Effect of Vitamin A, Retinoic Acid and Xanthophyll on Hematopoiesis and Tissue-Iron-Content of Rats.

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Introduction

Association of vitamin A deficiency with anaemia has been reported in several studies with animals[1-10] as well as with human subjects.[11-16] Although hematopoietic action of vitamin A was reported as early as 1922 by Findlay and Mackenzie[1], the problem was not considered with the seriousness it deserved until recently. Little has been done yet to elucidate a meaningful clue to their mode of interaction in biological system. The present animal study has been undertaken to investigate the action of vitamin A itself and two vitamin A analogs such as all-trans retinoic acid and xanthophyll on the process of hematopoiesis.

Materials and Methods

Post weaning rats of Long-Evans strain weighing around 90g were divided into four groups-Gr.A, Gr.B, Gr.C and Gr. D. Each group consisted of six rats, excepting that Gr. D consisted of twenty four rats. Animals in four groups were assigned to four dietary allotments such as (I) control (ad libitum fed), (II) control (pair fed), (III) vitamin A deficient and (IV) vitamin A deficient but fortified with all-trans retinoic acid. Basal diet for all groups was a semisynthetic diet deficient in vitamin A but complete in all other nutrients (Table-1). Control diet was prepared by supplementing the deficient diet with retinylacetate (8000 I.U/kg diet). Retinoic acid fortified diet was prepared by supplementing deficient diet with all-trans retinoic acid (800 µg/kg diet).

Rats of Gr. A and Gr. B were fed control diet, the former being provided ad libitum and the later in amounts per rat not greater than the average amount per rat consumed by the deficient animal.

Gr. C was provided with retinoic acid fortified diet, while Gr. D received vitamin A deficient diet. All animals were exposed to water ad libitum. Throughout the

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depletion period, small amount of vitamin A (30 I.U. per rat) was provided to the rats that received vitamin A deficient diet at an interval of 7-days. The dietary regime was continued for fifty days till the appearance of mild vitamin A deficiency amongst the rats fed vitamin A deficient diet, as was indicated by an initial weight plateau. On day 51, all rats of Grs. A-C and six rats of Gr. D were sacrificed by decapitation under mild chloroform anesthesia. Before sacrifice, blood was collected from tail-tip for the estimation of hemoglobin. On sacrifice, blood was collected for the enumeration of RBC, and serum was collected for the estimation of serum iron, serum-TIBC and serum vitamin A. Liver, spleen, heart, kidney, lung, brain and muscle from hind-leg were harvested, rinsed in isotonic saline, blotted dry and weighed. The samples were then collected in acid washed glass containers and preserved in deep freeze at -18°C in order to determine tissue iron and liver vitamin A. This phase of the experiment was accomplished as a base-line study for the second phase of the experiment.

In the second phase, rest of the deficient rats of Gr. D were divided into three groups-Gr.D_1 Gr.D_2 and Gr. D_3, in order to supplement them with vitamin A, all-trans retinoic acid and xanthophyll. Supplementation was made orally by feeding them in a single bolus of 0.1ml soy bean oil. Gr. D_1 was supplemented with retinylacetate (100 µg/rat/day), Gr. D_2 was supplemented with all trans retinoic acid (50 µg/rat/day) while Gr.D_3 received xanthophyll (200 µg/rat/day Throughout the supplementation period, rats that received xanthophyll, still continued to receive a small dose of retinylacetate (30 I.U/rat/day), at an interval of 7-days. After three weeks on supplementation, rats of all treatments were sacrificed by decapitation under mild chloroform anesthesia. Blood, serum and tissue samples were collected and subsequent analysis were accomplished in a similar procedure as in base-line study.

