

The Impact of Alpha-Lipoic Acid on Hematological and Biochemical Values of Blood in Male Rats with Induced Anemia

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Abstract Anemia is a frequent multifactorial disease, defined by reduced red blood cell count or hemoglobin concentration, which compromises the delivery of oxygen to body tissues. Alpha-lipoic acid (ALA), a natural mitochondrial antioxidant, is well documented in its ability to combat oxidative stress and regulate a variety of metabolic processes. In this study, investigated the potential impact of ALA supplementation on blood and biochemical parameters of male albino rats with PHZ-induced anemia. Thirty rats were distributed randomly into three groups: healthy control group (C), PHZ-induced anemia (T1), and anemic group receiving ALA (T2). Several parameters were measured, including hematological parameters, indicators of iron status, thyroid hormones, and markers of oxidative stress (MDA, GSH, and CAT). The study findings confirmed that PHZ treatment resulted in the significant decrease of red blood cells, hemoglobin, hematocrit, GSH, CAT, iron, ferritin, hepcidin, and T3 and T4 level, but the significant elevation of MDA and TSH levels. It must be particularly noted that ALA supplementation significantly improved blood indices, restored iron homeostasis, normalized the level of thyroid hormones, and increased the level of antioxidant protection. Statistically processed data provided significant differences ($p \leq 0.05$) among treated groups and an untreated anemic group. Overall, these results suggest that ALA is of potential value as a protective agent against the cytotoxic effects of anemia and oxidative stress.

Keywords: Alpha lipoic acid, anemia, oxidative stress, hematological parameters, rats.

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Introduction Anemia continues to be one of the most prevalent health problems globally, irrespective of age and social strata of the population. It is characterized by low RBC count, Hb concentration, or Hct, leading to a diminished capacity of the blood to carry O₂, resulting in cellular metabolism and tissue and organ function compromise (1). Anemia has a multifactor origin, including nutritional deficiencies (iron, folate, vitamin B12), chronic diseases, genetical and hemolysis agents. Of the compounds that are used to induce anemia experimentally, phenylhydrazine (PHZ) is commonly used in animal models. PHZ induces hemolysis by giving rise to oxidative stress via lipid peroxidation of erythrocyte cell membranes, resulting in premature destruction of RBCs as well as the hampering of erythropoiesis (2, 3). While resultant oxidative damage to the RBC that adversely affects red cell survival, the oxidative modifies develop priming signals that serve to initiate inflammatory

and metabolic cascades that contribute to development of systemic sequelae.

Oxidative stress, defined by an imbalance between generation of reactive oxygen species (ROS) and antioxidant defenses, is crucial to the development of anemia and related tissue injury. Hence, antioxidant therapy may be increasingly conceived as one of the basic factors, crucial for reducing the severity and sequelae of anemia (4). Alpha-lipoic acid (ALA) or thioctic acid is a naturally-occurring disulphide compound that is chemically produced in the mitochondria from octanoic acid endogenously. First identified by Reed and coworkers in 1951, ALA was first characterized as a vitamin-like substance before its realization as a powerful antioxidant (5, 6). ALA has two optical isomers: the active R-form and the inactive S-form produced during chemical synthesis (7).

As an antioxidant, ALA is special because it can function inside and outside the cell since it is both hydrophilic and lipophilic, which enables it to

scavenge free radicals throughout the body. It scavenges ROS directly; recycles other key antioxidants, e.g., glutathione, vitamins C and E; and chelates redox-active metals ions to prevent oxidative damage and maintaining redox homeostasis (8, 9).

Clinically, ALA has been studied in the treatment of a number of diseases associated with oxidative stresses, such as diabetic neuropathy, hepatic disorders, CVD, neurodegenerative disorders (e.g., Alzheimer's disease) and some malignancies (10-13). Its ability to attenuate inflammation, increase glucose uptake, augment endothelial function and promote mitochondrial bioenergetics further suggests its widespread therapeutic potential (14, 15).

