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Probing the Individual and Synergistic Effect of Calcium and Zinc against Lead Induced Hematological Alterations and Oxidative Stress

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Abstract

Introduction: Lead (Pb) exposure is a worldwide unavoidable environmental problem because of its presence in various useable appliances causing severe health problems. Different minerals like Zinc (Zn) and Calcium (Ca) are known to exert competitive behavior with Pb because of structural analogy thus hinder Pb absorption in blood.

Objective: To determine individual as well as synergistic potential of Zn and Ca in inhibition of Pb induced adverse impact on rat hematology and antioxidant enzymes.

Methodology: In controlled environment, *Sprague dawley* rats received Lead acetate 20 mg/kg of Body Weight (bw), zinc sulphate 20 mg/kg bw and calcium carbonate 7500 mg/kg bw with different combinations via nasogastric tube feeding for 35 days. Blood samples collected from rats were tested for complete blood count and antioxidant enzymes in blood.

Results: Lead exposure significantly decreased the red blood cells, hemoglobin, hematocrit, monocytes, granulocytes, platelet count ($P<0.01$) white blood cells and red blood cell distribution width ($P<0.05$). Antioxidant enzymes including superoxide dismutase and catalase were also found significantly reduced ($P<0.01$) after Pb exposure. These alterations in hematological and antioxidant enzymes concentrations were lessened by Zn, Ca and Zn+Ca supplementation, with Zn being more effective than others.

Conclusion: Results of this study clearly shows that Zn and Ca either alone or in combination possess ameliorative potential against the hallmarks of Pb induced oxidative stress but Zn alone showed greater impact than Ca and Zn+Ca supplementation. So it can be inferred that Zn and Ca supplementation could act as potential preventive strategy to hinder Pb induced hematological alteration and oxidative stress.

Keywords: Hematology; Lead toxicity; Synergistic; Zinc; Calcium; Oxidative stress

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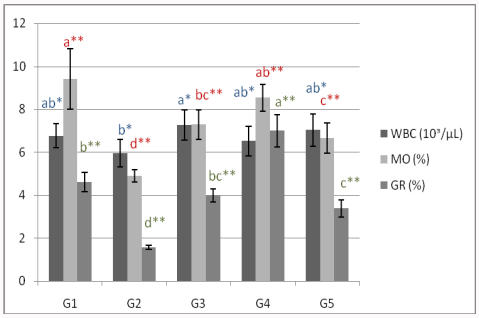
Introduction

Lead (Pb) is being used vastly by many industries like food packaging. The other notable sources for Pb are contaminated air, soil and water ultimately increasing its exposure to all living beings. It can also penetrate in human body directly or through different food chains [1]. It has been found genotoxic and cytotoxic, once it makes its entrance into physiological system, where it interacts with compounds containing nitrogen, sulfur and oxygen elements, thus impairs metabolism and induces oxidative stress [2]. Primary indications of Pb toxicity are its increased levels in blood [3]. Center for Disease Control and Prevention (CDC) has adopted 5 µg/dl as the reference value to differentiate between children who have exposure and need case management. Almost 4.5 million children in US have BLL higher than this CDC developed level [4]. Institute for Health Metrics and Evaluation made estimation that 540,000 deaths have been caused by Pb exposure in 2016, which means a loss of almost 14 million years of healthy life globally because of Pb's prolonged effects on health. Its effects are mostly in underdeveloped countries having low socioeconomic status [5]. In Pakistan BLLs of Karachi's population were recorded approx. 10.82 µg/dL on average in 2010 [6].

Pb has different mechanisms for disturbing enzyme system and different cellular processes of the body. Mostly researches which are being made on Pb include its toxic effects on renal, cardiovascular, hematology and neurotoxicities. More emphasis was given to its neurological effects [7]. Hematological system can suffer from its effects even at very lower concentrations i.e.,



G_1: Control **G_2:** Lead Acetate (20 mg/kg bw)
G_3: Lead Acetate (20 mg/kg bw) + Zinc Sulphate (20 mg/kg bw)
G_4: Lead Acetate (20 mg/kg bw) + Calcium Carbonate (7500 mg/kg bw)
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Figure 1: Concentration of White Blood Cells, Monocytes and Granulocytes of Pb intoxicated and Ca & Zn supplemented rats;
Results are stated as Mean ± SD. Superscripts with different letters differ significantly (P<0.05).

