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ALTERATIONS IN DEVELOPING RBCs AFTER PRENATAL AND POSTNATAL EXPOSURE TO LEAD ACETATE AND VITAMINS

Ragini Sharma, Khushbu Panwar* and Sheetal Mogra

Environmental and Developmental Toxicology Laboratory, Department of Zoology, University College of Science, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

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Correspondence to Author:

Khushbu Panwar

Research Scholar, Environmental and Developmental Toxicology Laboratory, Department of Zoology, University College of Science, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

E-mail: khushbu999@yahoo.com

ABSTRACT:

Purpose: The current investigation was carried out to study the hematopathological changes in the developing red blood cells of neonate of Swiss albino mice exposed with lead acetate and vitamins.

Methods: Lead acetate was administered orally at 8, 16, 32 mg /kg/BW to selected pregnant females from 10th day of gestation to 21st day of lactation. Vitamin C (166 mg/kg BW) and vitamin E (133mg/kg BW) individually and in combination with lead were also administered from 10th day during the same period. Hematopathological alterations in the RBCs were examined in neonates after birth at postnatal days 1, 7, 14 and 21.

Results: The examination of blood smears demonstrated that lead exposure during gestation and lactation led to various hematological disorders in red blood cells of neonates and abnormal types of RBCs like schistocytes, stomatocytes, codocytes, echinocytes with prominent hypochromasia, anisocytosis, macrocytosis etc. were also noticeable. The antioxidants vitamin C and E also induced structural abnormalities independently and also with co-administration of lead in developing RBCs in forms of dacrocytes, elliptocytes and other atypical forms.

Conclusion: Lead may interfere in heme biosynthesis apparently characterized by several enzyme blockades and exerts the negative effect on hematopoietic system and give rise to abnormal blood cells. This study clearly demonstrates that gestational and lactational exposure to lead is extremely hazardous in causing alterations in the neonatal peripheral red blood cells. Vitamins supplementation also induces negative influence during this highly susceptible period.

INTRODUCTION: Lead intoxication is a complex disorder that affects several cells and organs, including functional and structural alteration of erythrocytes [1].

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One of the most prominent effects of lead is exerted on the biosynthesis of heme, the prosthetic group present in hemoglobin, cytochromes, catalases and peroxidases [2]. The role of blood leukocytes in reproductive functions and in immune processes lead us to suspect that lead may affect these functions, changing the proportions, levels and activity of the different leukocytes. Prepubertal rat exposure to lead also affects blood neutrophil and eosinophil leukocyte levels and induces eosinophil degranulation [3].

Moderate lead levels of 100 micrograms/L can inhibit fetal heme synthesis and erythropoiesis [4]. It has long been known that hematopoiesis and heme synthesis is affected by lead poisoning [5]. Lead can cause damage in the erythrocyte, originating defective cells, preventing them from carrying oxygen and it also produces high blood pressure that increases the risk of heart attack [6]. At levels of 30μg/dL, elevation in erthyrocyte protoporphyrin may be seen. Finally, at levels of 40 μg/dL, reduced hemoglobin synthesis may be found [7]. Even though manifestations of Pb poisoning in humans are non-specific, they are always accompanied by oxidation [8].

The largest part of circulating lead is bound to hemoglobin in erythrocytes, in which the concentration of lead is about 16 times greater than in plasma [9]. Almost all (99%) blood lead is associated with erythrocytes, and 50% of erythrocyte lead is bound to hemoglobin. Inorganic lead ion is not metabolized in the body and has a potential to be conjugated with glutathione [10].

Lead is firmly bound to red blood cells, enhancing transfer from maternal circulation through the placenta to the fetus. As in adults, the lead can also be found in fetal blood, soft tissue and bone. The fetus is more sensitive to lead because the fetal blood-brain barrier is more permeable and the fetus has less bone tissue for sequestering lead.

A pregnant woman's elevated blood lead level can lead to miscarriage, prematurity, low birth weight, and problems with development during childhood [11]. Lead induces disturbances in the development of different types of WBCs during postnatal development and lead to an abrupt neutrophilic degeneration, immature cells, abnormal neutrophils, reactive and plasmacytoid lymphocytes [12].