Indeed, recent studies showed that ALA supplementation can improve hematological parameters including iron metabolism, EPO sensitivity and can attenuate oxidative damage to erythrocytes as well as ameliorating antioxidant defense system (16-18). These features make ALA an attractive candidate in the therapy of anemias, e.g. those associated with oxidative stress and hemolysis. In the light of this, a study was undertaken to investigate the possible therapeutic role of alpha-lipoic acid on hematological and biochemical derangements induced in male albino rats treated with an inducing agent, phenylhydrazine. Furthermore, investigate whether ALA treatment can alleviate oxidative injury, rectify hematological homeostasis, enhance iron status and promote overall physiological performance in this anemic model.

Material and Methods

Ethical Approval

All experimental procedures involving animals were performed in accordance with the ethical guidelines for animal studies and were approved by the College of Education, University of Al-Qadisiyah, Iraq Scientific and Ethical Committee (Approval No. 85 - 5/1/2025). All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to achieve the study goals.

Experimental Protocol and Animal Model

This study was conducted to study effects of alpha-lipoic acid (ALA) on hematological and biochemical parameters in phenylhydrazine (PHZ)-induced anemic male albino rats. This experiment was performed in the Animal House of Science College, Al-Qadisiyah University. The study was conducted on thirty healthy adult male albino rats, aged 3–4 months and weighing 200–270g. Animals were maintained in plastic cages with sawdust bedding, the bedding being renewed and disinfected every 3 days. During the whole experiment they were kept in constant temperature (23–27°C) and 12 h light/dark cycle conditions. Rats had free access to food and

water and one week acclimation before experimental treatment.

Chemicals and Preparation

Phenylhydrazine (PHZ) was used to induce anemia in rats and was obtained from a local pharmacy in Baghdad. It was dissolved in distilled water to achieve a final concentration of 40 mg/kg body weight, and 1 ml of the solution was injected intraperitoneally for each rat (20).

Alpha-lipoic acid (ALA) was employed as the treatment drug within this study, with a concentration of 60 mg/kg body weight was prepared using the method outlined by (21) the ALA was solved in distilled water, and 1 ml of the solution was administered orally to each rat once daily through a special syringe with a hooked end. The study period from October 1 to December 1, 2024.

Induction of Anemia

Phenylhydrazine (PHZ) was administered intraperitoneally in a dose of 40 mg/kg body weight for two consecutive days to cause hemolytic anemia in the T1 and T2 groups. The anemia model was induced considering the oxidative activity of PHZ on red blood cells (RBCs) leading to their degradation and eventual induction of anemia (20).

Experimental groups

The rats were randomly divided into three groups (n = 10 per group) as following (with prior acclimation): Group (C): Was injected IP with 0.9% NaCl and received oral gavages of distilled water once daily for 30 days.

Group T1 (Anemic Control): Phenylhydrazine (40 mg/kg body weight, i.p.) was given for two days for induction of anemia (20), thereafter received distilled water orally for 30 days.

T2 group (ALA-treated): After making anemia using PHZ as explained previously, the rats were treated with 60 mg/kg/day alpha-lipoic acid (21) by gavage for 30 days consecutively.

Blood Sample Collection

At the end of the experimental period (30 days), animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (0.3 ml/kg) and xylazine (1.0 ml/kg). Cardiac puncture was performed to draw blood from the heart with a sterile syringe. The blood was alternatively divided into two fractions:

The first fraction was collected in tubes with an anticoagulant (EDTA) in order to perform hematological studies.

The second fraction was deposited into tubes without EDTA, left to clot and spun at 3000 revs/min for 15 minutes to obtain serum. The serum samples were maintained at –20°C until used for biochemical, antioxidant, and hormonal estimations.

Parameters studied

Hematological Assessments

RBC count, Hb concentration, and PCV were measured using an automated hematology analyzer (GENEX) according to the manufacturer protocol.