Analytical Methods

Liver vitamin A was determined colorimetrically by using TCA as chromogenic reagent,[17] following extraction with petroleum ether (40-60°C)[26]. Serum vitamin A was estimated by HPLC using retinol as standard and retinylacetate as internal standard.[18]

Serum iron was determined by using orthophenanthroline as chromogenic reagent.[19] Tissue iron was determined by using bathophenanthroline.[20] Serum-TIBC was determined by the method of Ramsay.[21] Percent transferrin saturation was calculated from the values of serum iron and TIBC using the formula-100x (serum iron/serum-TIBC). Hemoglobin was determined by cyanmethemoglobin method,[22] using commercial kit (Sigma. Chem. Co. Ltd). RBC was enumerated by standard method.[23] Hemoglobin was determined by microcentrifugal method.[24] Statistical analysis was done following Student's t test between the means.[25]

Results

Table-2, Rats fed vitamin A deficient diet gained less weight as compared to either ad libitum fed or pair fed control. Weight gain for retinoic acid fortified rats was found to be comparable to that of ad libitum fed control.

Animals fed vitamin A deficient diet showed marked ($P < 0.001$) depletion of serum vitamin A and liver vitamin A, indicating a real vitamin A deficient state. Rats fed vitamin A deficient diet developed anaemia with a significant ($P < 0.001$) decrease in hemoglobin, hematocrit, RBC, serum iron and percent transferrin saturation (%TS) as compared to either pair fed or ad libitum fed control. Serum-TIBC, however, was found to remain unaffected in vitamin A deficient rats. There was found no significant difference between the hematological values of ad libitum fed and pair fed control. Rats reared on vitamin-A deficient diet but fortified with retinoic acid showed significant ($P<0.001$) depletion of serum vitamin A and liver vitamin A. Notably, retinoic acid fortified rats had significantly ($P<0.001$) larger store of liver vitamin A as compared to the vitamin A deficient animals, though the levels under both the conditions were still within the deficient range.

Moreover, retinoic acid fortified rats were found to have normal levels of hemoglobin, hematocrit, RBC, serum iron and percent transferrin saturation as compared to the control animals.

Table 3, Vitamin A deficient rats upon repletion with either retinylacetate or all-trans retinoic acid showed a complete restoration of hematopoiesis. Hemoglobin, hematocrit, RBC, serum iron and %TS were found

to be increased to the control level following the administration of retinylacetate or retinoic acid. Hemoglobin, hematocrit, RBC, serum iron, serum-TIBC and %TS of vitamin A deficient rats did not increase following the administration of xanthophyll.

Table 4, Animals fed vitamin A deficient diet was found to have significantly ($P < 0.001$) larger deposition of iron in liver, spleen, heart and muscle, but lower deposition of iron in kidney and lung as compared to either ad libitum fed or pair fed control. While, iron content of brain was found to remain unaffected in vitamin A deficient rats. No significant difference was found between the tissue iron content of pair-fed and ad libitum -fed control animals. Animals fed vitamin A deficient diet but fortified with retinoic acid showed normal concentration of iron in kidney, brain, heart, lung and muscle as compared to either ad libitum-fed or pair-fed control. While, they showed an insignificantly larger deposition of iron in liver and spleen as compared to the ad libitum-fed control, but significantly larger deposition as compared to the pair-fed control.

Table 5, Vitamin A deficient rats upon repletion with retinylacetate showed a significant reduction of iron accumulation in their liver, spleen, heart and muscle. Moreover, retinylacetate supplementation caused a simultaneous increase of iron in their kidney and lung; while iron content of brain remained unaffected. Vitamin A deficient rats upon repletion with all-trans retinoic acid showed a significant reduction of iron accumulation in their liver, spleen, heart and muscle. As compared to retinylacetate repleted rats, retinoic acid supplemented rats had still larger deposition of iron in their liver. Supplementation of xanthophyll to vitamin A deficient rats did not cause a decrease of iron in liver, spleen, heart and muscle; instead their iron content tended to increase further, though insignificantly. Brain iron, however, remained unaffected.

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

Ingredients	Percentage
Rice flour	50
Skimmed milk powder[b]	35
Soy bean oil	5
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. It was prepared according to Barua et al[4].

b. Skimmed milk powder was washed with hot ethanol.

c. Vitamin A free vitamin mixture was prepared according to Ambree et al.[26] Vitamins were premixed with a bulk of rice flour to provide the required amounts of vitamins per kg diet in 6% combination.

d. Salt mixture was prepared according to the recommendation of American Institute of Nutrition[27].

