Research Paper

Erythrocyte Morphology, Osmotic Fragility and Hematological Studies after Short Term Dietary Copper Deficiency in Male Wistar Rats

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Saini *et al.*: Short Term Dietary Copper Deficiency Effects Wistar Rat Erythrocyte

Trace element copper is involved in myriad of physiological activities vital for normal function of organs of animals and humans from fetal development onwards. Its deficiency might occur in infants, children as well as adults and has been reported as a global problem affecting developed and undeveloped countries. Copper is essential for efficient iron uptake and mobilization. The present study evaluates the effect of dietary copper deficiency on erythrocyte of prepubertal male Wistar rats for 2, 4 and 6 w. Erythrocyte morphology, osmotic fragility, hematological parameters-hemoglobin, total leukocyte count, cell count (neutrophil, lymphocytes monocytes, eosinophils, platelets), packed cell volume, total red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were evaluated. Occurrence of stomatocytes, echinocytes, dracocytes, acanthocytes, Howell-Jolly inclusion, microcyte and macrocytes were observed after 2 and 4 w of copper deficiency when compared with their respective control and pair fed groups. The extent of morphological changes enhanced after 6 w of dietary copper deficiency with additional appearance of fragmented cells and few elongated cells. The percentage of altered erythrocyte morphology after 2 w deficiency was 0.051 % which enhanced subsequently as duration increased to 0.081 % (4 w) and 0.1709 % (6 w). Consequent to these changes significant ($p<0.05$) increase in erythrocyte osmotic fragility, eosinophil and lymphocyte count was observed while hemoglobin, neutrophil and platelet count, packed cell volume, total red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration decreased. Monocyte decrease was not significant. These changes were more pronounced after 4 and 6 w. Dietary copper insufficiency caused impairment of membrane, deregulation of hematopoiesis and altered the hematological profile indicating detrimental changes in properties of cellular membrane which may probably be due to enhanced oxidative stress.

Key words: Erythrocyte morphology, osmotic fragility, hematological parameters

Copper, a transitional trace element is involved in numerous physiological processes and is critical for both humans and several mammals particularly during the period of rapid postnatal growth[1-3]. Homeostasis of copper is regulated by cupric reductases[4-6], transporters[7], chaperones[8-9] and metallothionein[10]. Copper and iron, although required in miniscule amount being involved in numerous physiological functions, interaction exists due to same oxidation states as well as similar physiochemical properties. Ceruloplasmin–A copper protein, with a capacity to carry 95 % circulating copper[11], appears essential for iron oxidation after being released from liver, brain etc.,[12]. Besides iron, copper is also required for hemoglobin synthesis (mechanism unknown) probably being associated with iron import *via* transporters into and its utilization within mitochondria[1]. Copper may participate in the synthesis of haeme through cytochrome oxidase[13] *via* several mitochondrial proteins–COX17, SCO1 and SCO2[14].

Erythrocytes are sensitive cells and can serve as cellular indicators[15] to indicate onset of any physiological

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change occurring in the body. Dietary copper is essential for adequate supply as copper is involved in proper functioning of several enzymes particularly during pregnancy for normal development and growth[16]. Deficiency of copper leads to increased incidence of infection[17], failure of copper metalloenzymes[18] and copper trafficking proteins[19], imbalance of iron homeostasis[20], generation of free radicals[21] etc. The study was conducted as blood would be the one of the first organ besides liver which would be affected by the deficiency state and can easily be detected by testing before acute manifestations of symptoms occurs from affecting fetal development onwards. Hence, an attempt has been made to study the effect of dietary copper deficiency on erythrocyte morphology, osmotic fragility and hematological indices of prepubertal male Wistar rats.

MATERIALS AND METHODS

The basal diets were formulated using ICN Research Diet Protocol (1999). The ingredients of the diet (per kg diet) were egg white/albumin 180 g, corn oil 100 g, corn starch 443 g, sucrose 200 g, cellulose 30 g, choline chloride 2 g, DL-methionine 7 g, AIN-76 salt mixture 35 g, AIN-76C vitamin-antibiotic mixture 10 g. Copper contents of basal diet for each group were estimated at 324.8 nm in air acetylene flame on GBC 902 atomic absorption spectrophotometer and copper concentrations were adjusted to 126 nmol/g and 6.3 nmol/g of copper sulfate.

