Hematopoiesis and the Level of Riboflavin, Glutathione and Ceruloplasmin in Vitamin A Deficient Rats

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Introduction

Deficiency of vitamin A has consistently been found to be associated with a larger accumulation of iron in the liver and other tissues; and a marked depression in hematopoiesis. It is suggested that iron is arrested in storage in absence of vitamin A; and hemoglobin synthesis is impaired for want of iron[1,4]. Generally the increased iron storage is considered under two heads (a) the result of increased uptake of iron followed by subsequent storage and (b) the result of altered cellular metabolism giving rise to endogenous iron overload. In view of normal absorption of iron during vitamin A deficiency[2], disorder in cellular iron-metabolism seems to be the most probable cause for this iron-accumulation. Mobilization of iron from ferritin occupies an important area in intracellular iron metabolism. Its transport to and from tissues needs a smooth mobilization. Transport of iron out of the cells depends upon mobilization of iron from ferritin within the cytosol, from phagolysosomes[5], from effete red cell digested by reticuloendothelial cells[6] and from other sources. Some compounds have been implicated to the mobilization of iron from cellular stoage. These compounds are ceruloplasmin[7,9], ascorbic acid [10,11], riboflavin [12,13] and glutathione [14]. In consideratrion of the influence of ascorbic acid, riboflavin, glutathione and ceruloplasmin in cellular iron mobilization, it was assumed that vitamin A deficiency might have given rise to tissue-iron accumulation through its capacity, if there is any, to reduce the supply of these compounds in the system. Moreover, level of ascorbic acid has been reported to be decreased during vitamin A deficiency[15]. Thus the present study has been undertaken to investigate the effect of vitamin A deficiency on the status of riboflavin, ascorbic acid, ceruloplasmin and glutathione in rats. However, results related to ascorbic acid has been reported elsewhere [16].

Materials and Methods

Thirty six post weaning Long Evans

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rats (mean weight 75g) were collected from ICDDR, B, Mohakhali, Dhaka and housed in individual wire cages. Eighteen rats were provided with vitamin A deficient basal diet (Table-1), and the rest eighteen rats (control) were kept on same diet supplemented with vitamin A (8000 I.U/Kg diet). Vitamin A was supplemented in the form of retinol palmitate. Rats were provided with water ad libitum. Depletion was continued for 8 weeks, until the rats fed deficient diet showed an initial weight plateau, while control rats still continued to gain weight. Hemoglobin levels of all rats were determined by collecting blood from tail tips. Then rats were sacrificed in three batches of 12 rats (six deficient and six control). Rats were sacrificed under mild chloroform anesthesia by decapitation. First batch was sacrificed as a hematopoietic base line for others. After sacrifice, blood was collected for hematocrit. Serum, liver and spleen were collected and preserved at-18°C until further biochemical analysis for iron and vitamin A. Second batch was sacrificed to determine the level of riboflavin in liver, spleen and brain, and also ceruloplasmin in serum. Third batch was sacrificed to assess the level of glutathione in liver, spleen, brain and erythrocyte. Analysis of riboflavin glutathione and ceruloplasmin was accomplished immediate after sacrifice. Liver vitamin A and serum vitamin A were determined in the rats of all batches.

Analytical Methods- Glutathione was determined by following the method of Sedlak and Lindsay[17] for nonprotein bound sulfhydhyl with GSH (reduced glutathione) as a standard. Erythrocyte GSH was estimated by following the same method after extracting GSH in the metaphosphoric acid. Glutathione from erythocyte was extracted by following the method of Cho et al[18]. Total tissue riboflavin was determined fluorometrically[19]. Ceruloplasmin in serum was determined by following the method of Ravin et al[20]. Liver vitamin A level was determined colorimetrically by using TCA[21]. Serum vitamin A was determined by HPLC, following the method described by Bieri et al[22]. Hemoglobin was determined by cyanmethemoglobin method[23] using Commercial Kit (Sigma Chemicals, USA). Serum iron was determined colorimetrically by using orthophenantholine as chromogenic reagent[24]. Tissue iron was determined colorimetrically by using bathophenanthroline as chromogenic reagent[25]. Hematocrit was determined by standard microcentrifugal method [26].

Statistical Analysis- Difference between two groups was evaluated by students' t-test between the means.

Results

Table2 shows the effect of vitamin A deficiency on hematopoietic parameters of rats. Rats fed vitamin A deficient diet showed marked decrease in the level of hemoglobin, hematocrit and serum-iron

as compared to the control. Liver vitamin A and serum vitamin A also decreased significantly in rats kept on vitamin A deficient diet. There has been found a larger deposition of iron in liver and spleen of vitamin A deficient rats.

Table-3 shows the effect of vitamin A deficiency on the level of riboflavin, glutathione and ceruloplasmin of rats.

Riboflavin level in liver, spleen and brain did not differ significantly between vitamin A deficient and control rats.

Concentration of glutathione has been found to be markedly increased in liver, spleen, brain and erythrocytes of vitamin A deficient rats as compared to the control.