Table 2. Hematopoietic parameters and vitamin A status of control (adl), control (pair fed), vitamin A deficient and retinoic acid supplemented rats.

Parameters.	Groups of rats			
	Control (Adl) Gr.A	Control (Pair-fed) Gr. B	RA-supplemented Gr. C	Deficient Gr. D
Body weight (g)	267.26±7.15 ^{ab}	260.35±9.50^{a}	285.75±8.30^{b}	230.50±6.20^{c}
Hemoglobin (g/dl)	14.23±0.31^{a}	14.72±0.28^{a}	13.72±0.33^{a}	10.25± 0. 25^{b}
Hematocrit (%)	41.50 ±1.29 ^{a}	42.05± 1.22^{a}	43.31±1.04^{a}	33.44± 1.85^{b}
RBC (mill/mm^3)	5.89 ± 0.14 ^{a}	5.98± 0.15^{a}	5.62±0.12^{a}	4.42±0.13^{b}
Serum-iron (µg/dl)	162.45±8.84^{a}	154.01±9.33^{a}	156.56±15.09^{a}	98.69±10.64^{b}
TIBC (µg/dl)	431.32±21.62^{a}	408.05±28.92^{a}	423.43±25.00^{a}	416.98±18.74^{a}
% Trans-Saturation	36.37±2.56^{a}	37.30±1.73^{a}	38.82±1.26^{a}	24.22±1.73^{b}
Serum-vit A (µg/dl)	56.78±8.94^{a}	54.33±4.70^{a}	15.15±2.09^{b}	16.38±1.31^{b}
Liver-vit A (µg/g)	169.47 ±17.60^{a}	162.62±9.83^{a}	14.96±1.96^{b}	12.23±1.78^{c}

Values are mean ± S.D. (n=6). Values in the same line with different superscripts differ significantly ($p < 0.001$).RA - retinoic acid.

Table 3. Hematopoietic response of vitamin A deficient rats to the supplementations of vitamin A, retinoic acid and Xanthophyll.

Parameters.	Groups of rats			
	Base limiting (Gr.D)	Vitamin A deficient upon treatment with		
		Vitamin-A (Gr. D_1)	Retinoic-acid (Gr. D_2)	xanthophyll (Gr. D_3)
Hemoglobin (g/dl)	10.25.±0.25 ^{b}	14.09±0.35^{a}	13.88±0.40^{a}	10.62±0.26^{b}
Hematocrit (%)	33.44±1.85^{b}	42.53±1.60^{a}	43.77±1.72^{a}	34.20± 0. 46^{b}
RBC (mill/mm^3)	4.42 ±0.13^{b}	5.99± 0.16^{a}	5.84±0.17^{a}	4.32± 0.23^{b}
Serum-iron (µg/dl)	98.69 ± 10.64^{b}	149.76± 10.30^{a}	154.65±7.81^{a}	109.00±11.39^{b}
TIBC (µg/dl)	416.98±18.74^{a}	425.71±24.80^{a}	420.46±20.20^{a}	432.23±25.70^{a}
% Trans-Saturation	24.22±1.73^{b}	36.24±2.78^{a}	38.88±2.92^{a}	23.42±2.29^{b}
Serum-vit A (µg/dl)	16.38±1.31^{a}	53.28±5.18^{b}	16.02±1.12^{a}	13.87±0.89^{c}
Liver-vit A (µg/g)	13.23 ±1.78^{b}	120.13±12.51^{a}	14.05±1.02^{b}	12.99±1.04^{b}

Values given in the table are mean ± S.D (n = 6). Values in the same line with different superscripts differ significantly ($p < 0.001$).

Table 4. Tissue-iron content of control (adl), control (pair fed), vitamin-A deficient and retinoic acid supplemented rats.

