Journal of Clinical Research Letters
Journal of Clinical Research Letters
ISSN: 0976–7061 & E-ISSN: 0976–707X, Volume 2, Issue 1, 2011, pp-10-15
Available online at http://www.bioinfo.in/contents.php?id=60

THE ADAPTIVE CHANGES OF THE OXIDATIVE PENTOSE PHOSPHATE PATHWAY IN RESPONSE TO OXIDATIVE STRESS INDUCED BY COPPER AND ZINC DEFICIENCY IN LUNG OF FEMALE RATS

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Received: December 22, 2011; Accepted: December 29, 2011

Abstract- The purpose of the present study was to assess and examine the links among zinc and/or copper deficiency, oxidative stress and the adaptive changes of the oxidative phase of the pentose phosphate pathway in lung of adult female rats fed diets deficient in copper and/or zinc. Four groups each of ten adult female Albino rats (Sprague-Dawely) strain, mean weight varied between 80.2 to 82.5 g were fed on basal control diet (CD), copper deficient diet (-CuD), zinc deficient diet (-ZnD), and diet deficient in both of copper and zinc (-CuZnD). Serum and lung were analyzed for enzymatic and non-enzymatic primary defense system components. Serum and lung Cu and Zn levels were lowered significantly ($p<0.01$) in –CuD, -ZnD, and -CuZnD rats. The activities of erythrocyte CuZnSOD, lung catalase and GPx were reduced in all deficient rats. Lung content of GSH, GSSG, and ascorbate were significantly reduced by feeding Cu and/or zinc deficient diets. Serum MDA content was increased in rats fed diets deficient in copper and/or zinc groups. Lung activities of oxidative pentose phosphate pathway enzymes (G6PDH and 6PGDH) were significantly increased by Cu and/or zinc deficiency. The present study concluded that, the oxidative stress induced by feeding diets deficient in Cu and/or Zn result in changes in the oxPPP enzymes activities (G6PDH and 6PGDH). The oxPPP maintains the redox potential and adapts these changes to protect against oxidative stress by NADPH generation, which is required for detoxification of free radicals and peroxides.
Keywords- Oxidative stress, Pentose phosphate pathway, Copper-Zinc deficiency

Introduction

The pentose phosphate pathway (PPP) comprises two separate branches, oxidative and non-oxidative. Reactions in the oxidative branch are irreversible, whereas all reactions of the nonoxidative branch are fully reversible. The oxidative branch primarily depends on the rate-limiting enzyme glucose 6-phosphate dehydrogenase (G6PDH) [1]. Glucose-6-phosphate dehydrogenase (G6PDH) activity is needed in all cell types for the production of NADPH and for control of carbon flow through the pentose phosphate pathway. G6PDH catalyzes the first reaction of this pathway, oxidizing glucose-6-phosphate to 6-phosphogluconolactone and in the process reducing NADP to NADPH. This reaction is the rate-determining step of the oxidative portion of the pentose phosphate pathway. Together with 6-phosphogluconate dehydrogenase (6PGDH), these enzymes provide NADPH for reductive biosynthetic reactions, such as fatty acid, cholesterol, and amino acid synthesis, and for maintenance of reduced glutathione concentrations. The oxidative pentose phosphate pathway (oxPPP) is a major source of reducing power and metabolic intermediates for biosynthetic processes. The oxPPP is one of the main pathways of NADPH generation, and maintains the redox potential necessary to protect against oxidative stress [2]. NADPH protects against oxidative stress directly by neutralizing reactive oxygen intermediates (ROIs) or indirectly via regenerating reduced glutathione (GSH) from its oxidized form GSSG. Physiological conditions resulting in oxidative stress can also result in changes in G6PDH activity. NADPH is required for detoxification of free radicals and peroxides. G6PDH gene expression is essential for protection of the cell against even mild oxidative stress [3].