30 prepubertal male Wistar rats (30-40 d; 35-50 g) were divided into four groups of 10 each Group 1: Copper Control (CC) group-Wistar rats were fed with diet containing 126 nmol Cu/g. Tap water was provided *ad libitum*; Group 2: Pair Fed (PF) group-Wistar rats were fed 126 nmol Cu/g diet but the amount of feed given was equal to the feed consumed by Copper Deficient (CD) group the previous day to account for stress and starvation effect. Tap water was provided *ad libitum* and Group 3: CD group-Animals were fed 6.3 nmol Cu/g diet and demineralized water was provided *ad libitum*.

Male Wistar rats were housed individually in polypropylene cages with stainless steel grills. Cages and water bottles were washed with detergent solution, demineralized water and finally rinsed in 1 % Ethylenediamine Tetraacetic Acid (EDTA) solution prepared in demineralized water for removal of copper traces. Experiments were carried out for 2, 4 and 6 w and approved by Department Research Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSEA-1678/GO/Re/S/12). After completion of experiments, animals were anesthetized using sodium thiopentone (intravenous) and blood was collected by cardiac puncture in EDTA coated vials. Blood smear were prepared for the morphological as well as altered erythrocyte morphological studies in percentage. The altered morphological percentage was obtained by dividing it by the number of normal erythrocytes calculated (smear) and result multiplied by 100. Hematological parameters-Hemoglobin (Hb), Total Leukocyte Count (TLC), neutrophil, eosinophil, lymphocyte, monocyte and platelet count, Packed Cell Volume (PCV), Total Red Blood Cell (TRBC) count, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) were also evaluated.

25 µl blood was added to a series of 2.5 ml saline solution ranging from 0.00-0.80 g/100 ml in 5 mM phosphate buffer, pH 7.4. After gentle mixing it was incubated for 15 min at room temperature, centrifuged at 500 xg for 10 min and the absorbance of supernatants were measured at 540 nm on Systronics Spectrophotometer 169 (Serial No. 827). The Percent (%) haemolysis was calculated using formula[22]

% Haemolysis=Optical density of test/Optical density of distilled water (No NaCl)×100

Statistical analysis:

Data presented in the tables are given as mean± Standard Error of Mean (SEM). One way Analysis of Variance (ANOVA) was carried out separately for 2, 4 and 6 w experimental groups followed by post hoc test (Tukey's multiple comparison test) if the difference was found to be significant. Data were analyzed using GraphPad Prism Version 7.0 e. $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Deficiency of copper a global problem has been reported to affect other elements especially iron metabolism as ceruloplasmin and hephaestin having ferrioxidase activity is dependent on copper availability[1,23]. CC (fig. 1) and PF (fig. 2) group blood smear revealed normal histoarchitecture. Blood smear of CC (fig. 1) and PF (fig. 2) group of 2 w experiment revealed normal histoarchitecture. After 2 w of CD, morphological alteration in red blood cells were evident in the form of stomatocytes, acanthocytes, echinocytes (burr

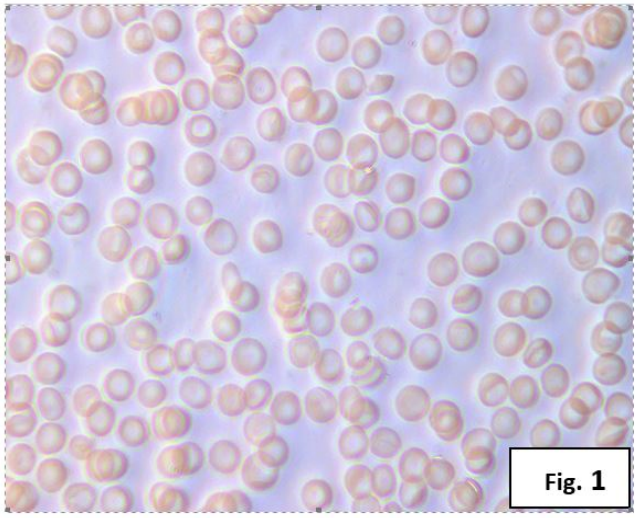

Fig. 1: Microphotograph of prepubertal Wistar male rat CC blood smear after 2 w showing normal histoarchitechture 1000x