below 10 µg/dL [8]. Several studies have reported that Pb causes anomalies in Red Blood Cells (RBCs) by denaturing cell membranes *via* lipid peroxidation and it also inhibits heme synthesis [9]. Mostly employed therapeutic strategies to fight against are promoting its excretion by sequestering agents like dimercaptosuccinic acid and ethylenediaminetetraacetic acid. However, they impart several side effects thus render them non suitable for long term treatments [10]. So more researches are being made to find out secured dietary therapies to cope up with Pb induced injurious effects. Several nutrients have been reported for their positive role in ameliorating Pb toxicity but very few researchers tried to correlate dietary modifications and lead acetate induced haematopathological alterations. Metals like Zn and Ca with similar structural and chemical properties to that of Pb compete at binding sites of enzymetic and absorptive proteins in intestine, blood circulation and tissues [11]. Keeping in view this scenario, the current study was planned to testify ameliorative potential of individual and combined administration of zinc and calcium against lead induced oxidative stress and heamatopathological alterations.

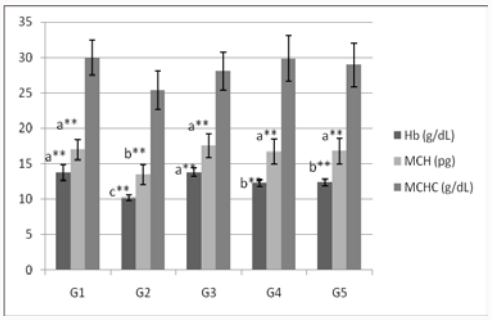
Material and Methodology

Chemicals: Lead acetate (>99.5% purity), zinc sulfate (98% to 99% purity) and calcium carbonate (>99.5% purity) were purchased from Sigma Aldrich (Darmstadt, Germany). Distilled water, phosphate buffer, riboflavin, Nitroblue Tetrazelium (NBT), triton-X, methionine and phosphate buffer for analysis of antioxidant enzymes in blood.

Animal study: For the purpose of current study, 25 *Sprague-Dawley* rats were kept in Animal Room of National Institute of Food Science and Technology, University of Agriculture, Faisalabad. Rats were acclimatized to environmentally controlled room and were fed on laboratory standard diet & water *ad-libtum.* After period of 24 hours (maintenance period) animals were divided into 5 groups, each group containing randomly selected & equally distributed 5 rats. During 35 days trial G_1 served as normal group and was given standard laboratory diet, G_2, the positive control group, received normal diet with 20 mg/kg/day lead acetate in 0.5 mL solution administered through GIT. G_3 received Pb+Zn and G_4 was given Pb+Ca solutions by same route of administration while G_5 was treated with Pb+Zn+Ca for the evaluation of synergistic effects of Zn and Ca on Pb absorption. After completion of trial, the twelve hours

Table 1: Treatment plan.

Groups	$ZnSO_4$ mg/kg/day	$CaCO_3$ mg/kg/day
G_1	-	-
G_2	-	-
G_3	20	-
G_4	0	7500
G_5	20	7500



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G_5: Lead Acetate (20 mg/kg bw) + Zinc Sulphate (20 mg/kg bw) + Calcium Carbonate (7500 mg/kg bw)

Figure 2: Concentration of Hemoglobin, Mean Corpuscular Hemoglobin and Mean Corpuscular Hemoglobin Concentration of Pb intoxicated and Ca & Zn supplemented rats;
Results are stated as Mean ± SD. Superscripts with different letters differ significantly (*=P<0.05, **=P<0.01).

fasted animals were anesthetized. Venous blood was collected, after 35 days of 12 hours fasted rats, in heparinized tubes for hematological analyses as well as in serum separating tubes to measure the antioxidant enzymes levels to test the effectiveness of given minerals against lead induced adverse impacts (Table 1).