Concentration of ceruloplasmin in serum increased significantly in vitamin A deficient rats as compared to the control.

Table 1 : Composition of vitamin A deficient basal diet[a]

Ingredients	Percentage
Rice flour	50
Skimmed milk powder[b]	35
Soybean oil	5
Vitamin A free-vitamin mixture[c]	6
Salt mixture[d]	4

a. Vitamin A deficient basal diet is a semisynthetic diet rich in all nutrients except vitamin A and carotene. It was prepared according to Barua et al [27].

b. Skimmed milk powder was procured from local market and washed with hot ethanol.

c. Vitamin A free vitamin mixture was prepared according to Ambree et al [28]. Vitamin was premixed with a bulk of rice flour to provide the required amounts of vitamins per Kg diet when mixed in 6% combination.

d. Salt mixture was prepared according to the recommendation of American Institute of Nutrition[29].

Table 2 : Effect of vitamin A deficiency on hematopoietic parameters of rats.

Measures	Groups of rats	
	Control	Vit A deficient
Hemoglobin (g/dl)	14.40 ± 0.51^{a} (18)	11.67 ± 0.24^{b} (18)
Hematocrit (%)	43.66 ± 1.31^{a} (18)	32.75 ± 1.45^{b} (18)
Serum-iron (µg/dl)	153.83 ± 11.15^{a} (6)	$105.03 + 12.47^{b}$ (6)
Splenic-iron (µg/g)	268.16 ± 16.54^{a} (6)	373.17 ± 21.52^{b} (6)
Liver-iron (µg/g)	197.12 ± 13.7^{a} (6)	275.87 ± 15.20^{b} (6)
Liver vitamin A (µg/g)	165.50 ± 15.60 (18)	12.31 ± 1.30^{b} (18)
Serum Vitamin A (µg/dl)	62.35 ± 6.15^{a} (18)	14.25 ± 1.50^{b} (18)

Values depicted in the table are mean ± SD. Number in the parenthesis indicates the number of rats. Values within the same line not bearing common superscript letter are significantly($p<.001$) different.

Table 3: Effect of vitamin A deficiency on the level of riboflavin, glutathione and ceruloplasmin in rats.

Measures	Groups of rats	
	Control	Vit A deficient
Riboflavin:		
Liver riboflavin (µg/g)	190.78 ± 19.73[a] (6)	198.82 ± 13.48[ab] (6)
Splenic riboflavin (µg/g)	19.19 ± 1.55[a] (6)	20.76 ± 2.56[ab] (6)
Brain riboflavin (µg/g)	28.5 ± 3.89[a] (6)	32.24 ± 4.29[ab] (6)
Glutathione (GSH):		
Liver-GSH (mg/100g)	152.3 ± 15.47[a] (6)	248.23 ± 20.65[b] (b)
Splenic-GSH (mg/100g)	150.0 ± 12.70[a] (6)	234.88 ± 29.86[b] (6)
Brain-GSH (mg/100g)	47.94 ± 8.71[a] (6)	81.31 ± 9.84[b] (6)
Erythrocyte-GSH (µ Mole/g)	53.98 ± 6.06[a] (6)	84.32 ± 9.55[b] (6)
Serum Ceruloplasmin:		
Serum ceruloplasmin (Units)	0.43 ± 0.02[a] (6)	0.61 ± 0.03[b] (6)

Values are mean ± S. D (n=6). Values within the same line not bearing common superscript letters are significantly ($p<0.001$) different. Ceruloplasmin unit - PPD- Oxidase activity in terms of change in O.D/0.1 ml serum.

Discussion

Lower levels of vitamin A in serum and liver of rats kept on vitamin A deficient diet indicate that they were depleted of vitamin A (Table-2) Lower levels of hemoglobin and hematocrit in vitamin A deficient rats suggest that vitamin A is needed for normal hematopoiesis and its deprivation leads to anemia despite an adequote intake of iron through diet. This result is in agreement with the findings of other investigators who claimed in their studies with animals and human subjects that vitamin A is needed for normal hematopoiesis[1-4,30]. Larger storage of iron in liver and spleen, along-with a simultaneous decrease of iron in serum or otherwise in transport suggests that in vitamin A deficiency iron is somehow sequestered in liver and spleen, resulting in decreased amount of iron in transport. This culminates in impaired synthesis of hemoglobin for want of iron. Mejia et al[1] also reported a larger accumulation of iron in liver and spleen alongwith a lower concentration of iron in serum of vitamin A deficient rats. In one of our previous studies, we observed a larger storage of iron in liver, spleen, heart and muscle of vitamin A deficient rat[4]. It is suggested that vitamin A somehow, either directly or indirectly, helps the mobilization of iron from the storage in liver and other tissues. If the action would have been an indirect one, it was assumed that vitamin A could have done it through reducing the supply of the compound(s) suggested to be needed for the mobilization of iron. Thus in present study, we sought to investigate the effect of vitamin A deficiency on the level of ascorbic acid, riboflavin, glutathione and ceruloplasmin. Results with ascorbic acid has been published elsewhere [16], where ascorbic acid level has been found to be decreased due to the action of vitamin A deficiency. But oral ascorbic acid supplementation to vitamin A deficient rats could neither correct the anemia nor could reduce the iron accumulation, indicating that iron-storage in vitamin A deficiency is unrelated to the status of ascorbic acid.