The prime targets to lead toxicity are the heme synthesis enzymes, thiol-containing antioxidants and enzymes (superoxide dismutase, catalase, glutathione peroxidase, glucose 6-phosphate dehydrogenase and antioxidant molecules like GSH) and it is observed that the low blood lead levels are also sufficient to inhibit the activity of these enzymes and induce generation of reactive oxygen species and intensification oxidative stress. Hence, oxidative stress plays important role in pathogenesis of lead-induced toxicity and pathogenesis of coupled disease. It is known that *α*-tocopherol (vitamin E) and l-ascorbic acid (vitamin C) can modulate many biochemical processes as antioxidants.

Many studies conducted in adults proved that an oxidant-antioxidant balance may play a role in lead induced toxicity in living beings. Ali *et al.,* [13] studied effect of vitamin C against lead toxicity suggested that vitamin C is a good antioxidant to overcome lead toxicity. Qureshi *et al.,* [14] also revealed that vitamin C and E supplementation rebalanced the alterations caused by lead in adult mice.

The present study was carried out to study the alterations in red blood cells due to lead acetate toxicity and interference through vitamins in neonates, which passes from adult pregnant female during gestation and lactation.

MATERIALS AND METHODS:

Ethics: The proposed experiments were conducted during 2009-2011 in the Department of Zoology, University College of science, Mohanlal Sukhadia University, Udaipur and the experimental protocols were approved by the Institutional Animal Ethical Committee of the University NO.CS/Res/07/759.

Test Chemicals:

- Lead acetate: Laboratory reagent lead acetate, manufactured by 'S.D. Fine Chem. Ltd.', Mumbai was used for the experiments.
- Vitamin C: 'Limcee' capsule of vitamin C containing 500 mg of ascorbic acid, manufactured by 'Sarabhai Chemicals', Vadodara was used for the investigation.
- Vitamin E: 'Evitam-400' capsules of vitamin E containing 400 mg of tocopheryl acetate (vitamin E), manufactured by 'Strides Arcolab Ltd.', Bangalore was used for the present investigation.

Animals: Sexually mature Swiss mice in age group of 6-7 weeks, weighing 30 gm were used during the entire experimental period. The animals were fed on a standard diet and water ad libitum. Female mice showing vaginal plugs were separated and the day on which sperms were detected in the vaginal smear was counted as day 1 of pregnancy.

Experimental protocol: All the selected animals were divided into the control, lead, vitamin C and E and with combinations with lead treated groups. The dose of lead acetate were given to pregnant Swiss mice at a concentration of 8, 16 and 32 mg (266, 533, and 1066 mg/kg/BW) and vitamin C (166 mg/kg BW) and vitamin E (133mg/kg BW) from 10^{th} day of gestation to 21^{st} day of lactation individually and with combinations of lead+ vitamin C and lead+ vitamin E.

Structural changes in red blood cells were observed for hematological investigation in all treated groups on postnatal day (PND) 1, 7,14 and 21. For determination of hematological parameter, blood samples were obtained from the tail of pups from each litter of control and treated mice from different experimental groups at each postnatal day after birth. The tip of the tail was cleaned with spirit before being cut with a sharp blade and was not squeezed to avoid dilution of blood by tissue fluid. These blood films were fixed in absolute methanol for 15 minutes and stained with freshly made Giemsa stain, the washed slides were allowed to dry and the hematological observations were taken.

Experimental groups: The selected pregnant females were divided into twelve (12) equal groups as follows:

Group I: control group (distilled water)

Group II: 266 mg/kg BW (8 mg/animal/day) lead acetate

Group III: 533 mg/kg BW (16 mg/animal/day) lead acetate

Group IV: Exposure of 1066 mg/kg BW (32 mg/animal/day) lead acetate

Group V: 166 mg/kg BW vitamin C

Group VI: 266mg/kg BW lead acetate +166mg/kg BW vitamin C (8+C)

Group VII: 533mg/kg BW lead acetate +166mg/kg BW vitamin C (16+C)

Group VIII: 1066mg/kg BW lead acetate +166 mg/kg BW vitamin C (32+C)

Group IX: 133mg/kg BW vitamin E

Group X: 266mg/kg BW lead acetate +133mg/kg BW vitamin E (8+E)

Group XI: 533mg/kg BW lead acetate +133mg/kg BW vitamin E (16+E)