Group	Iron(µg/g wet.wt) present in						
	Liver	Spleen	Kidney	Brain	Heart	Lung	Muscle
Control (adl)	174.41.±16.15 ^{ab}	288.63.±17.85 ^{ab}	57.31.±3.85 ^{a}	23.43.±2.69 ^{a}	59.68.±5.97 ^{a}	40.61.±4.42 ^{a}	13.75.±1.20 ^{a}
Control (Paid-fed)	158.00 ±6.16 ^{a}	259.03.±14.77 ^{a}	56.69 ±6.91 ^{a}	21.98 ±1.99 ^{a}	50.02.±6.93 ^{a}	38.22.±3.68 ^{a}	13.81.±1.10 ^{a}
Retinoic acid	192.27±11.11 ^{b}	312.66.±15.59 ^{b}	58.96±5.32 ^{a}	25.88.±3.64 ^{a}	57.56.±6.36 ^{a}	43.93.±4.93 ^{a}	12.96.±0.53 ^{a}
Deficient	286.51±17.94 ^{c}	416.01±21.66 ^{c}	40.78±3.78 ^{b}	23.66.±3.42 ^{a}	88.25.±6.85 ^{b}	26.44.±3.36 ^{b}	19.35.1.30 ^{b}

Values in the table are mean ± S.D. (n=6). Values within the same column with different superscripts differ significantly ($p < 0.001$)

Table 5. Response of tissue-iron of vitamin A deficient rats to the supplementations of vitamin A, retinoic acid and xanthophyll.

Group Treatment	Iron(µg/g wet.wt) present in						
	Liver	Spleen	Kidney	Brain	Heart	Lung	Muscle
Gr.D ----------	286.51±17.94 ^{a}	416.01±21.66 ^{a}	40.76.±3.78 ^{a}	23.66±.3.42 ^{a}	88.2±6.85 ^{a}	26.44±3.36 ^{a}	19.35±1.30 ^{a}
$Gr.D_1$ Vitamin- A	202.40.±11.85 ^{b}	312.15.±15.37 ^{b}	62.82±6.82 ^{b}	24.99.±2.71 ^{a}	60.10±5.04 ^{b}	44.85.±4.70 ^{b}	12.69.±1.71 ^{b}
$Gr.D_2$ Retinoic acid	235.37±10.50 ^{c}	332.44.±15.06 ^{b}	58.45±5.09 ^{b}	25.80.±3.69 ^{a}	66.36±5.69 ^{b}	46.73 ±4.13 ^{b}	13.56.±1.02 ^{b}
$Gr.D_3$ Deficient	316.56±12.55 ^{a}	445.57±17.83 ^{a}	34.86±4.47 ^{a}	22.08.±3.13 ^{a}	97.02±8.72 ^{a}	25.77.±3.20 ^{a}	21.66±3.14 ^{a}

Values given in the table are mean ± S.D. (n=6). Values within the same column with different superscripts differ significantly ($p < 0.001$ and higher)

Discussion

Results of the present study suggest with enough confidence that vitamin A is needed for normal hematopoiesis and its deprivation may lead to anaemia, despite an adequate intake of iron through diet. This observation is consistent with several previous studies which claimed an association of vitamin A deficiency with anaemia.[1-16] Iron accumulation in liver, spleen heart and muscle is another feature of this anaemia. Iron accumulation in tissues alongwith a lower concentration of iron in serum or otherwise in transport suggests a probable imbalance in the metabolism of iron in absence of vitamin A. Mejia et al [6,7] and Hodges et al[13] also observed similar iron accumulation in liver and spleen of vitamin A deficient rats with lower level of serum

iron. These authors suggested a probable immobilisation of iron from the stores in absence of vitamin A.

Iron is carried in blood in combination with a glycoprotein-the transferrin. Since serum-TIBC represents 90% of serum transferrin, the normal serum-TIBC in vitamin A deficient rats obviously indicates the normal synthesis of transferrin during vitamin A deficiency, even though glycoprotein synthesis has already been reported to be depressed by the deficiency of vitamin A[28]. This is in accordance with a similar finding by Mejia and Arroyave[15] who reported normal level of transferrin in human serum with less than normal level of retinol. Thus synthesis of iron transport protein is not impaired by the action of vitamin A deficiency.

