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Developmental defects of hematopoietic system of mice offspring influenced by maternal vitamin C intake during pregnancy

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ABSTRACT

The effect of lower intake of vitamin C during pregnancy and lactation of pregnant mice on the hematopoietic development of pups was evaluated. Dams were provided low (0.15 g/kg diet), normal (0.50 g/kg) dietary vitamin C level. Analysis of HPLC indicated significant decrease in the amount of Vit C in the bone marrow tissue extract of the Vit C deficient pregnant mother's pup. The complete blood count indicated approximately 20% decrease in RBC count and approx 25% decrease in the count of WBC in peripheral blood of offsprings of vit C deficient pregnant mother values too. BM from control mice showed normal tissue morphology whereas in vitamin C deprived newborn bone marrow showed abnormality in tissue morphology. It showed the detachment from the bone structure and significant reduction in the hematopoietic cellular mass. In vitro colony-forming assays showed that the number of colonies formed by control pup BM was significantly higher of approximately 32% than deficient bone marrow. ROS level was significantly increased in Vitamin C deficient new born mice ($p< 0.01$) bone marrow by about 30% compared to the new born offspring of control pregnant mother. It can be concluded that Vitamin C is an essential micronutrient of the diet of pregnant mother and deficiency of which can cause the defective developmental profile of bone marrow hematopoietic tissue of the offspring.
The effect of vit C deficiency in bone marrow of newborn mice on the expression of marker of oxidative stress was shown in figure 3.

KEYWORDS vitamin C, mice , pregnancy, bone marrow haematopoiesis

1. Introduction:

Hematopoiesis starts in the yolk sac in the mammalian fetus. It then shift little later to the liver and spleen and finally to the bone marrow in the seventh month of the fetal development. [1,2] The bone marrow remains the site of morphogenesis of blood cells throughout life of the individual. It takes about 10 to 14 days for an event affecting the earliest stage of blood cell formation to be reflected in the peripheral blood count. The maintenance of hematopoiesis is a complex process, developmental programme of which depends on its microenvironmental conditions that regulate the differentiation of hematopoietic stem cells (HSC_S) into the required number of mature blood cells.[3,4,5]

Vitamins are essential for fetal health and development. Although micronutrient deficiencies during pregnancy have been associated with adverse pregnancy outcomes.[6] their effects on the long-term health of the offspring are not well understood .[7]

The classical symptoms of vitamin C deficiency is Scurvy and it have been known for long time. However during last few decades more detailed knowledge on diverse.[8,9,10] biological functions of ascorbic acid have been obtained

Apart from the importance of ascorbic acid for collagen synthesis[11] and bone remodelling[12,13] but it has now possible to correlate the biochemical metabolism of ascorbic acid with hematopoietic tissue development of the offspring related to the status of this vitamin in mother's blood.

The focus of this study is to evaluate the impact of maternal Vitamin C intake during pregnancy on the plasma Vit C level of newborn and effect on hematopoietic tissue development.

1. Materials and methods:

Animals and Diet

Virgin female mice , 4-6 weeks of age and weighing (25) g were provided free access to vitamin C rich diet , to which vitamin C was added (the powdered vitamin C L-ascorbate) (0.50 g /kg diet) as well as natural vitamin C sources, such as lemon juice tomato were added and vitamin C deficient group of mice were provided low vitamin c powder (0.15 g / kg diet) . The experimental diet with the assigned vitamin C concentration was fed for at least 2 months before mating and throughout pregnancy. The low vitamin C intake was also adequate to achieve normal weight gain during pregnancy and lactation period.[14,15] The animal room was provided with a 12-hr light and dark cycle and at controlled temperature 22 to24^{0}C. Animals were bred using established procedure with one female being placed with one male during the receptive time .

Collection of samples

Bone marrow tissues from all new born pups from each litter were collected within 24 hr after delivery. Pups were weighed and then killed using an overdose of CO. Blood was removed from the left ventricle of the heart using a heparinized syringe. Plasma was obtained and mixed with an equal volume of 1 M metaphosphoric acid (MPA) containing 0.54 mmol/L disodium ethylenediaminetetraacetate (Na_2EDTA). After centrifuging at 1000 g (Beckman Instrument) and 50 µl of plasma were added to 150 µl of sample buffer (0.1M sodium dihydrogenphosphate and 0.2 mM Na_2EDTA, adjusted to pH 3.1 with orthophosphoric acid). This mixture was centrifuged at 1000 g for 10 min at 0°C. The resulting supernatant was stored at -20°C and a 10 µl aliquot was injected for analysis within 3 h.