Iron Profile

ELISA kits for serum ferritin and hepcidin measurements were provided by BioSystem (USA). The serum iron was measured by spectrophotometry with the BioSystem kit. Fe²⁺ were dissociated from transferrin under acidic and reduced conditions and then reacted with bipyridine to yield a pink complex, which was detected at 520 nm.

Hormones Parameters

The serum levels of triiodothyronine (T3), thyroxine (T4), and thyroid stimulating hormone (TSH) were estimated by using an enzyme-linked immunosorbent assay (ELISA) kit following the method of Wisdom (22) as absorbance values at 450 nm. Rapid Hormone kits were purchased from Biocheck, Inc.

Oxidative Stress Biomarkers

Estimation of MDA concentration Lipid peroxidation was estimated by measuring the levels of MDA, a lipid peroxidation product, by the method of thiobarbituric acid reactive substances (TBA) as described by Guidet & Shah (23). The colored product was quantified at 532 nm absorbance.

Estimation of Glutathione (GSH): The GSH level of serum was determined according to the method of James et al. (24) by Ellman’s reagent at 412 nm absorbance.

CAT enzymatic assay: The determination of CAT activity was based on the reduction of H₂O₂ measured spectrophotometrically following the method of Mueller et al. (25).

Statistical Analysis

All the results of the study were analyzed statistically to find out the significant differences between the averages, and for this purpose the Statistical Package for the Humanities (SPSS) version 27 was used. According to the study data, the arithmetic mean and standard error were calculated for each indicator and the one-way analysis of variance (ANOVA) test was used with the Least Significant Difference (LSD) value to know the differences between the averages, and significant differences were determined at a probability level (P < 0.05) (26).

Results

Hematological Parameters

The effects and statistical comparisons of hematological indices among groups are provided in Table 1. Phenylhydrazine-induced anemia (T1 group) caused a significant (p ≤ 0.05) decrease in RBC count, Hb concentration, and PCV as compared to the control group. The average RBC count in the control

group was 7.24×10¹²/L, and decreased to 4.10×10¹²/L in the T1 group. Likewise, Hb level fell from 13.7 g/L in control subjects to 7.7 g/L in anemic subjects. PCV followed a similar pattern falling from 41.82% to 27.4%.

Treatment group with alpha-lipoic acid (T2 group) significantly reversed these parameters with an RBC of 5.14×10¹²/L, Hb of 10.76 g/L, and PCV of 34.36 %, showed mild ~~some~~ recovery of hematological function on treatment.

Table (1): Hematological parameters (RBC, Hb, PCV) of the experimental groups.

| Groups | Parameters | | |
|--------|---------------------------|-----------------|-----------------|
| | RBC (10 ¹² /L) | HB (g/L) | PCV (%) |
| C | 7.24±0.05 A | 13.7±0.23 A | 41.82±0.09 A |
| T1 | 4.10±0.08 C | 7.7±0.07 C | 27.4±0.13 C |
| T2 | 5.14±0.15 B | 10.76±0.11 B | 34.36±0.18 B |
| LSD | 0.512 | 0.352 | 0.895 |

C: control group, T1: Anemic Control T2: ALA-treated. The letters refer too significant differences between treatment

Iron Profile

The serum iron, ferritin, and hepcidin levels are shown in Table 2. The iron, ferritin, and hepcidin concentrations in the anemic rats (T1 group) were significantly lower than in the control group (iron 32.6 mg/L vs. 95.4 mg/L; ferritin 45.3 mg/mL vs. 125.6 ng/mL; hepcidin 18.7 pg/mL vs. 42.6 pg/mL, respectively).

While, serum iron (65.7 mg/L), ferritin (83.4 ng/mL), and hepcidin (34.9 pg/mL) were significantly higher by alpha-lipoic acid group than the anemic group, but lower than the control group. The results indicated that ALA supplementation improved iron homeostasis and promoted the recovery of erythropoiesis.