Group XII: 1066mg/kg BW lead acetate +133mg/kg BW vitamin E (32+E)

RESULTS: The hematologic abnormalities are used to aid in diagnosis and understanding of the pathophysiologic mechanisms of lead poisoning. Erythrocyte shape abnormalities, basophilic stippling, porphyrin abnormalities and normoblastosis have been associated with lead ingestion in many species. Review of blood smear at different postnatal days revealed numerous changes in the normal biconcave structure of RBC due to lead poisoning in both animals and human beings. The red blood cells in our study are pinkish, biconcave discs very numerous in control animals. Usually, they measure 6-7µm in diameter. Early postnatal RBCs are large at birth and the cell size decreases gradually during development (**Table 1, fig. 1**).

Influence of vitamin C shows several spherocytes and vitamin E reveals elliptocytes with small number of schistocytes and echinocytes (**Table 1, fig. 19, 8, 12 and10**). In peripheral blood smear, lead treated groups show abnormal types of RBCs with reference to shape and size, although the normal development of erythrocytes is not perturbed. In few groups there are structural changes in red blood cell, with large RBCs (macrocytes) and small RBCs (microcytes) indicating pathological condition (**Table 1, fig.4 and 5**). This is the indication of macrocytic anemia, in which the RBCs are enlarged and microcytic anemia having small RBCs in lead and lead + vitamin treated groups.

Except macro and microcytes, the lead treated RBCs also exhibit hypochromasia representing small amount or concentration of hemoglobin due to expanded central pallor zone and hollow space (**Table 1, fig. 15**). However, this type of hypochromic RBCs are not seen in vitamins and lead + vitamins treated groups.

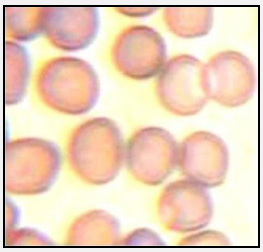
It is not uncommon to see variation in the shapes in RBCs of neonates. Irregular shaped cells are more frequent in newborns than in adults, making it difficult to discriminate between normal variants and pathological changes during postnatal period. In our study this phenomena of anisocytosis is also seen at 3rd and 4th week of lactation in lead treated groups which does not seems a normal condition (**Table 1, fig. 6**). RBCs with abnormal morphology may be removed from the circulation leading to anemia. Therefore, certain types of anemia may be characterized by poikilocytosis also observed in lead treated groups (**Table 1, fig. 18**).

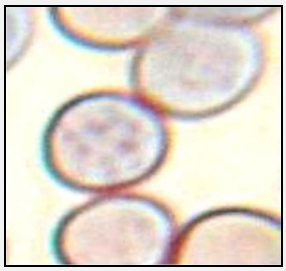
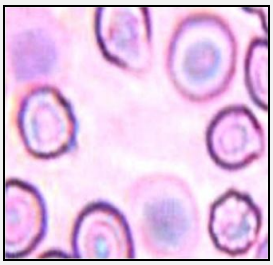
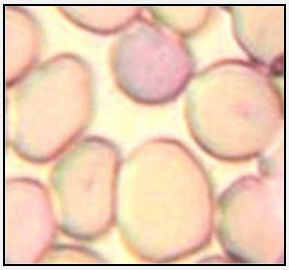
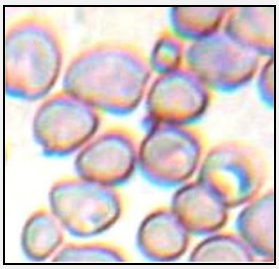
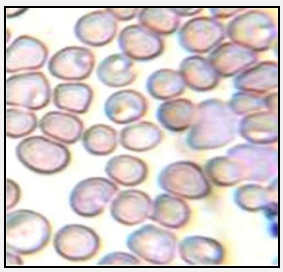
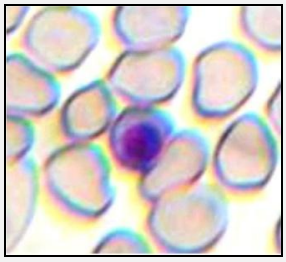
Basophilic stippling describes the presence of small granular bodies within the red cell cytoplasm and occurs due to disordered and accelerated erythropoiesis, indicating red cells with immature cytoplasm are released into the circulation as a feature of lead poisoning and anemia. This phenomenon is frequently seen in lead treated groups and remains observable within the combinations of vitamins + lead treated groups (**Table 1, fig. 2**). A hemolytic process causes RBCs to be destroyed before the end of their expected life spans, termed as schistocytes, is also observable in the blood smear of lead and both vitamins treated groups. Schistocytes are red blood cell fragments that results from membrane damage encountered during passage through vessels which normally occur in severe burns, uremia, and hemolytic anemia sometimes referred as "bite cells" (**Table 1, fig. 12**).