Copper and zinc are two of the most abundant trace elements found in the human body and are intricately involved in the metabolism of oxygen and the biochemistry of redox reactions. Both Zn and Cu are cofactors in the metalloenzyme CuZn-superoxide dismutase (CuZnSOD), which provides the first line of defense against activated oxygen species by dismutation of the superoxide anion radical [4]. Dietary Zn deficiency has been shown to cause an increase in free radical generation in lung microsomes [5]. Zn-deficient as well as Cu-deficient rats had increased amounts of lipid peroxidation. Dietary Zn deficiency has also been shown to cause an increase in NADPH-dependent H2O2 production in the microsomes of lung [6]. The lung contains the largest endothelial surface area of any organ, which makes the lung a major target site for circulating oxidants and xenobiotics. It is one of the major

organs for free radical detoxification because lung can generate free radicals enzymatically during cell metabolism. Detoxification of activated oxygen species is particularly important in the long as this organ is exposed to high oxygen concentrations [7]. The purpose of the present study was to assess and examine the links among zinc and copper deficiency, oxidative stress and the adaptive changes of the oxidative phase of the pentose phosphate pathway in lung of adult female rats fed diets deficient in copper and zinc.

Materials and Methods

Experimental animals and diets

Four groups each of ten adult female Albino rats (Sprague-Dawely) strain, mean weight varied between 80.2 to 82.5 g were fed on basal control diet (CD), copper deficient diet (-CuD), zinc deficient diet (-ZnD), and diet deficient in both of copper and zinc (-CuZnD) throughout the experimental period (28 days).. The animals were 4 weeks old at the beginning of the experiment. They were obtained from Helwan breeding farm, Cairo, Egypt. Animals received human care, housed individually in stainless steel cages with wire mesh bottoms and maintained at temperature 25°C ±5°C, humidity 50%± 10% and light dark cycle held constant 12/12 h. Food and water were provided ad libitum during the experimental period (28 days). The rats were fed on diets according to AIN, 1977 [8] and classified as follows: Group I: the basal control diet (CD)

Group II: Copper deficient diet (- CuD)

Group III: Zinc deficient diet (-ZnD)

Group IV: Copper and zinc deficient diet (-CuZuD)

Biochemical Measurements

Blood and lung were analyzed for enzymatic and non-enzymatic primary defense system components, copper and zinc levels, and the enzymes activities of the oxidative pentose phosphate pathway [glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH)]. Lipid peroxidation products measured as malondialdehyde (MDA) content.

Blood Analysis

At the end of the experiment (28 days), rats were fasted for 12 hrs, then the animals were scarified under ether anesthesia and blood samples were taken from hepatic portal vein in centrifuge tubes. The tubes centrifuged at 10000 x g for 20 minutes to provide serum needed for the biochemical analysis. Serum was separated immediately and stored at -20°C until analysis. Fresh serum was tested for content of ascorbate by colorimetric method [9]. The concentration of serum malondialdehyde (MDA) was determined by colorimetric procedure using kit of Biodiagnostic [10]. Serum zinc (Zn) and copper (Cu) contents were measured using an ATI-UNICAM 929 model Atomic Absorption Spectrophotometer [11]. The activity of superoxide dismutase (CuZnSOD) in erythrocyte was determined by colorimetric method kit developed by Biodiagnostic [12].

Lung Analysis

Determination of the enzymatic and nonenzymatic defense system components

For determination of the enzyme activities, lung tissues were homogenized with a laboratory mortar grinder in various buffers according to the assay procedures for glutathione peroxidase (GPx) catalase (CAT) and glutathione reduced and oxidized (GSH,GSSG). The homogenate was centrifuged at 10000 x g for 30 minutes and the supernatant was used in the enzyme assays. GPx activity was determined by colorimetric method kit developed by Biodiagnostic [13]. Catalase activity was measured as the decomposition of hydrogen peroxide by colorimetric method kit developed by Biodiagnostic [14]. Reduced and oxidized glutathione levels were measured by colorimetric method kit developed by Biodiagnostic [15].