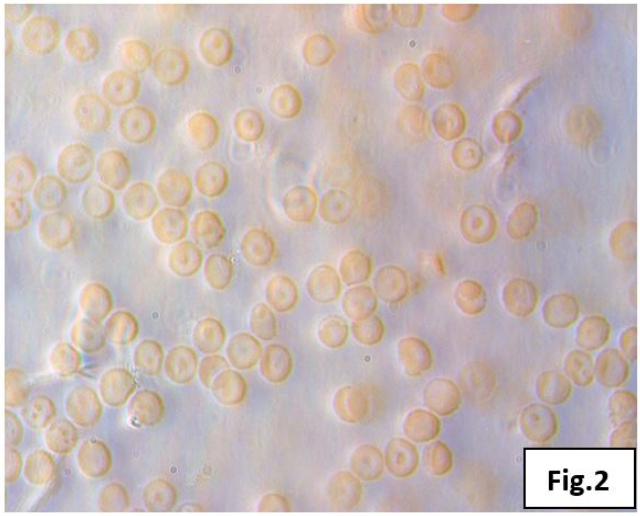

Fig. 2: Microphotograph of prepubertal Wistar PF male rat blood smear after 2 w showing normal histoarchitechture 1000x

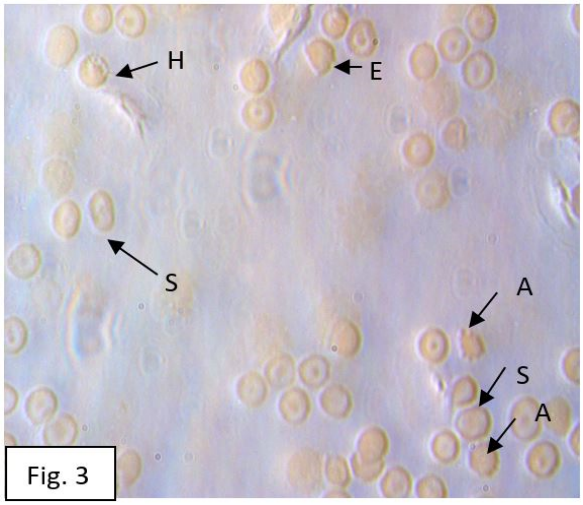

Fig. 3: Microphotograph of prepubertal Wistar CD male rat blood smear after 2 w showing (S) stomatocytes; (A) acanthocytes; (E) echinocytes and (H) Howell-Joly inclusion type 1000x

cells) and rarely Howell–Jolly type inclusion (fig. 3). Analysis further revealed significant altered erythrocyte morphology to be 0.0519 % in deficient animals (Table 1). Blood smear of 4 w experiment CC (fig. 4) group did not exhibit any change in morphology. PF group revealed few acanthocytes,

TABLE 1: ALTERED MORPHOLOGICAL ERYTHROCYTES (%) IN CC, PF AND CD MALE WISTAR RATS AFTER 2, 4 AND 6 W OF DIETARY CD (mean±SEM)

Weeks	CC group	PF group	CD group
2 w	0.0089 %	0.0092 %	0.0519 %[b*c*]
4 w	0.0098 %	0.0107 %	0.0811 %[b*c*]
6 w	0.0101 %	0.0176 %[a*]	0.1709 %[b*c*]

Note: [a]CC *vs.* PF; [b]CC *vs.* CD and [c]PF *vs.* CD, *Significance level $p<0.05$

Multiple comparison procedures were performed for 2, 4 and 6 w experimental sub-groups