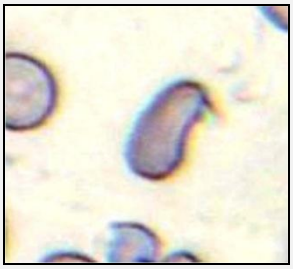
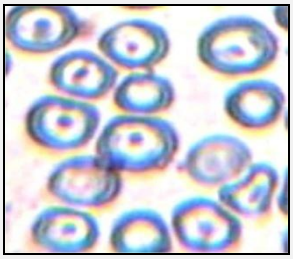
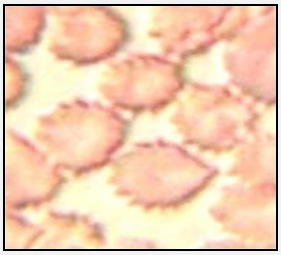
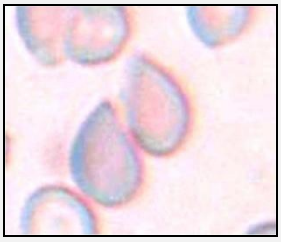
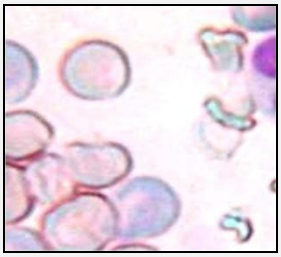
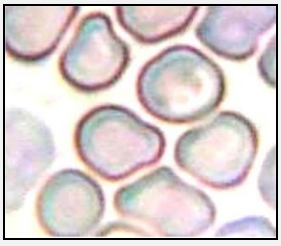
Acanthocytes (irregularly shaped erythrocytes), crenated cells (exhibits small, delicate, regular shaped spines), target cells (have a "lump" of hemoglobinized cytoplasm within the area of normal central pallor), stomatocytes (elongated (mouth-like) area of central pallor) are also prominently seen in all lead treated groups with small fraction in other groups (**Table 1, fig. 3, 9 and 16**).

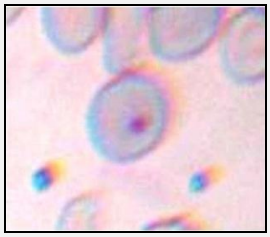
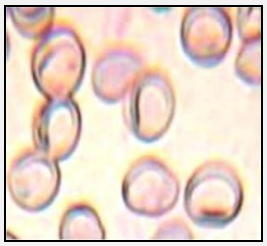
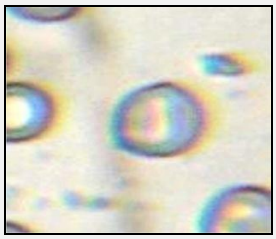
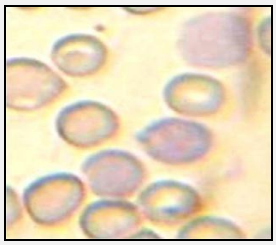
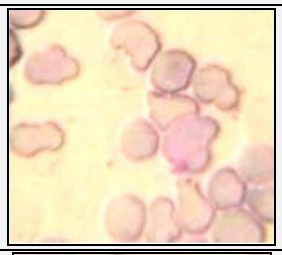
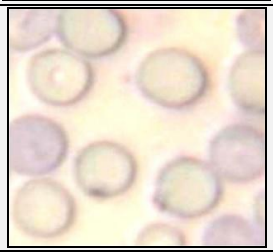
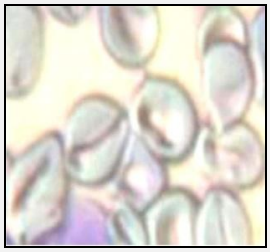
With structural changes in the periphery of erythrocytes, there are also some special red blood cell inclusions observed in lead and lead + vitamin treated groups. Spherical blue-black inclusions of red blood cells “Howell-Jolly bodies” are seen in lead treated peripheral blood smears. These are nuclear fragments of condensed DNA, normally removed by the spleen. A rounded often refractive projection from the surface of the red blood cell due to oxidation and denaturation of hemoglobin termed as “Heinz body” is seen in lead + vitamin C treated groups. Dacrocytes, also known as ‘teardrop cells’ is a remarkable feature in lead + E treated group (**Table 1, fig. 14, 13 and 11**). The various types of red blood cells observed in all groups are described as follows:

TABLE 1: MORPHOLOGICAL AND STRUCTURAL EVALUATION OF RED BLOOD CELLS IN CONTROL AND ALL EXPERIMENTAL GROUPS FROM BIRTH (PND1) TO THE TERMINATION OF LACTATION (PND 21)

S. No.	Structural appearance	Types of Cells	Description of cells	Presence in groups
1		Normal red blood cell	Pink, biconcave, disc-shaped cell with a flattened center on both sides without nucleus	Control groups

S. No.	Structural appearance	Types of Cells	Description of cells	Presence in groups
2		Basophilic stippling	The presence of small granular bodies within the red cell cytoplasm	Lead, lead + vitamin c treated groups
3		Acanthocytes (spurr cells)	Irregularly shaped erythrocytes containing blunt membrane spicules that are unevenly distributed around the red cell surface	Vitamin C and lead treated groups
4		Macrocytosis	Presence of abnormally large red cells	Lead, Lead + vitamin E, lead + vitamin C treated groups
5		Microcytosis	Decrease in the size of red blood cell	Lead treated groups
6		Anisocytosis	Increase in the variability of red cell size	Lead and vitamin treated groups
7		Nucleated red blood cell (nrbcs or normoblasts)	Immature RBC having small basophilic nucleus	At PND 1 and 7 in all groups, but number increased in lead treated groups and can be seen till third week

S. No.	Structural appearance	Types of Cells	Description of cells	Presence in groups
8		Elliptocyte (cigar cell/ Ovalocyte)	Oval (ovalocyte) or cigar shaped cells	Vitamin E treated groups
9		Codocytes/ Leptocytes/ Mexican hat cells (target cells)	Red cells with a central area of increased staining	Lead, lead + vitamin C, lead + vitamin E treated groups
10		Crenated cells /Burr cells (echinocyte)	Red blood cell with, multiple, small, delicate, regular shaped spines distributed evenly around the membrane	Lead, vitamin C, vitamin E lead + vitamin C, lead + vitamin E treated groups
11		Dacrocytes (tear drop cells)	Red cells shaped like a tear drop or pear	Lead + vitamin E treated groups
12		Schistocytes (fragmented cells)	Red cells fragmented by their passage through intravascular strands of fibrin	Lead, vitamin C, vitamin E treated groups
13		Heinz bodies	Rounded often refractive, projection from the surface of the red blood cell	Lead+ vitamin C, treated groups

S. No.	Structural appearance	Types of Cells	Description of cells	Presence in groups
14		Howell-Jolly bodies	Spherical blue-black inclusions of red blood cells	Lead, lead + vitamin C treated groups
15		Hypochromia	Impaired staining of red cells	Lead treated groups
16		Stomatocyte (mouth cells)	Oval or rectangular area of central pallor, sometimes referred to as a "mouth" arises as a result of loss of concavity on one side	Lead, lead + vitamin C, lead + vitamin E treated groups
17		Polychromasia	The heterogeneous staining of red blood cells of different ages	Lead treated groups
18		Poikilocytosis	Variation in cell shape	Lead treated groups
19		Spherocytes	Overly-round or spheroid red cells	vitamin C, vitamin E lead + vitamin C, lead + vitamin E treated groups
20		Eccentrocyte (folded cell)	A red blood cell with a crescent shape area that is eccentrically placed	Lead + vitamin E treated groups

DISCUSSION: There are many evidences which conclude that lead toxicity contributes to the interference in the development of the fetus via entering through the placenta and this toxicity also continues into neonates; hence it is dangerous for newborns and their future generations. Lead stored in the bone as a result of childhood lead poisoning moves into the blood, increasing the mother's blood lead level and passing to the fetus. Pregnancy related hormonal changes affect calcium metabolism and also cause lead to leave the bone and enter the blood. Whenever maternal blood lead becomes elevated, it is easily obtainable to the fetus and can negatively impact the fetal development [15].