Table (2): Iron profile markers (Serum Iron, Ferritin, Hepcidin) in the studied groups.

| Groups | Parameters | | |
|--------|---------------|------------------|------------------|
| | Iron (mg/L) | Ferritin (ng/mL) | Hepcidin (pg/mL) |
| C | 95.4±8.2 A | 125.6±10.2 A | 42.6±0.45 A |
| T1 | 32.6±5.7 C | 45.3±7.5 C | 18.7±0.12 C |

| | | | |
|-----|---------------|---------------|----------------|
| T2 | 65.7±6.3 B | 83.4±9.1 B | 34.9±0.18 B |
| LSD | 3.562 | 4.251 | 2.014 |

C: control group, T1: Anemic Control T2: ALA-treated. The letters refer to significant differences between treatment

Thyroid Hormones

Relative to non-anemia, the levels of thyroid hormone were significantly disturbed in anemia condition (Table 3). The T1 category exhibited low concentrations of T3 and T4 (0.68 ng/mL and 3.42 ng/mL, respectively) and high levels of TSH (1.43 ng/mL), which are similar to a hypothyroid status. Whereas, the levels of T3 (1.02 ng/mL) and T4 (5.21 ng/mL) were significantly improved, and TSH was reduced (0.77 ng/mL) with the treatment of alpha-lipoic acid in the T2 group. This indicates an inhibitory action of ALA on TH function in anemic rats.

Table (3): Thyroid hormones (T3, T4, TSH) of the experimental groups.

| Groups | Parameters | | |
|--------|----------------|----------------|----------------|
| | T3 (ng/mL) | T4 (ng/mL) | TSH (ng/mL) |
| C | 1.45±0.12 A | 6.87±0.45 A | 0.65±0.08 C |
| T1 | 0.68±0.09 C | 3.42±0.32 C | 1.43±0.15 A |
| T2 | 1.02±0.10 B | 5.21±0.36 B | 0.77±0.10 B |
| LSD | 0.0551 | 0.324 | 0.0145 |

C: control group, T1: Anemic Control T2: ALA-treated. The letters refer to significant differences between treatment

Oxidative Stress Markers

Oxidative stress markers (Table 4) exhibited a higher MDA concentration (8.76 μmol/L) and lower levels of GSH and catalase (8.3 and 18.3 μmol/L, respectively) for T1 in contrast to control.

Whilst, in treated group with ALA, these changes were back almost to near normal (MDA 4.93 μmol/L, GSH 16.7 μmol/L, catalase 32.5 μmol/L). These findings emphasize the powerful antioxidative effect of ALA in ameliorating oxidative stress during PHZ-induced anemia.

Table 4: level of oxidative stress markers (MDA, GSH, and Catalase) in study groups.

| Groups | Parameters | | |
|--------|----------------|---------------|-------------------|
| | MDA (μmol/L) | GSH (μmol/L) | Catalase (μmol/L) |
| C | 2.45±0.22 C | 24.6±2.1 A | 42.6±3.5 A |

| | | | |
|-----|----------------|---------------|---------------|
| T1 | 8.76±0.67 A | 8.3±0.7 C | 18.3±1.6 C |
| T2 | 4.93±0.38 B | 16.7±1.4 B | 32.5±2.7 B |
| LSD | 0.754 | 1.478 | 1.044 |

C: control group, T1: Anemic Control T2: ALA-treated. The letters refer to significant differences between treatment

Discussion

Hematological Parameters

In present our study, PHZ treatment exhibited most severe haematological disruption such as profound decrease in red blood cell (RBC) count, haemoglobin (Hb) concentration and packed cell volume (PCV) with respect to control was observed. These results are in agreement with previous reports stating that phz induces its hemolytic effect by inducing oxidative damage to the erythrocyte membranes and hemoglobin leading to hemolysis and subsequent anemia (27, 28).