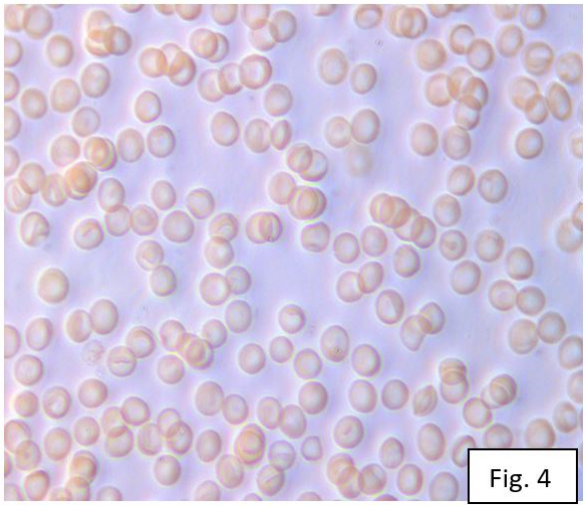

Fig. 4: Microphotograph of prepubertal Wistar male rat CC blood smear after 4 w showing normal histoarchitechture 1000x

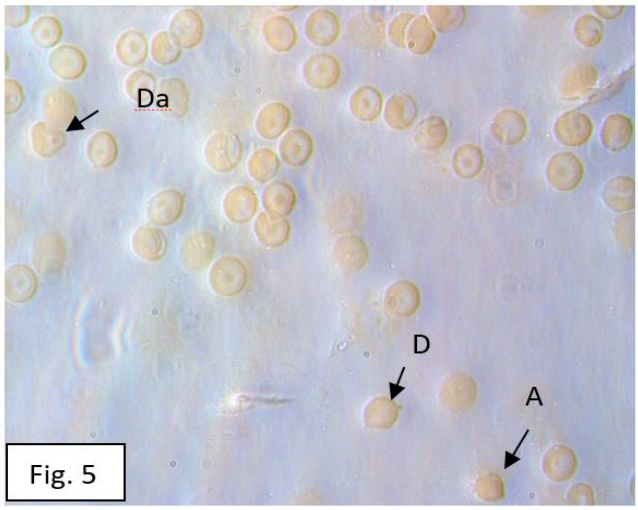

Fig. 5: Microphotograph of prepubertal Wistar PF male rat blood smear after 4 w showing (A) acanthocytes; (D) dracocytes and (Da) damaged RBC 1000x

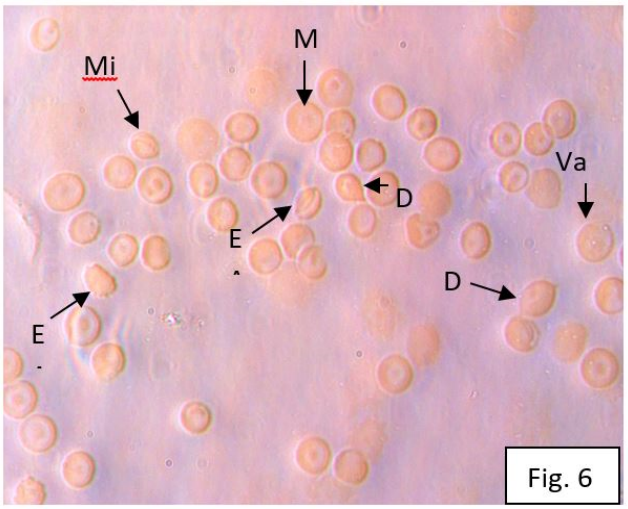

Fig. 6: Microphotograph of prepubertal Wistar CD male rat blood smear of 4 w showing (E) echinocyes; (D) dracocytes; (M) macrocytes; (Mi) microcytes and (Va) vacuolization in RBC 1000x

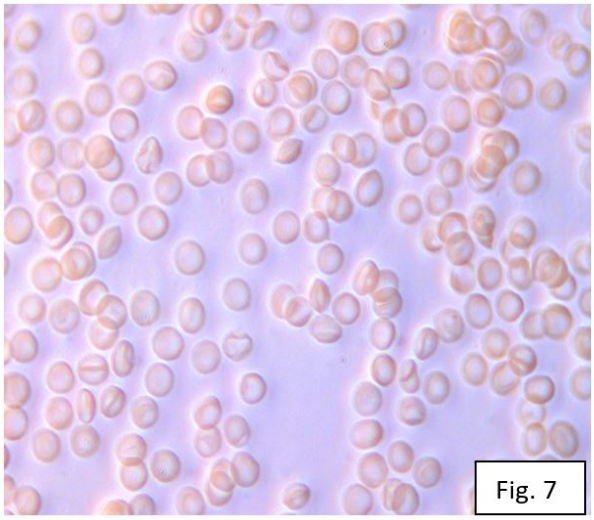

Fig. 7: Microphotograph of prepubertal Wistar male rat CC blood smear after 6 w showing normal histoarchitechture 1000x

