ORIGINAL ARTICLE
Expression of Vascular Endothelial Growth Factor Receptor Increases in Fetal Liver after Administration of High Doses of Vitamin A to Pregnant Mice

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ABSTRACT

Background: Vitamin A belongs to an array of retinoids, which have a pivotal part in the development of embryo and fetus and is important in themorphogenesis and cellular differentiation of developing liver. Vascular endothelial growth factor (VEGF) and its receptor the vascular endothelial growth factor receptor 2 (VEGFR2) are known to regulate the formation of vasculature and organogenesis of liver. For the contribution of better enlightenment of the modulating effects of vitamin A on the developing liver we investigated the effect of vitamin A on the histology and immunohistochemical expression of VEGFR2 in the liver from the fetuses.

Methods: For this purpose, eighteen pregnant albino mice were divided into three groups having six mice each. Group A served as control and was given 1ml of olive oil whereas group B and C were experimental groups and were given 0.6mg/kg and 1.8mg/kg of vitamin A diluted in 1ml of olive oil. Doses were given at 7th, 8th and 9th gestational days (GD). All animals were sacrificed at 18th GD and the livers from the fetuses were collected to observe the histological parameters such as inflammation, hepatic vacuolar degeneration, central vein diameter and hemorrhage in central veins along with immunohistochemical expression of VEGFR 2.

Results: Results showed that vitamin A affected the histology of liver. Inflammation, hepatic vacuolar degeneration (HVD) and the central vein hemorrhages were increased significantly ($p<0.001$), while diameter of the central vein decreased ($p<0.001$) in group C as compared to group A and B. The immunohistochemical expression of VEGFR2 decreased in sinusoids of group C ($p<0.001$) as compared to group A and B.

Conclusion: Thus, the maximum tolerated dose of vitamin A given to group C tends to affect the microarchitecture of the developing liver and expression of VEGFR2.

Keywords: Vitamin A, Vascular endothelial growth factor, VEGF, vascular endothelial growth factor receptor 2,

INTRODUCTION

Vitamin A plays an essential role in a large number of physiological functions that includes vision, epithelial integrity, growth, reproduction, hematopoiesis, and immunity (Coates et al., 2010). Vitamin A and its related compounds including retinol, retinal, retinoic acid and retinyl esters play a unique role in embryonic and fetal development and are crucial for both cellular differentiation and morphogenesis (Spiegler et al., 2012).Possible functions of vitamin A during embryogenesis were first inferred by studying its teratogenic effects, i.e. how the administration of excess doses of RA, interferes with normal developmental processes (Chagas et al 2003).The studies have been performed in a wide range of species including amphibians, zebra fish, chick and rodents(Maia et al 2019). Gene knockout studies then confirmed the crucial functions of RARs in mouse development(Mark et al., 2006).

Among the known mechanisms, modulation of VEGF expression by vitamin A has been described in plasma and lungs of fetuses and neonates of mice(Pinto et al., 2007). The authors also have demonstrated that vitamin A recovers the expression of VEGF and its receptors which were reduced in congenital diaphragmatic hernia(Schmidt et al., 2012).

The vascular endothelial growth factor (VEGF) family contains of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (Kowanetz and Ferrara, 2006). VEGF is indispensable for the development of hepatic vasculature(Carpenter et al., 2005). Its receptor VEGFR2 is highly expressed in the vascular endothelial cells of developing liver. VEGF/VEGFR2 signaling plays a pivotal role in the regulation of formation of hepatic vasculature derived from endocardium and hence involved in the organogenesis of liver(Zhang et al., 2016).Differential signaling of VEGF through its receptors VEGFR1 and VEGFR2 highlights the intricacies of this communication system in the liver. Signaling through VEGFR1 induces SECs (Sinusoidal endothelial cells) to release various cytokines that

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stimulate hepatocyte proliferation and signaling through VEGFR2 stimulates SEC proliferation (Lecouter et al., 2003).

Considering the role of VEGF and its receptors in liver development and a strong relationship between vitamin A and VEGF signaling, there is limited data available regarding both in the development process of liver.

Therefore, this study had been designed to determine the effects of antenatal administration of vitamin A on histology of developing liver in mice fetuses and on the expression of VEGFR2.