Iron accumulation may stem from (I) excessive absorption, (II) hemolysis, (III) depressed synthesis of serum transferrin and (IV) defects in the mobilization of iron from the store.

Mejia et al[6] reported normal absorption of iron in vitamin A deficient rats. Gardner et al[30] reported normal absorption of iron in vitamin A deficient rats. Gardner etal[30] reported that iron accumulation during vitamin A deficiency is not due to increased hemolysis. Present finding, in accordance with the observations of Mejia and Arroyave[15], suggests a normal synthesis of transferrin in vitamin A deficient rats. Moreover, increased absorption and hemolysis are generally associated with a higher level of serum iron. In contrast, serum iron in vitamin A deficient rats has consistently been found to be less than the control values in present study as well as in previous studies[7]. In view of these observations, iron accumulations during vitamin A deficiency appears to be related to some defects in the mobilization of iron from the store.

Liver is the major site for parenchymal iron storage. Spleen and muscle are the important sites for reticuloendothelial store of iron.[31] Thus iron accumulation in liver, spleen and muscle indicates a defect probably in both parenchymal and reticuloendothelial release of iron, which ultimately culminates in impaired hematopoiesis. Impairment in reticuloendothelial release of iron appears to be more relevant in this regard, because reticuloendothelial reutilization of iron is in the mainstream of body's total iron exchange for hematopoiesis.

Since kidney is the site for RBP breakdown after its normal life span[32]; this organ continues to be furnished with a constant supply of retinol from RBP degradation, even when other organs become virtually depleted of retinol. Thus lower level of kidney iron might be related to a raiding of iron out of kidney with the help of retinol whatever is made available in kidney from RBP breakdown products.

The concept that vitamin A helps hematopoiesis through mediating the mobilization of iron is further substantiated by the fact that vitamin A deficient rats upon repletion with retinylacetate achieved a complete restoration of hematopoiesis alongwith a concomitant decrease of iron-accumulation in liver, spleen, heart and muscle. Increased serum iron indicates the increased amount of iron in transport for being used up in subsequent hematopoiesis.

All-trans retinoic acid almost paralleled retinylacetate in its capacity to sustain as well as to promote hematopoiesis in vitamin A deficient rats. Since retinoic acid can replace vitamin A in many physiological functions, it is thus most likely that it would be able to replace vitamin A in hematopoiesis too. Moreover, retinoic acid has also been reported to be active in the development of human erythroid cells in an invitro study.[29]

Retinoic acid is very often considered to be a common metabolite of vitamin A. If not active itself, it is considered to be more nearer to the active form of vitamin A.[32] Though within the scope of present study, it is not possible to interprete the relative efficiency of vitamin A and retinoic acid in the process of hematopiesis or otherwise in iron metabolism, it is atleast suggested that retinol or retinoic acid either themselves or in some other form (s) potentially convertible from both retinylacetate and retinoic acid, are active in the process of hematopoiesis, most probably through their capacity to mobilize iron from the store. Inertness of xanthophyll in this regard indicates the need for specific vitamin A function for the mobilization of iron from the store.

Summary

Vitamin A deficiency caused a marked depression of hematopoiesis in rats. Hemoglobin, hematocrit, RBC, serum iron and percent

transferrin saturation (%TS) decreased significantly (P<0.001) in rats made deficient in vitamin A. Serum- TIBC, however, remained unaffected by vitamin A deficiency. Vitamin A deficient rats exhibited a larger deposition of iron in liver, spleen, heart and muscle, but a lower deposition in kidney and lung as compared to either adlibitum fed or pair fed control. Iron content of brain remained unaffected by vitamin A deficiency. Vitamin A deficient diet fortified with all-trans retinoic acid sustained normal growth and normal hematopoiesis. Vitamin A deficient rats upon repletion with either retinylacetate or all-trans retinoic acid showed a complete restoration of hematopoiesis. Iron accumulation in liver, spleen, heart and muscle decreased significantly following the administration of retinylacetate or all-trans retinoic acid. Administration of xanthophyll under similar condition could neither promote hematopoiesis nor could reduce iron accumulation in the tissue.

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