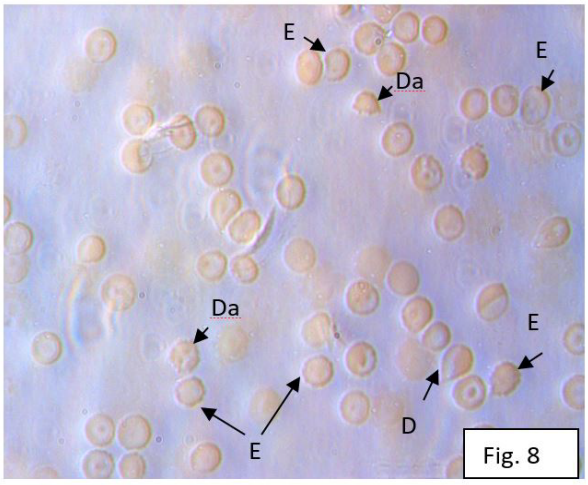

Fig. 8: Microphotograph of prepubertal Wistar PF male rat blood smear after 6 w showing (D) dracocytes; (E) echinocytes and (Da) damaged RBC 1000x

dracocytes and damaged RBC (fig. 5). After 4 w of dietary CD, acanthocytes, echinocytes, dracocytes (tear drop), microcyte, macrocyte, degenerated RBC with vacuolization or with clear halo on one side were observed (fig. 6). Observed altered erythrocyte morphology revealed 0.0811 % in deficient group which was significant when compared with CC and PF group (Table 1). Blood smear of 6 w experiment CC (fig. 7) group revealed no change in blood cells. However, PF group revealed appearance of dracocytes and echinocytes (fig. 8). CD diet for 6 w exhibited large number of stomatocytes, acanthocytes, echinocytes, dracocytes, macrocytes, microcytes, fragmented RBC and few elongated cells (fig. 9). Significant altered erythrocyte morphology after deficiency (0.1709 %) and PF (0.0176 %) was observed (Table 1). Morphological changes observed in erythrocytes after CD can be due to several factors including osmotic fragility and changes in hematological indices taken into account in the present study. Changes observed in PF groups probably accounts for starvation and stress. Authors[24,25] reported decreased superoxide dismutase activity–copper dependent enzyme resulting in increased generation of superoxide radical which has the potential to cause damage to cell membrane. Structural integrity as well as mobility of integral proteins in RBC is maintained by spectrin and band 3-A most abundant protein involved in anion transport and lipids[15,25]. Western blot technique with anti-deoxyribonucleoprotein antibody revealed that during oxidative stress prominent 240 kDa and 210 kDa protein bands were detected

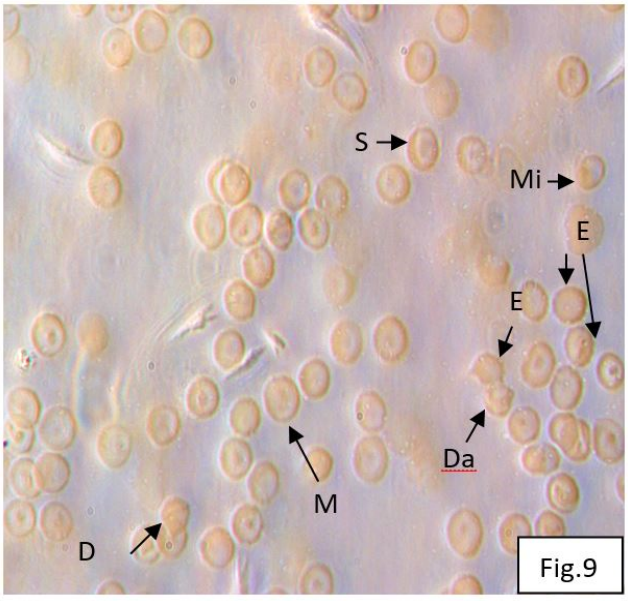

Fig. 9: Microphotograph of prepubertal Wistar CD male rat blood smear after 6 w showing (S) stomatocytes; (E) echinocytes; (D) dracocytes; (M) macrocytes; (Mi) microcytes and (Da) damaged RBC 1000x