Determination of copper and zinc

Lung tissue samples were dried to a constant weight and lung ash was dissolved in nitric acid. After dilution with deionized water, zinc and copper contents were measured using an ATI-UNICAM 929 model Atomic Absorption Spectrophotometer [11].

Determination of oxidative pentose phosphate pathway enzymes activities

Approximately 1 g of lung tissue was homogenized in 10 mL of 0.25M sucrose buffer (in 0.1 M phosphate buffer, pH 7.4) using homogenizer. Homogenates were centrifuged at 10000 x g for 30 minutes, and the supernatants were used for enzyme assays. Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activities were determined by spectrophotometric methods [16, 17].

Statistical Analysis

Analysis of variance (ANOVA; F-test) followed by the least significant difference (LSD) test was used to determine statistical significance ($P<0.01$) between the treatment groups. Statistical analysis was done by using SPSS 11.5 statistical software.

Results

Effect of dietary copper and zinc deficiency on serum and lung copper and zinc levels

Absolute and relative lung weights of zinc and copper deficient groups were significantly reduced ($p<0.01$) as compared to control rats (Table 1). Serum and lung Cu concentrations were lowered significantly ($p<0.01$) in both -CuD and -CuZnD rats compared to the control rats (CD). The -ZnD group had a similar value of serum and lung zinc as control group (Table 1 and Fig.1). However, serum and lung Zn levels were reduced significantly ($p<0.01$) in -ZnD and -CuZnD rats compared with CD rats (Table 1 and Fig.2). The lowest values of serum and lung Cu and Zn were observed significantly in –CuZnD rats when compared with other groups.

Effect of dietary copper and zinc deficiency on enzymatic activities of the primary defense system components

Copper and zinc deficiency caused significant reduction ($p<0.01$) in the activities of erythrocyte superoxide

dismutase (CuZnSOD), lung catalase (CAT) and lung glutathione peroxidase (GPx) enzymes in all tested groups (-CuD, -ZnD, and –CuZnD) as compared to control group. Erythrocyte CuZnSOD activity was lowered significantly ($p<0.01$) in the -CuD and -CuZnD groups than in the -ZnD group. The results showed that Cu and Zn deficiency resulted in significant reduction ($p<0.01$) in lung catalase activity in –CuD, -ZnD, and –CuZnD groups by 16%, 25%, and 29% respectively when compared to control group. Lung GPx activities in –CuD and –CuZnD groups were approximately 8% lower than in the control group. However, GPx activity in –ZnD group was lowered by 4% as compared to CD group (Table 2).

Effect of dietary copper and zinc deficiency on non-enzymatic components of the primary defense system and malondialdehyde (MDA) contents

Lung concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), and ascorbate were significantly reduced ($p<0.01$) by Cu and/or zinc deficiency as compared to control group (Table 3). Serum ascorbate concentration was significantly lower in -CuD rats than in -ZnD group. The percentages of reduction in serum ascorbate level in rat groups fed diets deficient in Cu and/or Zn (-CuD, -ZnD, and -CuZnD) were 55.9%, 48.6%, and 57.3% respectively. The results showed that Cu and/or Zn deficiency resulted in significant reduction ($p<0.01$) in lung GSH content in -CuD, -ZnD, and -CuZnD groups by 18%, 25%, and 30% respectively when compared to control group. On the other hand GSSG/GSH ratio was increased by Cu and Zn deficiency as compared to control group (Table 3). Lipid peroxidation was measured as the amount of malondialdehyde (MDA) formed. The results showed that serum MDA content was increased significantly ($p<0.01$) in rats fed diets deficient in copper and/or zinc when compared to control group (Fig.3).