Treatment with alpha-lipoic acid (ALA) resulted in a significant amelioration of these haematological parameters. These results indicate that ALA has a cytoprotective activity against oxidative damage to RBC.

The beneficial role of ALA could be mediated by its strong antioxidant action. ALA and its reduced form, dihydrolipoic acid (DHLA), are able to directly eliminate ROS, regenerate other endogenous antioxidants such as glutathione (GSH) and vitamin E, as well as to provide protection for lipids and proteins in membranes against oxidative stress (7, 9). Its ability to modulate oxidative stress probably helps preserving erythrocyte membrane integrity and, indirectly improves erythropoiesis leading to arms increase in hematologic parameters.

In addition, previous reports have demonstrated that ALA supplementation can protect erythrocytes from death under an oxidative stress and improve hematopoietic recovery through modulation of inflammatory cytokines and elevation of antioxidant defenses (16, 29).

Iron Profile

The current results indicated that the injections of phenylhydrazine (PHZ) resulted in a significant decrease in the serum values of iron, ferritin, and hepcidin as compared with those in control subjects. These results correspond to previous reports suggesting that hemolysis induced by PHZ causes an abnormal degradation of erythrocytes, resulting not only in an iron deficiency but also in an abnormal iron metabolism (30, 31).

The reduced serum iron level might be ascribed to the oxidative damage that the circulating red blood cells underwent as a result of the action of PHZ, their

hemolysis, liberation of free heme and iron, in addition to sequestration of iron in the macrophages and liver tissue, to counterbalance systemic cytotoxicity (32, 33). Furthermore, the inflammatory response following hemolysis might also deregulate iron homeostasis by leading to the production of cytokines that inhibit iron transport and absorption (28).

The serum iron, ferritin and hepcidin levels in the ALA treatment group were significantly higher than the anemic group. This enhancement in all probability is due to the strong antioxidative and anti-inflammatory properties of ALA and maintenance of erythrocyte integrity, and the promotion of iron recycling and utilization. ALA has been demonstrated as enhancing the bioavailability of iron, mainly because it prevents oxidative damage of iron-transport proteins including transferrin and promoting the repletion of iron regulatory pathways (16, 17).

In addition, ALA indirectly enhances erythropoiesis by inhibiting oxidative stress and increasing erythropoietin (EPO) sensitivity to enable more effective use of existing iron stores for RB cell synthesis (18). These results suggest a therapeutic ability of ALA to regulate iron homeostasis and to alleviate anemia induced by oxidative hemolysis.

Thyroid Hormones

The findings of current study demonstrated that the levels of serum triiodothyronine (T3) and thyroxine (T4) were significantly lower in PHZ-induced anemic rats, while the level of serum thyroid stimulating hormone (TSH) was obviously higher, compared to the control rats. These changes point to a thyroid derangement/ dysfunction as a suppressive or inhibition effect of anemia, which have been reported earlier (34, 35).

Suppression of T3 and T4 levels in the anemic group may be on account of oxidative stress and hypoxia induced by the anemia, as these would impede the activity of the deiodinase enzymes that convert T4 to the biologically active form T3 (36). Iron deficiency from anemia may contribute to thyroid dysfunction, further as the iron acts as a cofactor for Thyroid Peroxidase (the enzyme involved in thyroid hormone synthesis) (34).

Treatment with alpha-lipoic acid (ALA) greatly stabilized the thyroid hormone balance as measured by an increase in serum T3 and T4 levels and a decrease in TSH concentration in ALA-administered group (T2). These results indicate that ALA has a protective effect on thyroid function in anemic condition.

The positive effect of the ALA on the changes in thyroid hormones may be mediated through its strong antioxidative action, which inhibits the oxidative

damage in the thyroid tissue and rejuvenates the deiodinase enzymatic process (37). In addition, ALA optimizes mitochondrial function and cellular energy metabolism, which are essential to the normal synthesis and action of thyroid hormone (38, 39).