Hematological analysis: To analyze the ameliorative potential of zinc and calcium against PbAc induced alterations on blood composition, complete blood count (CBC) test was performed. Whole blood (2 mL) was taken in lead free and EDTA containing polyethylene tubes & was analyzed by using BC-5500 Automatic Blood Cell Analyzer. Blood indices i.e., Hemoglobin (Hb), Red Blood Cell Count (RBCs), RDW (Red Blood Cell Distribution Width), MCH (Mean Corpuscular Hemoglobin), Hematocrit (Hct), MCV (Mean Corpuscular Volume), Platelet Count (Plt), PDW (Platelet Distribution Width) and MPV (Mean Platelet Volume) were counted [12].

Superoxide Dismutase (SOD): Functioning of SOD was estimated analyzing its capacity to suppress NBT's photo reduction by following the protocols of Giannopolitis and Ries [13]. Solution prepared for this reaction was containing 15 ml of deionized water with 0.2 gm methionine, 17.5 ml deionized water with 0.015 of NBT, 17.5 ml dist. Water with 0.037 conc. of triton-X, 17.5 ml of dist. Water containing 0.013 riboflavin & buffer solution (10.2 M).

The reaction solution in test tubes was kept under UV lamp for 15 min prior to addition of riboflavin. By using spectrophotometer at 560 nm wavelength the absorbance of solution was estimated. Single unit of SOD functioning was considered to be its concentration that suppressed NBT photo reduction by 50%.

Catalase (CAT): Functioning of CAT was determined by

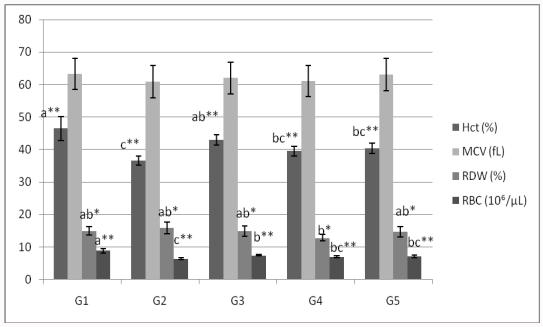

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Figure 3: Concentration of Hematocrit, Mean Corpuscular Volume, Red blood cell Distribution Width and Red Blood Cells of Pb intoxicated and Ca & Zn supplemented rats;
Results are stated as Mean ± SD. Superscripts with different letters differ significantly (*=P<0.05, **=P<0.01).

following procedures of Maehly and Chance with little modifications [14]. Reaction solutions used for this analysis contained 5.9 mM of H_2O_2, 0.1 ml extract of enzyme and 50 mM phosphate buffer having pH as 7. Enzyme extract was added to initiate the reaction. After each 20 secs, the alteration in reaction solution's absorbance at 240 nm wavelength was measured. Single unit of functioning of CAT recorded that shows absorbance alterations of 0.01 units/min.

Statistical analysis: Data obtained was subjected to analysis of variance for statistical evaluation. Comparison of all treatment means was done using Tukey's HSD at 5% significance level. Data was analysed by using software "statistics 8.1" [15].

Results

White Blood Cells (WBCs), Monocytes (MO) and Granulocytes (GR): Figure 1 shows that WBCs count of lead exposed rats was found significantly lower (P<0.05) than control and treatment groups. Decreased levels of WBCs with a mean of 5.95 (10^3/µL) and Standard Deviation (SD) of ± 0.64 were measured in lead exposed rats in comparison to mean of 6.76 (10^3/µL) with SD of ± 0.56 in control. These values were restored in all treatment groups but best recovery was seen in case of Zn supplementation as 7.26 ± 0.70 (10^3/µL).

MOs are the type of WBCs in blood. It is obvious from the above mentioned results of WBCs levels that over all WBCs level were reduced by the lead exposure in rats. The same trend has been seen in case of monocytes in Figure 1. Pb group, had significantly (P<0.01) lower levels of measured MO concentration as 4.902% mean value with SD ± 0.30 while in control group mean values were recorded as 9.414% (SD = ± 1.40). Simultaneous administration of Zn, Ca and Zn+Ca increased MO concentration but best concentrations were calculated in Ca supplemented rats as 8.538 ± 0.62%.

Significant decrease (P<0.01) in GR, another type of WBCs, has been measured because of Pb exposure with mean value of 1.568% (SD = ± 0.10) than mean values recorded for control group 4.6120 ± 0.46%. Supplementation of Zn and Ca normalized the Pb induced decreased levels of GR as shown in Figure 1. Best outcomes were seen in Ca supplemented rats where mean values of GR were measured as 7.006% (SD = ± 0.75).