MATERIALS AND METHOD

Pregnant mice were randomly divided into three groups (A, B and C) containing six female mice each. In these pregnant mice positive vaginal plug was considered as a sign of confirmed conception, and the subsequent morning was taken as gestational day 0.For the purpose of tagging the cages, cage cards indicating the group name, number of mice and day 0 of gestation were used (Table 1). 1ml of olive oil was administered to group A. Group B was given Recommended daily allowance (RDA) calculated to be 0.6mg/kg (WHO 2011). Whereas group C was given Maximum tolerated dose (MTD) dose of vitamin A calculated to be 1.8mg/kg (Dibley and Jeacocke, 2001).

Table 1: Animal grouping and strategy for experimental intervention

Groups	Intervention and dosage	Route of administration	Duration	Day of sacrifice
A	1ml of olive oil	Orally	3 days (7th -$9^{th\ GD}$)	18th
B	Vitamin A 0.6mg/kg dissolved 1ml of olive oil	Orally	3 days (7th -$9^{th\ GD}$)	18th
C	Vitamin A 1.8mg/kg dissolved 1ml of	Orally	3 days (7th -$9^{th\ GD}$)	18th

olive oil			

Hepatic vacuolar degeneration (HVD) or Steatosis was marked by presence of empty spaces in the cytoplasm of hepatocyte. It was scored by calculating subjectively the percentage of the total number of hepatocytes with clear spaces in the observed fields (Table 3). Five random fields for each section were observed and then the mean score was taken. (Takahashi and Fukusato, 2014).

Table 2: Scoring of Inflammation.

Lobular inflammation	Score
No foci	0
< 2 foci	1

Table 3: Scoring of hepatic vacuolar degeneration (Steatosis)

Hepatic Vacuolar Degeneration (Steatosis)	Score
< 5%	0
5%-33%	1
> 33%-66%	2
> 66%	3

The diameters of central veins and the maximum diameter were measured for each central vein. Diameter of five central veins were measured from three random fields of each of the section of the liver for each fetus making it total of fifteen measurements from each fetus liver. The number of central veins with dense collection of red blood cells (RBCs) corresponding to hemorrhages were counted in five random fields for each fetus under 40X objective lens. The central veins with no hemorrhage were designated with score 0. The central veins partially filled with RBCs were designated with score 1 while completely filled with RBCs were designated with score 2.

The immunohistochemical expression of VEGFR2 was analyzed by cytoplasmic staining, with a granular or clustered distribution, of the endothelial cells, lining the sinusoids and other vessels, with different colors fluctuating from faint yellow to dark brown,. The number of positive sinusoids was counted per millimeter square in 5 high power fields for each fetus. The staining intensity varying from low to high was also noted.

The data was entered and analyzed using SPSS 21.0. Mean SD was calculated for numeric variables (diameter of central veins). Normality of data was checked with empirical formula. One-way ANOVA was applied to compare means of all three groups. Post-Hoc Tukey test was applied to compare difference of mean among groups. Kruskal wallis test was applied where data was non normally distributed (Scores of inflammation, hepatic vacuolar degeneration and central vein hemorrhage).

RESULTS AND OBSERVATIONS

General Histological Features of Fetal Liver

The H & E stained slides of the liver showed hepatocytes with central nucleus and they were radiating from the central vein in the form of cords, the portal triads, however, at the angles of lobule were not well formed in all three groups. In some sections hepatocytes were also present in clusters with solitary hematopoietic fociamong them. Granulocytes and lymphocytes were seen scattered throughout the liver.
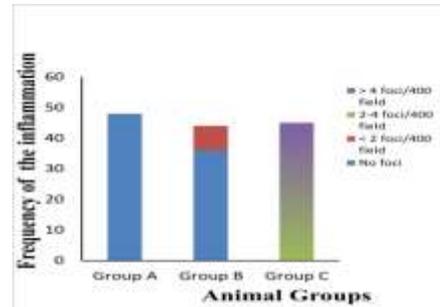

Figure 1: Frequency distribution of hepatocyte inflammation among the groups A, B and C. No focus of inflammation was seen in group A while in group B mean inflammation score was 0.1±0.39 and in group C it was 2±0.25. This difference was statistically different when analyzed with Kruskal-Wallis test (p<0.001)