with their molecular weights comparable with alpha and beta-spectrin chains with high carbonyl content suggestive of being targeted during such state[26]. Enhanced oxidative stress would have an effect on RBC on account of high level of polyunsaturated fatty acids, intracellular oxygen as well as hemoglobin[27]. Studies using Superoxide Dismutase 1 (SOD1), $SOD^{-/-}$ mice revealed that increased oxidative stress in erythrocytes enhances the production of auto-antibodies causing immune response[28]. Deficiency of copper has been reported to cause up regulation of Cyclooxygenase 2 (COX-2)–A pro-inflammatory enzyme which is also indicative of the fact that inflammation may function independently of SOD enzyme[29]. The occurrence of stomatocytes, acanthocytes, dracocytes, microcytes, vacuolization etc., in the present study indicates loss or defect in the RBC membrane. Deformability of RBC which can be retained in certain conditions is not only an indicator of pathological state, but also a determinant of blood viscosity[30-32]. The osmotic fragility increased significantly ($p<0.05$) with increase in Sodium Chloride (NaCl) (%) concentration in CD groups (2, 4 and 6 w) when compared with respective controls. 50 % osmotic fragility was observed at 0.25 % NaCl concentration (2 CC, 2 PF, 4 CC and 4 PF), 0.3 % NaCl concentration (2 CD and 6 PF), 0.35 % NaCl concentration (4 CD) and 0.38 % NaCl concentration (6 CD) (Table 2). Changes in RBC morphology enhance the susceptibility of erythrocyte to osmotic fragility–An indicator of hemolysis as observed in the present study. The occurrence of stomatocytes in CD groups is indicative of enhanced permeability of sodium leading to increase in osmotic fragility. Further, increased osmotic fragility may occur due to interaction of integral proteins as well as other proteins in RBC with reactive oxygen/nitrogen species.

Hemoglobin decreased significantly after CD (Table 3) indicating impairment of hemoglobin synthesis reducing oxygen carrying capacity leading to anemia. Moreover, increased osmotic fragility which reflects alteration of structural integrity of erythrocyte indicative of enhanced oxidative damage[33] as free radicals are also generated due to auto-oxidation of hemoglobin may also be the cause of anemia. World Health Organization (WHO)[34] reported iron deficiency anemia due to CD. Formation of hemosiderin, a proposed degradation product of ferritin, increased in animals deficient in copper[35]. CD anemia may possibly be due to: cytoplasmic vacuoles in the erythroid and myeloid precursors[36]; decrease in ceruloplasmin and cytochrome c involved in iron metabolism and transportation[23,37] and low hephastin in the intestinal mucosa and ceruloplasmin in liver due to which iron absorption decreases[38]. Vacuolization and ringed sideroblasts have been reported in early precursors of blood cells in bone marrow after CD[39,40]. Lee *et al.*[41] reported that deficiency of thioredoxin dependent peroxidases and peroxiredoxin II also causes anemia although exact mechanism is unknown. With increase in CD days/weeks anemia progressed along with damage to platelets probably due to enhanced oxidative damage to the cells. In the present study impaired hemoglobin synthesis is supported by the presence of microcytes, decreased total RBC count, PCV, MCV, MCH and MCHC (Table 3). Reeves *et al.*[23] also observed similar changes in hematological profile after CD diet to rats. However, no significant change was observed in monocyte count. Lymphocyte and eosinophil count increased although there was evident decrease in TLC (Table 3). The mechanism however remains unknown. Destruction of myeloid progenitor cells, reduced development and maturation of myeloid precursors, altered emigration of neutrophils from the bone marrow as well as increased clearance of neutrophils from the circulation may be associated with the formation of anti-neutrophil antibodies[42] which may explain decrease in neutrophil in the present study.