Concentration of riboflavin in liver, spleen and brain remained unaffected by the action of vitamin A deficiency. Serveral recent investigations would suggest that physiological release of iron from ferritin is mediated by a reductive process involving the participation of $FMNH_2$, which ultimately reduces the ferritin-Fe (III) to ferritin-Fe (II). This reductive step is necessary for the release of iron from ferritin [12,13,31]. Power et al[32] demonstrated a larger store of ferritin iron in the liver of riboflavin deficient rats. Moreover iron mobilization in vitro by hepatic mitochondria showed that riboflavin depleted animals had significantly lower mobilization rate of iron. The authors suggested that riboflavin deficiency might have impaired the mobilization of iron from ferritin stores. Hepatic ferritin-iron was also rerported to be increased in riboflavin deficiency by Thurnham et at [33]. In the context of probable role of riboflavin in iron release from ferritin, the present investigation was made to see the effect of vitamin A deficiency on riboflavin status, presuming that iron accumulation during vitamin A deficiency might be through some process involving the decreased concentration of riboflavin. In present study, normal level, of riboflavin in liver, spleen, and brain would indicate that iron accumulation during vitamin A deficiency is independent of riboflavin status.

Glutathione concentration in liver, spleen, brain and erythrocyte increased as a result of vitamin A deficiency. This observation is in accordance with the findings of Nair et al [34], who reported an increased level of intestinal reduced glutathione during vitamin A deficiency. In contradiction, Tom et al [35] reported a diminished concentration of glutathione in the lung of rats during either vitamin A deficiency or vitamin A excess state. Dogra et al [36] however suggested an inadequate supply of glutathione to lung tissue during vitamin A deficieny. Glutathione has been implicated to the release of iron from ferritin[12,14] and from transferrin[37]. Glutathione is considered to be a biologicl reductant like ascorbic acid. Thus, glutathone level during vitamin A deficiency was determined assuming that vitamin A deficiency could have caused iron accumulation through lowering the level of glutathione. In present study, in contradiction to our assumption, the concentration of glutathione rather increased in liver, spleen, brain and erythrocyte. Though the elevated level of glutathione cannot be interpreted in terms of its role, if these is any in the mobilization of iron, it may be inferred that iron accumulation during vitamin A deficiency is not due to lowered level of glutathione in the tissue.

Serum ceruloplasmia level increased due to vitamin A deficiency. Ceruloplasmin level has been reported to rise by about 30% as a result of vitamin A deficiency[38]. The importance of increased level of ceruloplasmin lies in the fact that keratin synthesis requires the participation of

copper ion. Since keratin synthesis (Keratinization) is increased due to vitamin A deficiency, there will be a greater demand for copper to satisfy the extra need. Thus, ceruloplasmin, the copper carrier protein is also increased during vitamin A deficiency.

Iron is transported through transferrin. Ceruloplasmin is needed for reductive incorporation of iron into transferrin, before it is carried to the erythroids for the synthesis of hemoglobin. Moreover, developing reticulocyte in bone marrow receives iron directly from transferrin-Fe (111)-complex, and not from other sources. Increased level of ceruloplasmin in present study clearly indicates that impaired iron mobilization during vitamin A deficiency is not due to impaired transport of iron through transferrin for want of ceruloplasmin. Moreover transferrin synthesis itself also remains unaltered by vitamin A deficiency[39]. Increased level of ceruloplasmin, however, cannot be interpreted in terms of its specific contribution, if there is any, to the metabolism of iron under present situation.

Results of the present study, however, at least suggest that accumulation of iron during vitamin A deficiency is not due to the lowered concentrations of glutathione, riboflavin and ceruloplasmin in the system.

Summary

Hematopoiesis in relationship to the levels of riboflavin, glutathione and ceruloplasmin was studied in vitamin A deficient rats. Rats were made deficient in vitamin A by feedig them vitamin A deficient basal diet for a period of 55 days. Rat fed vitamin A deficient diet showed marked decrease in the levels of Hb, hematocrit. serum iron, liver vitamin A and serum vitamin A as compared to the control. There was a larger deposition of iron in the liver and spleen of vitamin A deficient rats. Concentration of glutathione was found to be markedly increased in liver, spleen, brain and erythrocytes of vitamin A deficient rats. Vitamin A deficiency did not affect the concentration of riboflavin in liver, spleen and brain. Level of ceruloplasmin in serum increased as a result of vitamin A deficiency. It is suggested that iron-accumulation in liver and spleen of vitamin A deficient rats is not due to the lack of riboflavin, glutathione and ceruloplasmin in the system.

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