Effect of copper and zinc deficiency on lung glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activities

Lung activities of oxidative pentose phosphate pathway enzymes (G6PDH and 6PGDH) were significantly increased ($p<0.01$) by Cu and/or zinc deficiency as compared to control group (Table 4). Zinc-deficient rats had higher activities of oxPPP enzymes than Cu-deficient rats. The results showed that the percentage of increase in lung G6PDH activity in -CuD, -ZnD, and -CuZnD groups was 55%, 63%, and 75% respectively when compared to control group. However, 6PGDH activity in -CuD, -ZnD, and -CuZnD groups was increased by 38%, 45%, and 46% as compared to control group (Table 4).

Discussion

The PPP is regulated by a number of cellular processes, including oxidative stress. Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to detoxify the reactive intermediates or repair the resulting damage. G6PD is the key regulatory enzyme in the PPP and may provide an early marker of oxidative stress, since it responds rapidly to the increased demand for NADPH necessary for the maintenance of the cellular redox state [1]. The effects of Zn and Cu nutrition on various components of the primary free radical defense system were studied to investigate the specific effects of dietary Zn and/or Cu deficiency on oxPPP modulation. The highly significant reduction ($p<0.01$) of serum and lung Cu and Zn in -CuD, -ZnD, and -CuZnD rats indicate that severely Cu and Zn deficient rats were produced (Table1 & Fig.1). Components of the enzymatic defense system include superoxide dismutase (SOD) for the dismutation of the superoxide anion radical, catalase and glutathione peroxidase (GPx) for detoxification of hydrogen peroxide [18]. CuZnSOD activity, the first line of defense against activated oxygen species, responded directly to the concentration of Cu in the tissue. The reduction in CuZnSOD activity in the present study support the hypothesis that CuZnSOD activity is directly influenced by the tissue concentration of copper. It has been shown that the removal of Cu from the catalytic site of CuZnSOD results in loss of enzyme activity [19]. In the current study, CuZnSOD activity was higher in the lung of -ZnD rats than in -CuD rats (Table 2). The slightly increased copper content in the lung of -ZnD rats may cause the increase in CuZnSOD activity as compared to -CuD rats. GPx and catalase are located in different cellular compartments and function cooperatively in tissues in removing H2O2 and hydroperoxides [20]. The results of the current study showed significant reduction in the activities of GPx and catalase enzymes in the lung of Cu and/or Zn deficient rats (Table 2). The specific mechanism by which Cu deficiency depresses catalase activity is unknown, but it may be related to altered iron mobilization [21]. Malnutrition induced by Zn or Cu deficiency affects the homeostasis of iron metabolism. A homeostasis between rate of formation of free radicals and the rate of their neutralization if not maintained oxidative damage accumulates known as oxidative stress [22].

The results of the present study showed that the serum ascorbate concentrations were significantly depressed in -CuD, -ZnD, and -CuZnD rats (Table 3). Although many of the changes in the nonenzymatic components of the primary free radical defense system in -ZnD or -CuD rats were due to malnutrition, these changes may still affect the overall balance between free radical generation and defense. The potential toxicity of free radicals is counteracted by a large number of cytoprotective enzymes and antioxidants that limit the damage. This protective mechanism functions cooperatively in form of a cascade in which the cellular antioxidants α-tocopherol, ascorbic acid and reduced glutathione act in combination. α-tocopherol is a chain breaking antioxidant that by neutralizing a free radical gets converted to α-tocopheroxyl radical. It can be reduced back to α-tocopherol by ascorbic acid. Dehydroascorbic acid formed in this reaction can be reconverted back to ascorbic acid by reduced glutathione. It is important that

sufficient amounts of α-tocopherol, reduced ascorbic acid and reduced glutathione be present within the cell so as to provide protection against oxidative injury [23].

