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RESEARCH PAPER

TERATOGENIC EFFECT OF MATERNAL VITAMIN A CONSUMPTION ON THE LIVER, LIMBS AND OTHER MORPHOLOGICAL PARAMETERS OF THE PUPS OF WISTAR RATS

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ABSTRACT

Single doses of vitamin A administered at targeted gestation periods were observed to produce varying degrees of congenital malformations in the pups of rats. This study aims at evaluating the effect of high doses of vitamin A on the liver and gross structures of the pup of Wistar rats.20female rats were separated into 4 groups of 5 rats each. The rats were mated and given increasing doses of vitamin A-400,500and600mg/kg for groups B, C and D respectively for 14 days from day 1 of pregnancy while group A received only food and water after which the rats were allowed to litter. Physical examination was done on the pups using a magnifying hand lens on days 1,7and16 post- partum after which blood was collected. Serum analysis was done on blood samples to test for liver function enzymes AST, ALT and ALP and the livers were processed for histological studies. Results were computed using student T-test and reported as Mean ± Standard deviation at $P<0.05$.Histology of the liver showed no significant differences between groups B, C, D and the control, but results of the liver enzymes showed significant increase in serum ALP and AST which occurs in liver nephritis and is indicative of possible liver damage.

Keywords: Vitamin A, Teratogen, Liver enzymes, Morphological, Anti-oxidant.

INTRODUCTION

Vitamin A has been implicated as causing diverse kinds of birth defects in several studies including malformations of the limbs and the nervous system (Rutledge *et al*., 1994). This is an intriguing discovery considering the fact that there has been a lot of emphasis on Vitamin A consumption in recent years. Most of the common food additives like salt, sugar, vegetable oils etc. are specially fortified with Vitamin A and is prominently displayed on the packs having the diagram of an "eye with a big 'A' "inside it. More so, a lot of the common fruits consumed by the majority of the populace contain lots of vitamin A. Even the common table palm oil used is an important source of vitamin A. If this vitamin is as teratogenic as studies have shown, then somehow many people should have had malformed babies consequent to consumption of this vitamin. So, do we attribute the persistently high level of limb malformations reported by Vargession (2009) to ignorance in consumption of high doses of vitamin A? These among others were the question we set out to answer.

Vitamin A is the name of a group of fat-soluble retinoids, including retinol, retinal, retinoic acid, and retinyl esters (Institute of medicine, 2001). Vitamin A is involved in immune function, vision, reproduction, and cellular communication (U.S department of Agriculture, 2011). Vitamin A also supports cell growth and differentiation, playing a critical role in the normal formation and maintenance of the heart, lungs, kidneys, and other organs (Tanumihardjo, 2011). Common sources of vitamin A are food and dietary supplements. Two forms of vitamin A are available in the human diet: preformed vitamin A (retinol and its esterified form, retinyl ester) and provitamin A carotenoids (Otten *et al*., 2006; Tanumihardjo,

2011). Preformed vitamin A is found in foods from animal sources, including dairy products, fish, and meat (especially liver). By far the most important provitamin A carotenoid is beta-carotene; other provitamin A carotenoids are alpha-carotene and beta-cryptoxanthin. The body converts these plant pigments into vitamin A. It is also available as multivitamins and as a stand-alone supplement, often in the form of retinyl acetate or retinyl palmitate (U.S department of Agriculture, 2011).

The liver is the largest of the abdominal viscera, occupying a substantial portion of the upper abdominal cavity. It performs a wide range of metabolic activities necessary for homeostasis, nutrition and immune defense. It is composed largely of epithelial cells (hepatocytes), which are bathed in blood derived from the hepatic portal veins and hepatic arteries. There is continuous chemical exchange between the cells and the blood. Hepatocytes are also associated with an extensive system of minute canals, which form the biliary system into which products are secreted (Moore and Dalley, 2006). The liver is important in the removal and breakdown of toxic or potentially toxic materials from the blood. It regulates blood glucose and lipids, and plays a role in the storage of certain vitamins, iron, and other micronutrients as well as breaking down or modifying amino acids. It is involved in a plethora of other biochemical reactions. Since the liver is the primary site of detoxification and is generally the major site of intense metabolism, it is therefore prone to various disorders as a consequence of exposure to the toxins of extrinsic as well as intrinsic forms (Guyton and Hall, 2006).