Hemoglobin (Hb) and related hematological parameters:

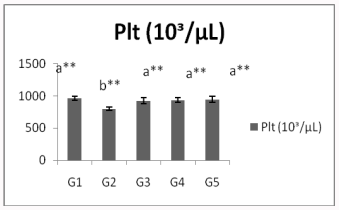

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Figure 4: Concentration of Platelet of Pb intoxicated and Ca & Zn supplemented rats;
Results are stated as Mean ± SD. Superscripts with different letters differ significantly (*=P<0.05, **=P<0.01).

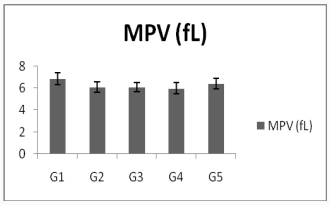

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Figure 5: Mean Platelet Volume of Pb intoxicated and Ca & Zn supplemented rats;
Results are stated as Mean ± SD.

Hemoglobin levels evaluated at the end of the study, showed that there was significant decrease in Hb levels (P<0.01) in Pb exposed rats than in all the other groups i.e., 10.176 ± 0.40 g/dL. Figure 2 shows that Zn and Ca supplementation have the potential to reverse the adverse effects of Pb on Hb levels in rats, while mean values of Hb in Zn group 13.732 ± 0.58 g/dL were precisely measured relevant to control group i.e. 13.732 ± 1.08 g/dL.

Mean Corpuscular Hemoglobin (MCH) is the measurement of the amount of hemoglobin per RBC. Figure 2 shows that there is a significant decrease (P<0.01) in MCH level in Pb exposed rats measured as 13.440 ± 1.44 pg in comparison to 16.956 ± 1.40 pg measured in control. Supplementation of Zn, Ca and Zn+Ca showed similar potential in restoring normal MCH levels, best precised values measured in Ca supplemented group as 16.704 ± 1.79 pg.

Mean Corpuscular Hemoglobin Concentration (MCHC) is the average amount of Hgb in specific volume of RBCs. Figure 2 shows that MCHC differs non-significantly in all the groups.

Hematocrit (Hct), Mean Corpuscular Volume (MCV), Red Blood Cells (RBCs) and Red Cell Distribution Width (RDW): Hct, the percentage of Red Blood Cells (RBCs), was found reduced after Pb exposure significantly (P<0.01) with mean value measured as 36.556 ± 1.42% than those measured in control 46.508 ± 3.66. Results of this study depicted in Figure 3 showed that Zn and Ca increased Hct percentage up to normal levels but effects were more efficient in Zn supplemented rats 42.98 ± 1.67%.

MCV represents average size of RBCs. Figure 3 shows non-significant variation in MCV values of all the groups which indicates

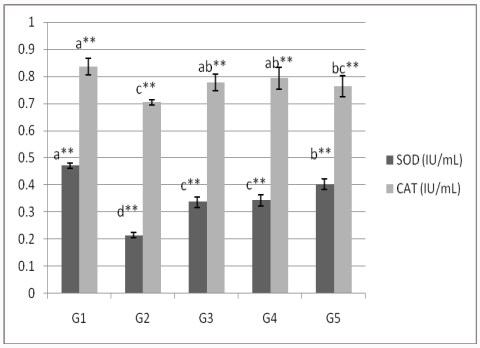

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Figure 6: Concentration of antioxidant enzymes; SOD and CAT of Pb intoxicated and Ca & Zn supplemented rats;
Results are stated as Mean ± SD. Superscripts with different letters differ significantly (*=P<0.05, **=P<0.01).

that Pb has no effect on the size of RBCs.