Dietary CD produced severe effects on erythrocyte membrane that are manifested by enhanced osmotic fragility and morphological changes in erythrocytes along with alterations in blood parameters. These changes increased/decreased with duration. Hence, exposure to insufficient copper during critical period of development would have substantial impact which may be more severe if the dietary deficient state persists. These adverse effects would have serious impact on human health.

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TABLE 2: OSMOTIC FRAGILITY OF ERYTHROCYTES IN CC, PF AND CD MALE WISTAR RATS AFTER 2, 4 AND 6 W OF DIETARY CD (mean±SEM)

NaCl Conc. (g/ml)	2 CC group	2 PF group	2 CD group	4 CC group	4 PF group	4 CD group	6 CC group	6 PF group	6 CD group
0	100	100	100	100	100	100	100	100	100
0.1	90.88±0.59	96.26±0.16^{a*}	98.04±0.23^{b*c*}	89.01±0.24	93.96±0.21^{a*}	96.39±0.18^{b*c*}	87.60±0.39	93.11±0.38^{a*}	95.830.13^{b*c*}
0.15	78.98±0.93	94.36±0.14^{a*}	96.07±0.23^{b*}	78.34±0.08	86.24±0.24^{a*}	93.98±0.15^{b*c*}	76.22±0.29	86.83±0.21^{a*}	92.06±0.21^{b*c*}
0.2	66.42±0.39	86.54±0.30^{a*}	90.55±0.20^{b*c*}	65.19±0.17	82.21±0.39^{a*}	89.23±0.25^{b*c*}	64.25±0.16	81.17±0.21^{a*}	86.40±0.45^{b*c*}
0.25	50.25±0.64	50. 60±0.31	75.81±0.14^{b*c*}	50.01±0.71	50.70±0.25	74.73±0.18^{b*c*}	50.29±0.50	66.43±0.34^{a*}	88.40±0.25^{b*c*}
0.3	46.72±0.19	48.08±0.23^{a*}	50.92±0.40^{b*c*}	46.08±0.28	48.12±0.35^{a*}	61.53±0.22^{b*c*}	44.67±0.67	50.31±0.17^{a*}	82.14±0.13^{b*c*}
0.35	44.54±0.33	45.85±0.03^{a*}	46.78±0.26^{b*c*}	41.52±0.32	44.92±0.16^{a*}	50.55±0.44^{b*c*}	38.11±0.20	41.83±0.24^{a*}	66.39±0.13^{b*c*}
0.38	34.31±0.67	38.11±0.23^{a*}	40.20±0.04^{b*c*}	32.87±0.32	36.22±0.14^{a*}	40.35±0.22^{b*c*}	29.53±0.09	33.12±0.21^{a*}	50.23±0.22^{b*c*}
0.4	27.86±0.27	35.98±0.22^{a*}	38.61±0.17^{b*c*}	26.45±0.25	35.81±0.29^{a*}	38.79±0.35^{b*c*}	25.39±0.15	33.92±0.25^{a*}	36.62±0.13^{b*c*}
0.48	25.83±0.28	28.13±0.20^{a*}	33.26±0.08^{b*c*}	23.71±0.22	28.06±0.24^{a*}	32.92±0.26^{b*c*}	21.00±0.19	25.25±0.23^{a*}	32.12±0.21^{b*c*}
0.5	24.49±0.21	26.34±0.16^{a*}	28.57±0.16^{b*c*}	22.56±0.08	25.58±0.28^{a*}	28.33±0.24^{b*c*}	20.98±0.22	23.38±0.13^{a*}	26.40±0.13^{b*c*}
0.6	23.26±0.29	25.75±0.13^{a*}	26.71±0.22^{b*c*}	21.69±0.32	25.75±0.32^{a*}	27.12±0.25^{b*c*}	16.21±0.19	20.70±0.26^{a*}	21.89±0.18^{b*c*}
0.7	19.88±0.23	23.42±0.16^{a*}	24.39±0.18^{b*c*}	18.97±0.13	21.67±0.24^{a*}	24.69±0.23^{b*c*}	13.03±0.19	17.27±0.18^{a*}	18.00±0.21^{b*c*}
0.8	18.24±0.17	22.75±0.10^{a*}	23.70±0.02^{b*c*}	15.93±0.27	22.08±0.27^{a*}	23.03±0.19^{b*c*}	9.67±0.23	14.81±0.07^{a*}	18.000.21^{b*c*}