The lung is constantly exposed to many atmospheric pollutants and is also at risk from oxidant injury by inhalation of high concentrations of oxygen. It contains the largest endothelial surface area of any organ, which makes the lung a major target site for circulating oxidants and xenobiotics. It is therefore no surprise that the human lung is one of the important storage areas for GSH, which detoxifies oxidants, free radicals, and organic poly aromatic hydrocarbons. Thus extracellular GSH in the lungs can protect pulmonary epithelial cells from oxidative stresses [24]. The results of the present study showed that Cu and/or Zn deficiency resulted in significant reduction in lung GSH content and an increase in GSSG/GSH ratio (Table 3). An increased GSSG/GSH ratio is considered indicative of oxidative stress [25]. The GSH redox system is crucial in maintaining intracellular GSH homeostasis, which is critical to normal cellular physiological processes and represents one of the most important antioxidant defense systems in the lung [24]. This system uses GSH as a substrate in the detoxification of peroxides such as hydrogen peroxide (H2O2) and lipid peroxides, a reaction that involves glutathione peroxidase (GPx). This reaction generates oxidized GSH (GSSG), which is subsequently reduced by glutathione reductase in a reaction that requires the pentose phosphate pathway utilizing NADPH. It has been proposed that the mechanism by which free radicals induce tissue injury is by stimulation of lipid peroxidation of biomembranes and (or) by modification of cellular proteins and nucleic acids [26]. Lipid peroxidation was measured as the amount of malondialdehyde (MDA) formed. The results showed that serum MDA content was increased significantly ($p<0.01$) in rats fed diets deficient in copper and/or zinc (Fig.3). Sulfhydryl oxidation-reduction by pentose phosphate pathway (PPP) generating NADPH is important cellular defense mechanisms by which the cell can withstand the deleterious effects of oxidant stress [26]. Further study suggested that the sulfhydryls exert a "coarse" control on PPP and that a "fine control is accomplished by NADPH [27].

The oxidative phase of pentose phosphate (oxPPP) pathway is one of the main pathways of NADPH generation [1]. G6PDH and 6PGDH are considered fundamental enzymes of the pentose phosphate pathway. These enzymes produce NADPH, which is required for many reduction systems [28]. It has been reported that when G6PD concentration is reduced to 10% of the normal level, the oxidative pentose phosphate pathway becomes defective, resulting in a lowered content of NADPH [29]. NADPH generation is also required for GSH production. When NADPH and GSH are deficient, the entrance of oxidizing compounds causes damage to the lipids and proteins and consequent cell destruction [30]. Physiological conditions resulting in oxidative stress can also result in changes in G6PD activity [31]. The results of the present study showed that, the lung activity of G6PDH, rate-limiting enzyme of oxPPP, was significantly increased by Cu and/or zinc deficiency (Table 4). The results suggested that the oxPPP play an important adaptive role in maintaining the redox potential necessary to protect against oxidative stress. G6PDH catalyzes the first reaction of this pathway, oxidizing glucose-6-phosphate to 6-phosphogluconolactone and in the process reducing NADP to NADPH. This reaction is the rate-determining step of the oxidative portion of the pentose phosphate pathway. G6PDH gene expression is essential for protection of the cell against even mild oxidative stress [32]. Glucose-6-phosphate dehydrogenase activity is generally found to be increased in the tissues of Cu-deficient rats, and this may be an adaptive response since the enzyme is known to be subject to tissue-specific regulation by oxidant stress [33].

The present study concluded that, the oxidative stress induced by feeding diets deficient in Cu and/or Zn result in changes in the enzymes activities of oxidative phase of the pentose phosphate pathway. The oxPPP play an important role in maintaining the redox potential necessary to protect against oxidative stress.