Assessment of Histological Parameters
Inflammation
Histological examination of the livers showed no focus of inflammation in group A while in group B mean inflammation score was 0.1±0.39 and in group C it was 2±0.25. This difference was statistically different when analyzed with Kruskal-Wallis test (Figure 1 and 2)
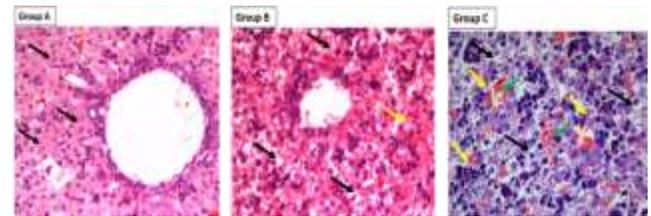

Figure 2: Showing the comparison of inflammation and hepatic vacuolar degeneration among group A, B and C. Inflammation was not present in group A, one focus (yellow arrows) could be seen in group B and three foci could be seen in group C. Black arrows showing cytoplasm of hepatocytes without any vacuoles in group A while in group B and C empty vacuoles could be seen depicting a fatty change (HVD). Note the presence of hemorrhages in the central veins and sinusoids in group C. H&E 40X.
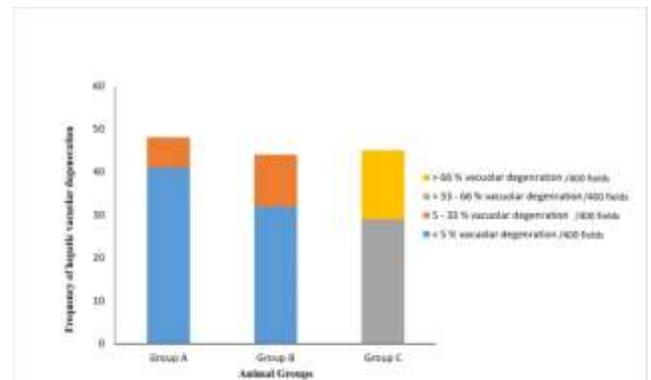

Figure 3: Frequency distribution of hepatic vacuolar degeneration among Groups A, B and C.

Figure 4: Showing the diameter of central veins (double arrow head) decreasing from group A to C and the central vein hemorrhages which are not present in group A but full (black asterisk) and partial (yellow asterisk) present in group B and C. H&E 10
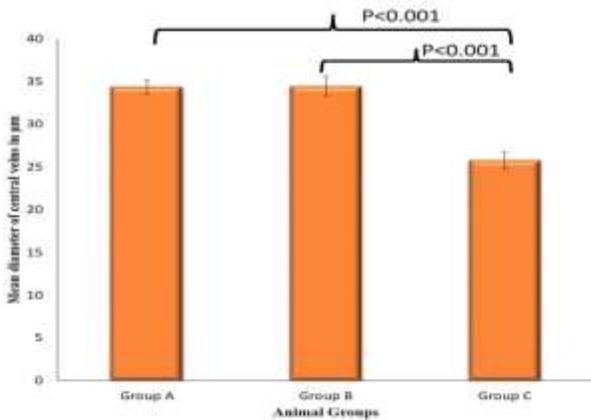
Figure 5: Comparison of central vein diameters among groups A, B and C. The overall significance was p<0.001.The error bars are showing ±SEM.
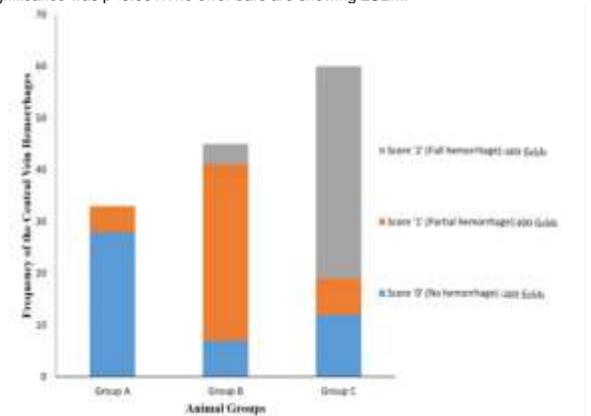

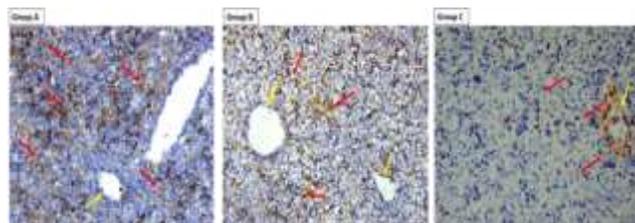Figure 6: Frequency distribution of central vein hemorrhages among the groups A, B and C.