Note: ^{a}CC *vs.* PF, ^{b}CC *vs.* CD, ^{c}PF *vs.* CD, *Significance level p<0.05
Multiple comparison procedures were performed for 2, 4 and 6 w experimental sub-groups

TABLE 3: HAEMATOLOGICAL PARAMETERS IN CC, PF AND CD MALE WISTAR RATS AFTER 2, 4 AND 6 W OF DIETARY CD (mean±SEM)

Parameters	2 CC group	2 PF group	2 CD group	4 CC group	4 PF group	4 CD group	6 CC group	6 PF group	6 CD group
Haemoglob (g/dl)	8.28±0.03	8.05±0.02	7.51±0.11^{b*c*}	9.18±0.09	8.73±0.06^{a*}	6.21±0.09^{b*c*}	10.43±0.06	9.35±0.10^{a*}	5.80±0.28^{b*c*}
TLC (Th/mm^3)	4.25±0.06	4.11±0.03	3.88±0.03^{b*c*}	4.40±0.04	4.08±0.03^{a*}	3.71±0.03^{b*c*}	4.35±0.05	3.81±0.03^{a*}	3.16±0.06^{b*c*}
Lymphocytes	47.33±0.21	47.67±0.21	48.67±0.21^{b*c*}	41.50±0.42	43.67±0.21	49.50±0.22^{b*c*}	46.50±0.22	47.00±0.44	58.50±0.22^{b*c*}
Monocytes	2.50±0.22	2.16±0.16	2.00±0.0	2.50±0.22	2.16±0.16	2.00±0.0 1	2.50±0.22	2.33±0.21	2.00±0.01
Eosinophils	2.50±0.22	2.50±0.22	3.33±0.21^{b*c*}	2.50±0.22	3.53±0.16^{a*}	4.90±0.22^{b*c*}	2.83±0.16	3.66±0.21^{a*}	5.50±0.22^{b*c*}
Neutrophils	47.67±0.21	46.67±0.21	44.83±0.30^{b*c*}	54.17±0.40	50.67±0.3^{a*}	47.17±0.30^{b*c*}	58.50±0.22	47.17±0.30^{a*}	36.17±0.16^{b*c*}
Platelet Count (Lakh/mm^3)	1.46±0.02	1.45±0.01	1.31±0.03^{b*c*}	1.76±0.02	1.66±0.02^{a}	1.45±0.02^{b*c*}	1.98±0.03	1.630.03^{a*}	1.55±0.02^{b*c*}
PCV (%)	24.50±0.16	23.72±0.15	23.42±0.11^{b*}	28.03±0.18	25.07±0.26^{a*}	21.00±0.15^{b*c*}	31.03±0.15	27.15±0.12^{a*}	14.07±0.25^{b*c*}
TRBC(MIL/CU-mm)	2.71±0.01	2.60±0.00	2.57±0.01	3.18±0.04	2.81±0.03^{a*}	2.40±0.04^{b*c*}	3.31±0.04	3.15±0.02	2.72±0.14^{b*c*}
MCV µm^3(fL)	75.90±0.23	74.85±0.06	72.43±0.11^{b*c*}	79.00±0.10	77.10±0.14	70.33±0.04^{b*c*}	79.72±0.19	76.50±0.09^{a*}	65.02±0.14^{b*c*}
MCH (pg)	25.22±0.09	25.03±0.07	24.22±0.09^{b*c*}	25.55±0.16	23.55±0.18	21.70±0.21^{b*c*}	25.80±0.11	24.30±0.13	18.07±0.18^{b*c*}
MCHC (g/l)	29.85±0.08	29.87±0.05	29.37±0.22	29.65±0.08	28.57±0.12	25.67±0.08^{b*c*}	30.15±0.09	29.62±0.10^{a*}	19.43±0.15^{b*c*}

Note: ^{a}CC *vs.* PF, ^{b}CC *vs.* CD, ^{c}PF *vs.* CD *Significance level p<0.05
Multiple comparison procedures were performed for 2, 4 and 6 w experimental sub-groups

Conflicts of interest:

The authors declared no conflicts of interest.

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