Changes in blood parameters are often one of the hallmarks of infection. These include changes in number and in cellular morphology. However, the changes encountered may vary significantly, depending on the type of infection and the host affected. The exposure to lead possesses the potentials to induce hazardous biological effects in both animals and human beings. Lead acetate severely affects the morphology and distribution of various blood cells. It crosses the placental barrier and its greater intestinal absorption in fetus results in developmental defects.

Blood smears examined illustrate that lead induces disturbances in the development of Red blood cells during postnatal development, leads to various changes in erythrocytes and induce an abrupt structural alteration. These alterations induced by lead are pathological. The results of the present study emphasize that prenatal lead exposure is extremely dangerous to developing fetus. The alterations in hematological changes serve as the earliest indicator of toxic effects on tissue [16].

Anemia is one of the early manifestations of lead poisoning, it results from reduction of the life span of circulating erythrocyte as well as by inhibition of the body's ability to make heamoglobin by interfering with several enzymatic steps in heme pathway. In this way, Ferrochelatase, which catalyze the insertion of iron into protoporphyrin IX, is quite sensitive to lead. Erythrocyte Na-K-ATPase is somewhat inhibited by lead suggesting a loss of cell membrane integrity, this may account for the shortened lifespan of erythrocytes, lead can also cause damage in the erythrocytes originating defective cells that are eliminated by spleen and their hemolysis [17, 18].

Anemia may result when the cell membranes of RBCs become more fragile as the result of damage to their membrane [19].

In our study, lead treated groups shows abnormal types of RBCs like macrocytes, microcytes showing hypochromasia, anisocytosis and as a certain types of anemia characterized by poikilocytosis is simply observed. Comelekoglu *et al.,* [20] also affirmed that some pesticides may provoke the alterations in size and surface shapes of erythrocytes.

Nikinma [21] suggested that toxic materials directly or indirectly damage the membrane structure, ion permeability and cell metabolism of erythrocytes thus may cause morphologically damaged erythrocyte formation. Structural defects and changes in surface shapes of erythrocytes have been reported by Koc *et al.,* [22] from endosulfan and malathion exposed rats. Isha *et al.,* demonstrates the high levels of lead exposure during gestation and lactation can severely damage heme synthesis and alter the number of RBC and WBC [23].

In present study, neonatal blood was examined from birth to lactation after exposure of their mother to various doses of lead and vitamins and hematological changes were observed. Basophilic stippling examined with presence of small granular bodies within the red cell cytoplasm was frequently seen in our study in all lead treated groups.

The combinations of vitamins with lead acetate were also detected with similar results. According to Bull [7] the basophilic stippling of red cells is due to the presence of aggregated ribosomes, which may also include mitochondrial fragments. Conditions, such as lead poisoning, can result in altered ribosomes to have a higher propensity to aggregate. After staining, this appears as increased basophilic granulation.

Schistocytes were also observed in the blood smear of lead and both vitamins tread groups. Tripathi and Srivastav [24] noticed fragmented red blood cells (schistocytes) after chlorpyrifos treatment to rats. This derives support from the previous findings of several investigators who have reported the occurrence of schistocytes after treatment with aluminium in rats [25] and phenylhydrazine in calf [26]. Schistocytes are one of the poikilocytes used to monitor toxicity.

It is suggested that glomerulonephritis is one of the factors for the formation of schistocytes. It is also reported that kidney and liver lesions observed after chlorpyrifos treatment to rats may be the reason for the presence of schistocytes. This can also be considered as supportive data for the other cell deformities (dacrocytes, acanthocytes) noticed in the present study.

It can be also speculated that chlorpyrifos caused alterations in the cytoskeleton (membrane proteins and/or lipids) of red blood cells thus affecting the surface area of the cell. This study was also in consistent with Gurer *et al.,* [27] who demonstrated a sign of anemia in lead exposed animals as evidenced by anisocytosis, poikilo-cytosis, and alterations in hemoglobin, hematocrit, and mean corpuscular volume.