It is the measurement of RBCs that are able to bind oxygen from blood and carry it to the tissues of body. Results of our study showed that Pb significantly decreased (P<0.01) RBCs level in group 2 as 6.424 ± 0.25 (10^6/µL) than those measured in control i.e. 8.860 ± 0.70 (10^6/µL). Figure 3 showed that simultaneous supplementation of Zn and Ca increased RBCs concentration, more precise results measured in Zn supplemented rats as 7.412 ± 0.18 (10^6/µL). RDW is the measurement of variation in sizes of RBCs. RDW calculated at the end of this study showed significantly (P<0.01) elevated in group 2 with mean value as 15.868 (SD=1.71). Figure 3 show that Zn and Ca supplementation have the potential to reverse the adverse effects of Pb on RDW. Ca restored RDW more effectively with mean values as 12.792 ± 0.98 than Zn and Zn+Ca supplemented rats in relevant to control values as 14.932 ± 1.28.

Platelet count (Plt): Figure 4 shows the platelet values of experimental rats were measured significantly lower (P<0.05) with mean value as 799.29 (10^3/µL) with SD ± 30 in Pb administered rats than those in control 962.72 (10^3/µL) with SD ± 30. Supplementation of Zn, Ca and Zn+Ca was found potent in increasing platelet count with equivalent extent.

Mean Platelet Volume (MPV): MPV i.e. average of the newly formed Plt, were found statistically non-significant (P>0.05) (Figure 5).

Antioxidant enzymes: Serum antioxidants i.e. SOD and CAT levels were measured at the end of the study to check the effect of lead on these enzymes.

SOD levels were found significantly (P<0.01) lower in group 2 than all other groups which means that lead has degraded the SOD enzymes in blood. Mean values of SOD measured lower in Pb exposed group as 0.213 IU/mL with SD ± 0.01 than those measured in control group 0.47 IU/mL (SD = ± 0.01). Figure 6 shows that zinc and calcium have potential to restore the SOD levels in lead exposed rats. Zn and Ca alone have similar effects in elevating SOD levels while their synergistic effect showed greater recovery in SOD values 0.40 IU/mL (SD = ± 0.02).

CAT levels were found to be significantly (P<0.01) decreased in group 2 than the treatment and control groups. Similar to SOD levels Pb also have degenerative effects on CAT enzymes. Mean values of CAT measured lower in Pb exposed group as 0.704 IU/mL with SD ± 0.01 than control values 0.836 IU/mL (SD = ± 0.03). Figure 6 shows that zinc and calcium have potential to restore the CAT levels in lead exposed rats. Zn and Ca alone showed similar effects in elevating CAT levels while their synergistic effect was measured lesser than their individual administration.

Discussion

In the current study we evaluated the ameliorative potential of Zn and Ca either with their separate or combined administration against Pb induced haematopathological disruptions as well as oxidative stress in blood cells of experimental rats.

Significant decrease (P<0.05) in WBCs was observed in lead intoxicated rats. In the similar way, granulocytes and monocytes concentrations decreased significantly (P<0.01). Altered leukocyte count is one of the major consequences of infections. Low leukocyte counts often reflect immunosuppression [16]. Faith and coworkers in their study reported lead induced immunosuppression after observing decreased levels of lymphocytes in lead intoxicated rats even at low dose of 25 ppm for 7 days [17]. Lead induced leukopenia has also been reported by Simsek et al., [18] and Wahab et al., [19]. Sharma et al., [8] also evaluated structural alteration in nucleus and cytoplasm of WBCs as the hallmark of chronic exposure to lead acetate in swiss mice. Adverse impacts of Pb on WBCs, monocytes and Granulocytes were significantly reestablished by Zn and Ca either alone or in combination but the reversal effects were more prominent in Zn supplemented rats than Ca and Zn+Ca. significant increase of WBCs in Ca supplemented rats was also noticed that shows that Ca inhibits Pb induced oxidative damages to blood cells. Ca intake reduces the Pb absorption from GIT as Pb & Ca competes for absorption at gastrointestinal receptors [20].

Decreased levels of hemoglobin (P<0.01) in Pb intoxicated rats were also measured. Pb disrupts hemoglobin synthesis by interacting with the sulphydryl enzymes i.e. ferrochelatase and alfa aminolevulinic acid dehydratase that are crucial for heme synthesis [21]. Caylak et al., [9] in their study reported decreased (P<0.01) levels of Hb in rats after chronic exposure of low dose of Pb. In connection with Hb, we also observed marked decrease in Hct, MCV and RBCs (P<0.01) levels. Decreased levels of HCT in lead exposed rats have also been reported by Sivaprasad et al., [22]. Pb decreases the life span of circulating RBCs by damaging causing destructions in their cell membrane [23].