Chromatography
Ascorbic acid analyses[16] were carried out with a Shimadzu Model, software LC-solution ,Degasser DGU-20A_3 Auto sampler SIL-20A HT absorbance detector operating at 254 nm at a sensitivity of 0.016a.u.f.s. A model 740 data module was used for peak integration all these systems were from Shimadzu, Japan. The separation was carried out on a Phenomenex-Luna (5 micron µ) c18 column(average particle size 5 micron,250x4.6 mm I.D). The mobile phase was buffer consisting of 0.1 M sodium dihydrogen phosphate (NaH_2PO4) and 0.2 mM Na_2EDTA, adjusted to PH 3.1 with orthophosphoric acid. The buffer was filtered through a Millipore HA 0.45 µm filter and degassed each day prior to use. The column was maintained to ambient temperature and the mobile phase was at a constant flow rate of 1.0 ml/minute. A standard stock solution of ascorbic acid was prepared in this buffer (1 mg/ml) and was further diluted in the same buffer.

Complete blood count
The number of leucocyte (WBC), erythrocyte (RBC), lymphocytes (LYM), neutrophilic granulocytes (GRA), intermediate cells (MON), and platelets (PLT) were counted.

Histology
Tibias were fixed in 4% paraformaldehyde at 4°C for 7 days. After fixation, bones were decalcified in 15% EDTA (ethylenediamine tetraacetic acid) for 1 week, dehydrated in progressive concentrations of ethanol, cleared in xylene, and embedded in paraffin. The entire tibia was then sectioned longitudinally in 5m per section. Sections from the center of the tibia were used for histologic staining with hematoxylin and eosin.[17]

Methylcellulose culture:
Cell suspensions were prepared from bone marrow in Iscove modified Dulbecco medium (IMDM) supplemented with 2% fetal bovine serum. Aliquots were then plated in a Methylcellulose medium with recombinant cytokines for colony assays of murine cells (Methocult M3434; Stem Cell Technologies, Vancouver,BC, Canada). This medium contained stemcellfactor, IL-3, IL-6, and erythroPoietin and allowed us to evaluate the activity of myeloerythroid progenitors. The plating was done according to the instructions of the manufacturer using between $2X10^4$ and$1x10^5$cells per 35 mm tissue culture dish. Cultures were placed in a humidified chamber and incubated at 37°C with 5% CO_2. The total number of colonies were counted between days 10 and 14 after plating.[17]

Determination of reactive oxygen species (ROS)
ROS levels in the mouse BM were determined[18] by DCFH-DA fluorescent assay with minor modifications. The tissue was taken in 100 Mm phosphate buffer, pH 7.2 and homogenized tissue was centrifuged at 5000 rpm for 20 minutes, at 4°C . Then the supernatant was collected for determination of ROS. Cells were counted using a hemacytometer and adjusted to a density of 10^7/mL using 1×PBS. DCFH-DA was then added into 300 µL cell suspension (10^6/mL) to form a final concentration of 10 µmol/L and incubated at 37°C for 30 min. After incubation, cells were washed with 1×PBS and re-suspended in 300 µL 1×PBS. The cell suspension (200 µL) was transferred into a 96-well microplate and the fluorescence intensity at 525 nm was measured under 488 nm excitation by a fluorescence reader.

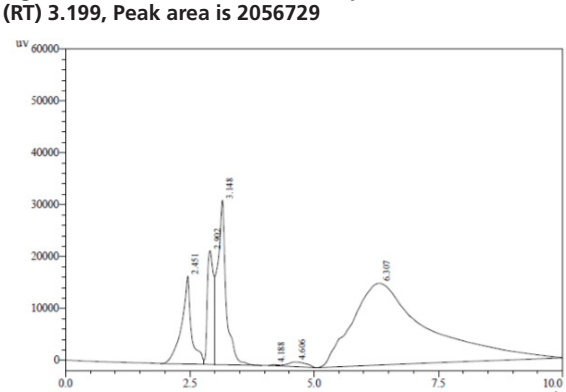
Statistical analysis
Analysis of variance (ANOVA) was applied on statistical analysis. The data was presented as the mean ± standard error of the mean. $p<0.05$ was considered as significant difference and $p<0.01$ was considered as extremely significant difference.

Result and discussion:
The result of HPLC (Figure 1) indicated significant decrease in the amount of Vit C in the bone marrow tissue extract of the Vit C deficient pregnant mother's pup. The result of complete blood count (Table I) showed approximately 20%decrease in RBC count and approx 25% decrease in the count of WBC in peripheral blood of offsprings of vit C deficient pregnant mother . There was a significant decrease in haemoglobin as well as hematocrit values too. The mouse BM histology was examined in femur of control newborn as well as vit C deprived mice (Figure 2). BM from control mice showed normal tissue morphology whereas in vit C deprived newborn bonemarrow showed abnormality in tissue morphology. It showed the detachment from the bone structure and significant reduction in the hematopoietic cellular mass. In vitro colony-forming assays were performed with bone marrow cells from pups of control and Vit C deficient pregnant mothers using media designed to support the growth of myeloid cell progenitors. The number of colonies formed by control pup BM was significantly higher of approximately 32% than deficient bone marrow .