Results of current study indicates that lead acetate induce alterations in erythrocytes and the other forms of red blood cells such as crenated, dacrocytes, schistocytes, echinocytes are observable in lead exposed pups. Suwalsky *et al.,* [28] have reported that human erythrocytes when incubated with aqueous extract of *Aristotelia chilensis* showed an alteration in erythrocyte morphology from the normal discoid shape to an echinocytic form. The presence of echinocytes (crenated cells) might influence the blood flow by increasing viscosity, resulting from the intermeshing of the spicules [29] thus obstructing the microcirculation.

Zeni *et al.,* [30] noticed occurrence of echinocytes in *Ictalurus melas* following exposure to an anionic detergent (sodium dodecyl benzene sulphonate) and suggested that echinocytes may result from cellular adaptation of physiological parameters involved in shape maintenance. Macrocytosis is a pathological alteration characterized by presence of abnormally large red cells was frequently seen in our investigation in lead treated groups and were also noticed in lead+ vitamin E and lead + vitamin C treated groups.

An increase in the cellular size of the red blood cells has also been recorded in rats post exposure to chlorpyrifos. This could be due to the membrane alterations. Chlorpyrifos may have impact on the cell flexibility and permeability by mechanisms not yet defined [24].

The increased cellular size observed in this study may derive support from the suggestions of Vives *et al.,* [31] who explained that the expansion of membrane increases the area/volume proportion and could allow the swelling of the cell, thus, reaching the largest volume before the lysis. An increase of erythrocyte size (MCV) has been associated with several factors but it is generally considered as a response to stress [32].

It is possible that lead accumulates in the erythrocyte membrane (a characteristic of divalent cations) due to the negative membrane surface charge density [33, 34] which may lead to a much higher lead concentrations in the membrane interface than in the bulk. Furthermore, there is evidence of inhibitory effects of lead on antioxidant enzymes [35, 36].

In addition to membrane peroxidation, lead exposure causes hemoglobin oxidation, which can also cause RBC hemolysis. The possible mechanism responsible for this reaction is lead-induced inhibition of ALAD. ALAD is the enzyme most sensitive to lead's toxic effects depressed heme formation. Lead also induces disturbances in the development of different types of WBCs during postnatal development and lead to an abrupt neutrophilic degeneration, immature cells, abnormal neutrophils, reactive and plasmacytoid lymphocytes [37].

In the present study, vitamin C and vitamin E was administered to pregnant females unaccompanied and along with lead acetate to determinate whether the given dose of vitamins can inhibit or reduce the alterations in blood cells; however the results of present investigation illustrated some unexpected outcome to analyze.

Influence of vitamin C shows several spherocytes and vitamin E reveals elliptocytes with small number of shistocytes and echinocytes. Dacrocytes/ Teardrop cells (abnormal form of RBC) were also noticed in lead + E treated groups at various places in the blood smear of neonate. Spherical blue-black inclusions of red blood cells, howell-Jolly bodies and Heinz body were seen in lead + vitamin C treated groups. Stomatocyte, spherocyte, eccentrocyte, target cells etc. and other abnormal RBCs were apparent in lead + vitamins treated groups seems no protection in lead intoxicated blood cells.

Vitamin C and E intake from food is generally considered safe during pregnancy. It is not clear if vitamin C and E supplementation in form of pharmacological doses exceeding Dietary Reference Intake recommendations is safe or beneficial in this critical period. The data are too small in number to say if vitamins supplementation separately or combined with other supplements is beneficial during pregnancy. Very limited studies have examined the role of vitamin C and vitamin E for ameliorating lead toxicity during postnatal development.

CONCLUSION: This study clearly demonstrates that gestational and lactational exposure to lead is extremely hazardous in causing alterations in the neonatal peripheral red blood cells. Despite the well-established relationship between oxidative stress and antioxidants, the present study did not perceive any risk reduction in lead induced blood abnormalities with the help of vitamin C and vitamin E supplementation and vitamins supplementation also induces negative influence during this highly susceptible period. Additional well-designed trials are needed before any conclusion can be made.

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