1182/blood.v108.11.3763.3763>.
- Shanmugarathinam A, Puratchikody A (2014). Formulation and Characterisation of Ritonavir Loaded Ethylcellulose Buoyant Microspheres. *J. Pharma. Sci. Res.*, 6 (8): 274–77.
- Steel, Robert GD, Torrie JH (1980). Principles and Procedures of Statistics : A Biometrical Approach. TA - TT -. 2nd ed. New York SE - xxi, 633 pages : illustrations ; 24 cm: McGraw-Hill New York. <https://doi.org/LK> - <https://worldcat.org/title/5494060>.
- Sun, Rongli, Juan Zhang, Lihong Yin, and Yuepu Pu. 2014. “Investigation into Variation of Endogenous Metabolites in Bone Marrow Cells and Plasma in C3H/He Mice Exposed to Benzene.” *International Journal of Molecular Sciences* 15(3):4994–5010. doi: 10.3390/ijms15034994.
- Rongli S, Zhang J, Yin L, Pu Y (2014). Investigation into variation of endogenous metabolites in bone marrow cells and plasma in C3H/He mice exposed to benzene. *Inter. J. Molec. Sci.*, 15 (3): 4994–5010. <https://doi.org/10.3390/ijms15034994>.
- Underwood C, Eps AWV (2012). Nanomedicine and veterinary science: The reality and the practicality. *Vet. J.*, 193 (1): 12–23. <https://doi.org/10.1016/j.tvjl.2012.01.002>.
- Liping W, He X, Bi Y, Ma Q (2012). Stem cell and benzene-induced malignancy and hematotoxicity. *Chemical Research in Toxicology*, 25 (7): 1303–15. <https://doi.org/10.1021/tx3001169>.
- Wang, XH, Cui FZ, Feng QL, Li JC, Zhang YH (2003). Preparation and characterization of collagen/chitosan matrices as potential biomaterials. *J. Bioactive and Compatible Polym.*, 18 (6): 453–67. <https://doi.org/10.1177/0883911503040434>.
- Weiss, Douglas J (2003). New insights into the physiology and treatment of acquired myelodysplastic syndromes and aplastic pancytopenia. *Veterinary Clinics of North America - Small Animal Practice*, 33 (6): 1317–34. [https://doi.org/10.1016/S0195-5616\(03\)00094-9](https://doi.org/10.1016/S0195-5616(03)00094-9).
- Younus, Maha A, Abdallaha BF (2023). Synthesis, Characterization and Anticancer Activity of Chitosan Schiff Base / PVP Gold Nano Composite in Treating Esophageal Cancer Cell Line. *Baghdad Sci. J.*, 21 (1): 95–106. <https://doi.org/10.21123/bsj.2023.7911>
- Youssef, Sayed F, El-Banna HA, Elzorba HY, Galal AM (2019). Application of some nanoparticles in the field of veterinary medicine. *Inter. J. Vet. Sci. Med.*, 7 (1): 78–93. <https://doi.org/10.1080/23144599.2019.1691379>
- Youssef, Sayed F, Elbanna HA, Elzorba HY, Galal AM, Mohamed GG, Ismail SH (2020). Synthesis and characterization of florfenicol-silver nanocomposite and its antibacterial activity against some gram positive and gram-negative bacteria. *Inter. J. Vet. Sci.*, 9 (3): 324–30. <https://doi.org/10.37422/IJVS/20.050>