The effect of vit C deficiency in bone marrow of newborn mice on the expression of marker of oxidative stress was shown in figure 3. ROS level was significantly increased in Vit C deficient new born mice ($p< 0.01$)bone marrow by 30% compared to the new born offspring of control pregnant mother.

Hematopoiesis is a tightly regulated process resulting in the production of blood cells and its disruption can lead to altered cell distribution and disease.[19] All blood cell lineages are produced in a hierarchical manner from HSC in BM and leukemia originates from damage induced in HSC.[20] One of the clinical consequences of damage to hematopoietic stem or progenitor cells is an alternation in circulating RBC, WBC and platelet counts.[21] Therefore, changes in the numbers of circulating blood cells in several lineages may reflect toxicity to BM HSC. In our study, significantly altered counts of WBC, RBC, hematocrit in Vit C deficient pregnant mother's pup are consistent with the developmental defects of some hematopoietic tissue . Direct evidence of BM developmental defects came from histological examination of the BMs of deficient and control pups, which revealed abnormalities induced by scarcity of availability of Vit C in the bone marrow microenvironment, The redox state of cells, the balance of oxidants, anti-oxidants and free radicals, plays an important role in cellular signaling, control of vascular tones, cell generation, and defense against microorganisms. ROS, including singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical, are induced by cellular aerobic metabolism, inflammation, or exposure to strees like deficiency of microelements like Vit C.[22,23] *In vivo*, there are a lot of anti-oxidants to delete ROS, the imbalance between ROS and antioxidant defenses has been described as oxidative stress [40]. The increased level of ROS in bone marrow tissue of Vit C deficient pups indicated the induction of adverse cellular effect.[24] on BM tissue in comparison to control one. Based on our data, oxidative stress and damage of BM were both demonstrated Vit C deficient pregnant mother's pup which indicates the direct relation of Vit C deficiency in mother's diet and developmental defects in the bone marrow hematopoietic tissue of the new born offspring.,

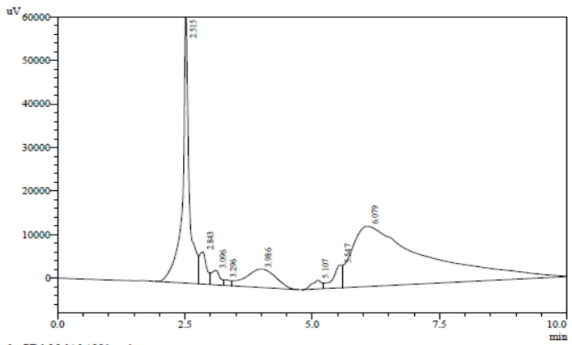
Fig: 1 (a) Standard ascorbic acid , peak at retention time (RT) 3.199, Peak area is 2056729



PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.451	234940	16959	9.393	19.388
2	2.902	192049	21907	7.679	25.045
3	3.148	382482	31753	15.292	36.301
4	4.188	1567	185	0.063	0.212
5	4.606	23028	873	0.921	0.998
6	6.307	1667051	15793	66.652	18.055
Total		2501116	87470	100.000	100.000

Fig 2: (b) . Peak of ascorbic acid from blood plasma of mice offspring(whose mother got dietary vitamin c during pregnancy and lactation s) RT is 3.148 and the peak area is 382482.



PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.515	560710	62177	23.723	62.476
2	2.843	70972	7362	3.003	7.398
3	3.096	38685	3305	1.637	3.321
4	3.296	11780	1332	0.498	1.339
5	3.986	171103	4221	7.239	4.242
6	5.107	26751	2014	1.132	2.024
7	5.547	64605	5248	2.733	5.274
8	6.079	1418921	13861	60.034	13.927
Total		2363527	99521	100.000	100.000

Fig 1(c) Small ascorbic acid peak is found at retention time 3.096, vitamin C deficient mice offspring group whose mother was vitamin c deficient during pregnancy.(retention time is 3.199 or 3.2 range).