Therefore, ALA supplement may help to ameliorate the damaging effects of anemia-mediated oxidative stress on thyroid axis and may protect towards full recovery of the endocrine balance.

Oxidative Stress Markers

According to these results, it is observed that PHZ application increased MDA levels and decreased the activities of GSH and catalase in serum of rats compared to control group. This finding shows that PHZ-induced anemia is closely associated with oxidative stress, which is consistent with previous reports showing that PHZ produces excessive reactive oxygen species (ROS) that promote lipid peroxidation, protein oxidation, and antioxidant defense consumption (28, 40).

The significant increase in MDA, a well-known end-product marker of lipid peroxidation, indicates high level of oxidative damage of cellular membranes, especially erythrocyte membranes. Also, the marked reduction in GSH and catalase activities was indicative of an overloaded endogenous antioxidant system and was unable to scavenge the high level of ROS produced as a result of hemolysis at the time (4). Alpha-lipoic acid (ALA) treatment significantly diminished oxidative stress, reflected by reductions in the MDA levels, and recovery of GSH and catalase activities to control levels. This antioxidant role of ALA is due to its antioxidant bi-functional properties: it acts as a free radical scavenger and also as a regenerator of other antioxidants (i.e.: GSH, vitamins C and E) (8, 9).

ALA also binds metals (i.e., metal-chelating), and has been shown to bind mainly redox-active metals and iron in particular and preclude Fenton type reactions (forms highly reactive $\bullet\text{OH}$) (7). In addition, ALA has been shown to up-regulate the expression of endogenous antioxidant enzymes, via the stimulation of transcription factors such as Nrf2, leading to prolonged antioxidant defense (38).

Therefore, the great antioxidative power of ALA is essential for the regulation of anemia-induced oxidative stress, maintaining the structure of the cell and tissue regeneration.

Altogether, the overall findings of the present study unambiguously indicate that phenylhydrazine-induced anemia is linked to severe deterioration of hematological indices, dysregulated iron homeostasis, impaired thyroid status, and exaggerated oxidative stress. These pathological changes would represent the central role played by

oxidative damage in the etiology and pathophysiology of anemia and its systemic complications.

Treatment of ALA prominently attenuated these adverse effects. The administration of ALA significantly corrected the parameters of the red cell indices (RBC, Hb, PCV), iron homeostasis (iron, ferritin, hepcidin), thyroid hormones (T3, T4, TSH) and ameliorated the oxidative stress markers (MDA, GSH, catalase). These results indicate that ALA was a strong antioxidant, anti-inflammatory and metabolic regulator.

Salvaging reactive oxygen species, recycling endogenous antioxidants, chelating transition metals, improving mitochondrial bioenergetics are the mechanisms which help in the therapeutic potential of ALA. The fact that ALA also modulates erythropoiesis, enhances iron utilization, and preserves endocrine activity demonstrates the potential wider application of its systemic actions beyond mere antioxidant protection.

In summary, the results of existing study suggest that alpha-lipoic acid exerts clear hematoprotective effects on anemia-induced alterations of both biochemical and hematological parameters. Whereas, ALA supplementation may offer a potential adjunct therapy for the treatment of anemia and oxidative stress-related conditions.

Conclusion

From the above investigation it can be concluded that phenylhydrazine induced anemia in rats resulted in remarkable hematological, biochemical and oxidative stress perturbations. Supplementation of alpha-lipoic acid greatly improved these pathological conditions by correcting red blood cell indices, iron metabolism data, thyroid hormones, as well as by restoring the antioxidant parameters. The strong antioxidative, anti-inflammatory, and metabolic effect of alpha-lipoic acid was crucial in ameliorating the negative effects of anemia and the oxidative stress. These findings provide evidence of the potential therapeutic value of alpha-lipoic acid as an adjuvant agent in the treatment of anemia and oxidative stress diseases. Its potential efficacy and possible mechanism in clinical practice needs further investigation.

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Conflict of interest

No conflict of interest is disclosed by the authors.

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