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Table 1- Effect of dietary copper and zinc deficiency on lung and serum copper (Cu) and zinc (Zn) levels

Treatments	**Absolute lung weight (g)**	**Relative lung Weight (%)**	**Lung copper (Cu) (µg/g dry wt)**	**Lung zinc (Zn) (µg/g dry wt)**
CD	1.15^{a}±0.05	1.32^{a}±0.06	6.68^{a}±0.25	98.32^{a}±4.1
-CuD	0.80^{b}±0.04	1.14^{b}±0.05	2.65^{b}±0.24	97.66^{a}±3.9
-ZnD	0.55^{c}±0.03	0.95^{c}±0.04	6.72^{a}±0.30	48.35^{b}±3.2
-CuZnD	0.50^{c}±0.03	0.83^{c}±0.04	2.55^{b}±0.21	46.29^{b}±2.8

**Values are expressed as mean± SD, n=10 CD= Control diet; -ZnD= Zinc deficient diet; -CuD= Copper deficient diet; -CuZnD= Copper Zinc deficient diet. There was no significant difference at p< 0.01 between means have the same alphabetic superscript in the same column*

Table 2- Effect of copper and zinc deficiency on enzymatic activities of the primary defense system components (erythrocyte superoxide dismutase; catalase; glutathione peroxidase).

Treatments	**Erythrocyte Superoxide dismutase (CuZn SOD) U/gHb**	**Lung Catalase(CAT) µmol/min.mg**	**Lung Glutathione Peroxidase (GPx) nmol/min.mg**
CD	236.25^{a} ±12.1	52.32^{a} ±4.2	383.2^{a} ±18.3
-CuD	46.15^{b} ±2.3	43.82^{b} ±3.9	350.7^{b} ±16.6
-ZnD	198.3^{c} ±13.6	39.18^{b} ±2.7	368.1^{c} ±13.9
-CuZnD	50.72^{b} ±4.7	37.12^{b} ±3.8	349.9^{b} ±15.5

**Values are expressed as mean± SD, n=10 CD= Control diet; -ZnD= Zinc deficient diet; -CuD= Copper deficient diet; -CuZnD= Copper Zinc deficient diet. There was no significant difference at p< 0.01 between means have the same alphabetic superscript in the same column*

Table 3- Effect of copper and zinc deficiency on non-enzymatic primary defense system components; lung reduced glutathione (GSH), oxidized glutathione (GSSG), GSSG/GSH ratio, and serum ascorbate levels.

Treatments	**Lung reduced glutathione (GSH) µmol/g protein**	**Lung oxidized glutathione (GSSG) µmol/g protein**	**GSSG/GSH ratio**	**Serum ascorbate µmol/L**
CD	25.02^{a}±2.7	4.13^{a}±1.8	0.165^{a}±0.05	65.2^{a}±6.7
-CuD	10.68^{b} ±2.1	2.62^{b}±0.52	0.245^{b}±0.32	28.7^{b}±3.2
-ZnD	13.12^{c} ±2.9	2.41^{c}±0.79	0.183^{c}±0.21	33.5^{c}±3.8
-CuZnD	9.35^{d} ±3.2	1.83^{d}±0.66	0.195^{d}±0.23	27.8^{b}±2.9

**Values are expressed as mean± SD, n=10 CD= Control diet; -ZnD= Zinc deficient diet; -CuD= Copper deficient diet; -CuZnD= Copper Zinc deficient diet. There was no significant difference at p< 0.01 between means have the same alphabetic superscript in the same column*

Table 4- Effect of copper and zinc deficiency on lung glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activities

Treatments	**Lung G6PDH (μmol NADPH/min.mg protein)**	**Lung 6PGDH (μmol NADPH/min.mg protein)**
CD	83.2^{a}±15.6	102.6^{a}±12.5
-CuD	129.3^{b}±13.6	142.2^{b}±18.2
-ZnD	136.2^{b}±22.7	148.8^{b}±14.5
-CuZnD	145.8^{b}±23.4	150.7^{b}±13.8

*Values are expressed as mean± SD, n=10 CD= Control diet; -ZnD= Zinc deficient diet; -CuD= Copper deficient diet; -CuZnD= Copper Zinc deficient diet. There was no significant difference at p< 0.01 between means have the same alphabetic superscript in the same column