Table I : Haematological parameters of control and vitamin C deficient mice . All data were represented as MEAN± SEM

Treatment	RBC $X10^{12}/L$	WBC X $10^{9}/L$	Hemoglobin g/dl	Hematocrit %
Control group(vitamin c supplied)	5.23± 0.24	8.03±0.10	11.48±0.8	38.06± 0.146
Vitamin c deficient group	4.08± 0.03	6.60±0.05	9.05±0.6	30.09±1.2

Table 3: Serum calcium level . All data are represented as MEAN± SEM

Experimental system	serum calcium level mg/dl
Control group	10.47±0.181
Vitamin c deficient	9.53±0.118

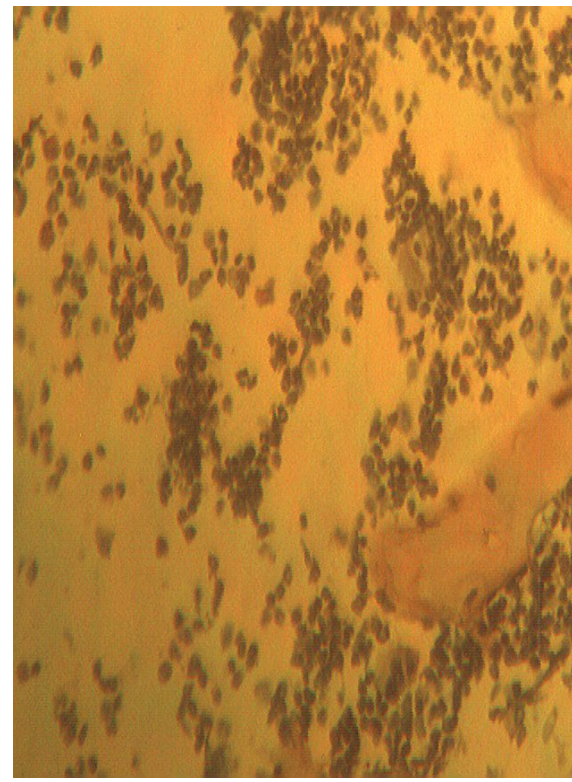
Table 4: DPPIV Enzyme assay from bonemarrow of control and vitamin C deficient mice. All data are represented as MEAN±SEM

Experimental system	DPPIV enzyme activity
control group	2.31± 0.055
Vitamin c deficient groups	1.60 ±0.028

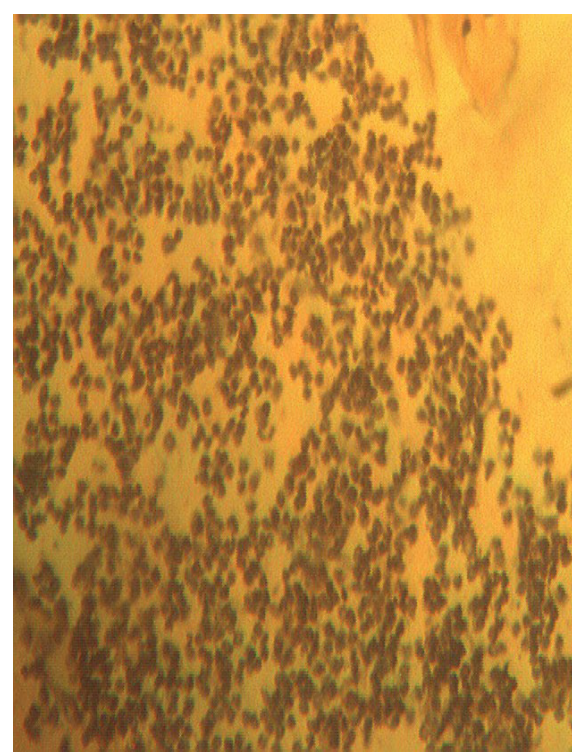
1 enzyme unit =0.01 ΔOD

Table 5: Measurement of bonemarrow alkaline phosphatase level. All data are represented as mean± SEM

Experimental system	Bonemarrow alkaline phosphatase level
Control group	10.34±0.017
Vitamin C deficient	9.05±0.02



A. Histological section of bone marrow of Vitamin c deficient mice (population of developing hematopoietic cells are less dense.(picture A)



B. Histological section of bone marrow of vitamin c supplied mice(Population of developing hematopoietic cells are more dense(picture B)

Discussion:

Hematopoiesis is dependent upon the tissue microenvironment composed of stromal cells, extracellular matrix and cytokines. Result of this study indicate that maternal vitamin C

intake influences vitamin C concentration of blood plasma of their pups of mice .Due to the integral role of vitamin C in collagen systhesis and bone remodelling the low maternal vitamin C intake during pregnancy compromise utero fetal development (27,28,29).For pups vitamin C concentration of plasma were higher in normal vitamin C intake compared with those of low maternal vitamin C group.

Low maternal vitamin C intake influences level of hematopoietic marker enzyme DPPIV , serum calcium level and bonemarrow alkaline phosphatase level. As vitamin C plays a crucial role in total body bone density , collagen systhesis, binemarrow extracellular matrix formation (30), so maternal vitamin C deficiency cause bonemarrow structural and microenvironmental alterations of the offsprings.

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