Figure 7: Immunohistochemical expression of VEGFR2 in liver section from group A, B and C. Red arrows indicating theendothelial cells lining the sinusoids stained positive for VEGFR2 protein. Note that the expression is high in sinusoids of group A and B whereas in group C expression has been decreased markedly. Yellow arrows shows expression of VEGFR2 in the central veins which is more marked in group C. IHC 20X.
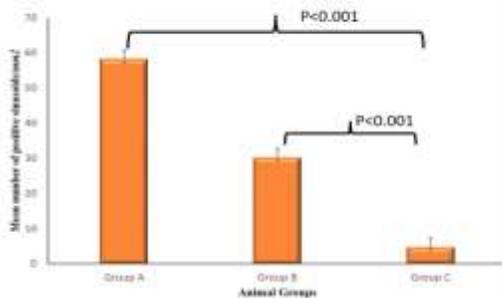

Figure 8: Comparison of Immunohistochemically stained positive sinusoids among the Groups A, B and C. The overall significance was p<0.001. The error bars are showing ±SEM.

Hepatic Vacuolar Degeneration: H & E stained sections of liver from each fetus were looked thoroughly for the presence of empty vacuoles in the hepatocytes which were designated as hepatic vacuolar degeneration (HVD) or steatosis. Slight steatosis was seen in groups A & B with mean scores of 0.1±0.3 and 0.2±0.4 respectively. It was significantly higher in group C with mean scores of 2.3±0.4 shown by Kruskal-Wallis test (p<0.001) (Figure 2 and 3)

Central Vein Diameter: Central vein diameter was seen to be decreased in Group C as compared to Group A and B (Figure 14). In group A it ranged from 32.62 to 36.03µm with a mean of 34.33±5.93 µm; in the group B it ranged from 32.16 to36.70 µm with a mean 34.43±7.55 µm of; in group C it ranged from 23.77 to 27.7264 µm with a mean of 25.75±6.57 µm. One-way ANOVA showed that difference was statistically significant (p<0.001). Post-Hoc Tukey tests revealed significant difference between groups A and C (p<0.001) and groups B and C (p <0.001) (Figure 4 & 5)

Central Veins with Hemorrhages: There was dense infiltration of RBC's in the central veins of treated groups B and C showing hemorrhages and congestion (Figure 5). Mean scores of central vein with hemorrhages in group A, B & C were 0.66±0.867, 1.04±0.515 and 1.91±0.288 respectively. According to Kruskal-Wallis test there was significant difference among the mean scores of the completely filled and partially filled central veins among the control and treated groups (p<0.001) (Figure 6).

Assessment of Immunohistochemical Expression

Effect of vitamin A on the expression of VEGFR2 was evaluated by thoroughly examining the immunohistochemically stained liver section of all the fetuses. The dose dependent loss of VEGFR2 expression was seen in the sinusoids of group B & C as compared to control group A. The mean number of positive sinusoids in Group A, B & C was 58.3±17.4, 31.4±16.8 and 4.8±3.9 respectively. ANOVA revealed that this difference was statistically significant (p<0.001). Post-Hoc Tukey showed significant difference between group A and C (p<0.001) and group B and C (p<0.001). Interestingly an increased expression of VEGFR2 in the central veins of group C was also observed whereas this expression in the central vein of groups A and B was low. However, this expression was found to be very weak in intensity in group C compared to Group A & B (Figure 7 & 8).

DISCUSSION

Vitamin A is being intensely investigated regarding its role in organogenesis and its teratogenic effects. Both the deficiency and excess of vitamin A can induce teratogenic effects(Suuberg, 2019) The current study was formulated to evaluate the outcomes of RDA and MTD of vitamin A on the histological features of livers of mice fetuses and to see the effect of these doses on immunohistochemical expression of VEGFR2.

The present study revealed that excessive doses of vitamin A produced inflammation and hepatic vacuolar degeneration (HVD) in the liver sections reflecting the evidence of liver injury. Our findings are in accordance with many of the previous studies where the chronic and excessive consumption of vitamin A caused hepatic steatosis and inflammation (Waheed, 2020). Vitamin A regulates release of inflammatory cytokines from hepatic stellate cells and hence is directly involved in hepatic inflammation(Haaker et al., 2020, Bourebaba and Marycz, 2021). These histopathological changes were attributed to the retinoids induced activation of leucocytes and TGFβ in hepatic cells(Haaker et al., 2020).