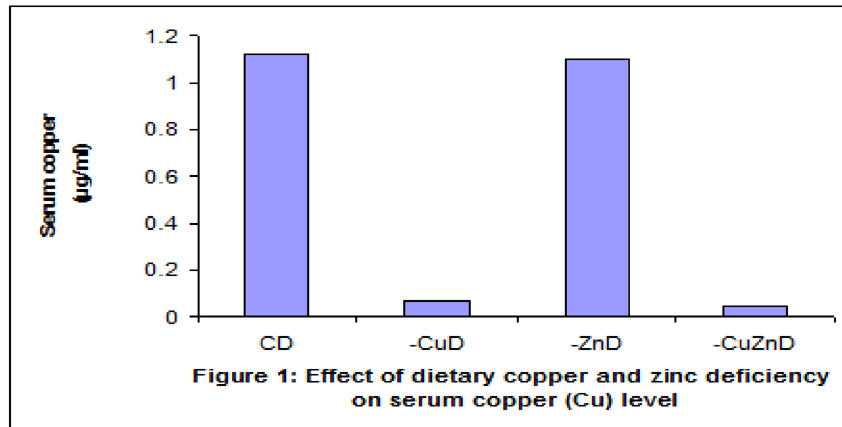

Figure 1: Effect of dietary copper and zinc deficiency on serum copper (Cu) level

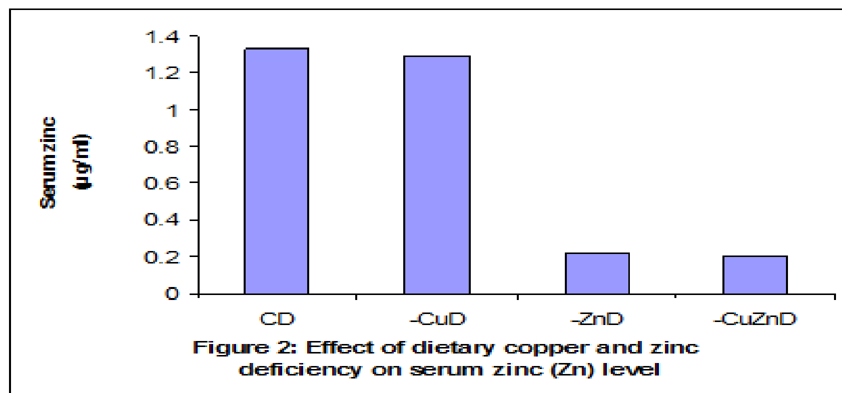

Figure 2: Effect of dietary copper and zinc deficiency on serum zinc (Zn) level

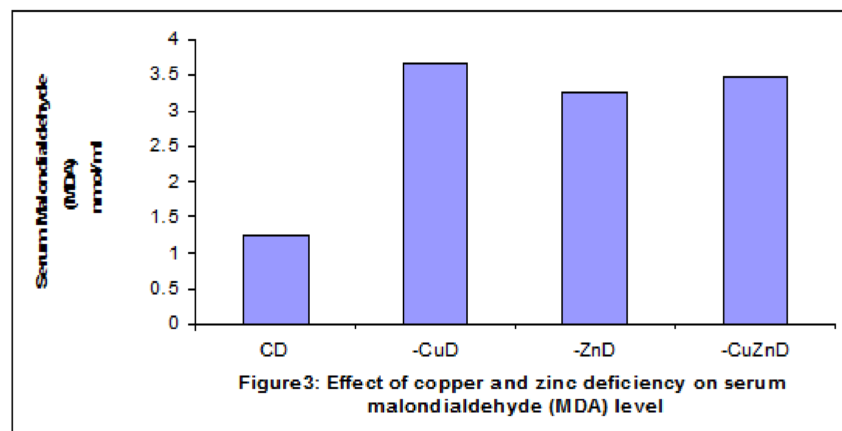

Figure3: Effect of copper and zinc deficiency on serum malondialdehyde (MDA) level