Results of our study reveal that variations in RBCs sizes are significantly higher (P<0.05) in lead exposed group. Increased RDW shows disruptions in RBC maturation and decreased antioxidant concentrations in serum [24,25]. All these harmful impacts caused by lead were significantly reversed by Zn, Ca and Zn+Ca supplementation. In case of HB, Hct, RBCs and MCV, the ameliorative impact of Zn was observed greater than Ca and Zn+Ca while for RDW Ca showed better results than Zn and Zn+Ca. Prasanthi et al., [26] have also reported that Zn or Ca can reduce Pb burden in blood when given simultaneously. Decreased levels of Hb and RBCs have also been shown by Zhai and coworkers in lead exposed mice and reversal of normal levels was observed after treatment with different antioxidant dietary supplements [10].

Although decrease in MCH and MCHC have also been reported

as a consequence of lead exposure but in current study we found them varying non-significantly (P>0.05) [19]. It might be justified with estimation that lead impacts their concentration in dose dependent manner. Platelet count and mean platelet volume were also assessed and results showed that platelet count in lead exposed rats was significantly (P<0.01) lower while MPV levels were found non-significantly different (P>0.05). Platelets are the blood cells specialized for blood clotting after any sort of injury. Barman et al., [27] in a survey based study reported reduced platelet levels in people working in specialized areas where lead-acid batteries were being manufactured. Pb disrupts endothelial lining of platelet cells and thus makes them fragile and inappropriate for blood clotting. Pb mimics Ca^{2+} actions and thus antagonizes Ca^{2+} dependent actions ultimately causing injury in endothelial tissues [27,28]. Simultaneous supplementation of Zn, Ca and Zn+Ca revived Pb induced reduced levels of platelets. Individual and symbiotic potential showed similar trend in normalizing platelet concentration. Das et al., [29] observed in their study the protective effects of simultaneous supplementation of different doses of Zn with cypermethrin, an active pesticide causing severe hematotoxicity. They found that Zn significantly improved Hb, Hct, RBCs, WBCs & Plt levels and oxidative stress biomarkers to their healthy levels in blood [29]. These results are compiled with our study.

To assess harmful impact of Pb induced oxidative stress, antioxidant enzymes including SOD and CAT levels in erythrocytes were measured. Observations of our study showed decreased concentrations of SOD and CAT in lead intoxicated rats. Supplementation of Zn, Ca and Zn+Ca significantly increased (P<0.01) SOD and CAT levels up to normal levels. In case of SOD, Zn+Ca while for CAT Zn alone showed greater impact in reviving their normal concentrations.

Several studies previously conducted have also reported decreased levels of SOD and CAT levels as a consequence of oxidative stress induced by lead [30,31]. Lodi et al., [8] have also reported similar association between lead and serum CAT levels. Haleagrahara et al., [32] reported marked decrease in SOD levels after intoxication of lead acetate in rats. Adi et al., [33] in their study observed the symbiotic effect of Zn+Ca and Vitamin E supplementation against cadmium induced oxidative stress damage. They reported increased CAT, SOD and glutathione activities in cadmium intoxicated rats and these effects were significantly reversed by Zn+Ca and Vitamin E supplementation [33]. Finally, it can be concluded from this study that Zn and Ca either alone or in combination possess ameliorative potential against the hallmarks of Pb induced oxidative stress.

Conclusion

Lead exposure is unavoidable these days because of its increased concentration in air, tap water, food packaging and in making of household useable. Lead induces its harmful impacts by reducing antioxidant enzyme content and increasing release of reactive oxygen species. We conducted this study to evaluate the ameliorative impacts of Zn, Ca and Zn+Ca supplementation against the adverse hematopathological alterations and oxidative stress induced by Pb. Observations gathered at the end of this study revealed that both Zn and Ca, alone and in combination improved hematological parameters and antioxidant enzymes in erythrocytes. Effects of greater content of recovery was seen in mostly parameters with Zn supplementation except for SOD and RDW for which better improvements were seen with Zn+Ca and Ca alone supplementation respectively.

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