The histological evaluation of MTD treated group C revealed decrease in the mean diameter of central veins whereas the number of congestion and hemorrhage in the central veins increased significantly. This agrees with the previous study where prenatal exposure to vitamin A affected the histology of livers of fetuses by inducing central vein hemorrhages (Mehrotra and Shah 2004). Another study showed that diameter of central veins is altered to increase the metabolic activity of liver to excrete the

toxin from body during detoxification process (Salahshoor et al., 2018). Excessive consumption of vitamin A produces a thick barrier in between sinusoids and hepatocytes due to expansion of space of Disse caused by deposition of basement membrane like substance and proliferation of Ito cells(Sanz-García et al., 2021, Kamm and McCommis, 2022). The barrier can compress the sinusoids and cause hepatomegaly and portal hypertension even when there is no cirrhosis (Ezhilarasan, 2022). The reduction in the size of central veins and congestion are the manifestation of portal hypertension (Forouhar et al., 1984).

Retinoid affects vascular development in organs by altering the VEGF/VEGFRsignaling (Schmidt et al.,2012). Our observations of the immunohistochemical expression of VEGFR2 among the groups revealed that prenatal administration of vitamin A decreased the expression of this protein in the sinusoidal endothelial cells in group B and C as compared to group A and the reduction was more marked in group C. Previously it is shown that VEGFR2 is expressed in different levels among the endothelial cells of sinusoids and other great hepatic vessels and specifically liver sinusoidal endothelial cells (LSEC's) show strong expression (Sugiyama et al., 2010). Ding et al. (2010) manifested that LSEC show a distinct type of VEGFR2 positive endothelial cells and the hepatocyte proliferation is inhibited by genetic ablation of VEGFR2 in LSEC (Ding et al., 2010). In another study the results were contrary to this where the prenatal administration RA improved vascularization and increased the expression of VEGF, VEGFR1 and VEGFR2 in endothelial cells of lung (Schmidt et al., 2012). The probable mechanism behind the RA induced anti-angiogenesis effects by changing the expression of VEGF and its receptors may be due to inhibition of inducible nitric oxide synthase (iNOS) (Cho et al., 2005). Reduction of the VEGFR2 expression in the experimental groups explains the other histological findings of the liver in the current study. It has been previously observed that endothelial component of the liver is disrupted by VEGF depletion with signs of congestive hepatic vasculature and impaired lipoprotein uptake (Carpenter et al., 2005) which is also shown in our study. We, therefore, can assume that excessive consumption of vitamin A is linked with decreased expression of VEGFR2 in the sinusoids which in consequence could lead to alterations in the histological structure of liver.

Another very interesting observation of the current study was the increase in expression of VEGFR2 in the ECs lining the central veins of MTD group. There is limited data available in respect to the expression of VEGFR2 in the central veins and the possible effects of vitamin A on it. As vitamin A decreased expression of VEGFR2 in sinusoids which could lead to defects in developing vasculature (Kostallari and Shah, 2016). Functionally the area around the central veins comprise the zone 3 where oxygenation is poor (Katz, 1992) and problems in the developing vasculature due to toxicity of vitamin A further increase the risk of hypoxia in this zone. Hypoxia induces production of HIF-1α which stimulate VEGF/VGFR2 signaling in the hypoxic zone (Lee et al., 2004). Thus HIF-1α induced VEGF signaling may be the possible mechanism behind increased VEGFR2 expression in the central veins. However, more work is needed to further explore the possible mechanisms behind the expression of VEGF and its receptors in central veins

Strengths & limitations: This is the first ever experimental model which showed interaction between the vitamin A and VEGF receptor during the developmental process of the liver. Hence it could be a productive model that can be used further for investigation of the role of vitamin A and VEGF signaling in liver development and disease. However, our model could not fully explain the molecular mechanisms behind this interaction, so more work is need to be done at molecular level to fully understand the intricacy and specificity of this complex signaling pathway during the hepatic development.

CONCLUSION

Vitamin A consumption during pregnancy has shown to induce changes in the microarchitecture of liver and the immunohistochemical expression of VEGFR2. The excessive consumption of vitamin A reduced the expression of VEGFR2, which led to the increase in inflammation, hepatic vacuolar degeneration, and central veins hemorrhages along with the reduced diameter of central veins. Therefore the dosage of vitamin A should not exceed the recommended daily allowance during pregnancy.

Conflict of interest: Nothing to declare

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