It is also site of biotransformation in which a toxic compound is transformed to a less harmful form to reduce toxicity (Hodgson, 2004). However, these results in progressive damage to the liver cells and produce hepatotoxicity. Alanine transaminase (ALT) is an enzyme that helps metabolize protein. When the liver is damaged, ALT is increased in liver and released in the bloodstream. Aspartate transaminase (AST) is an enzyme plays a role in the metabolism of the amino acid alanine. An increase in AST levels may indicate liver damage or disease. Aspartate transaminase is the mitochondrial enzyme, predominantly found in the liver, skeletal muscles and kidneys. Alanine transaminase is a cytosolic enzyme, which is more specific for the liver than aspartate transaminase (Hodgson, 2004). Alanine aminotranferase (ALT) and aspartate aminotransferase (AST) are located in liver cells and leak out into the general circulation when liver cells are injured. These two enzymes were previously known as the SGPT (serum glutamic-pyruvic transaminase) and SGOT (serum glutaic-oxaloacetic transaminase) respectively. ALT and AST are present in highest concentrations in cells from the liver, heart, skeletal muscles, and red blood cells (Infante, 2008).

MATERIALS AND METHODS

Materials: Well-ventilated cages, saw-dust to be used as animal bedding, pipettes, syringes, sample bottles, haematology bottles, heparinized capillary tubes, cotton wool, methylated spirit, normal saline, light microscope, glass slides, Bouin's fluid, 10% formal saline, test tubes, beakers (500ml, 200ml, 100ml), distilled water. The animals were fed on a daily basis with rat chow and water throughout the period of the experiment. Vitamin A was obtained from CLARION VITAMIN A manufactured in India by SOFT GEL HEALTHCARE PRIVATE LIMITED survey No. 20/1, Vandalur Kelambakkamroad,pudupakkamvillage,kancheepuram District,Tanilnadu-603 103,India. It contains palmitate BP 200,000 IU per gel.

Animal subjects, grouping and treatment: Twenty (20) adult female rats weighing an average of 150g (3 months old at time of purchase) were randomly separated into 4 groups A, B, C and D. The individual rats were marked for identification and allowed to acclimatize for two (2) weeks (14 days). From the 15th day, group D began to receive 100mg/kg of vitamin E which they continued for 21 days. From day 22 (7 days after group D started receiving Vitamin E) the rats were introduced to males for mating. From the next morning (day 23) the rats were tested for pregnancy using the vaginal lavage and microscopic examination for the presence of spermatozoa according to the method of Maconde et al., (2004). The first day of pregnancy was counted as day 1. From day 1 of pregnancy the rats received their corresponding dosages of Vitamin A for 14 days uninterrupted. Group A was the control and took only food and water. Group B received 400mg/kg/day of vitamin A, group C received 500mg/kg/day of vitamin A and group D received 600mg/kg/day of vitamin A.Note that all groups received the vitamin A for 14 days, counting day 1 from the day they were confirmed pregnant.

Data / Sample collection and analysis: Physical observation of the limbs of the mice was carried out immediately after their delivery with the aid of a hand lens. Physical examination was also employed to verify other observable anomaly in the different groups. Blood samples were collected from 5 pups ineach group into ethylene diamine tetra acetic (EDTA) acid treated sample bottles for the determination of serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) using standard procedures (Reitman & Frankel, 1957). Blood for haemalological parameters were obtained from the retro-ocular plexus from the pups using heparinized capillary tubes. The serum

chemistry was carried out at the Veterinary Pathology Laboratory of University of Ibadan. These five pups also had their livers harvested and fixed for histological analysis with H&E. The variation in the different groups was compared using the control as reference. The variations between the different groups for liver enzymes were compared using the student T-test. ALT was determinedusing Randox ALT kit following the manufacturer's instruction according to the method of Reitman & Frankel, (1957). Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazine formed with 2,4-diphenylhydrazine according to the reaction below:

$$\alpha\text{-oxoglutarate} + \text{L- alanine} \xrightarrow{\text{GPT}} \text{L- glutarate} + \text{Pyruvate}$$

AST was assayed using Randox AST kit following the manufacturer's instruction according to the method of Reitman & Frankel, (1957). AST is measured by monitoring the concentration of oxaloacetate hydrazine formed with 2,4-dinitrophenylhydrazine as shown in the equation below;

$$\alpha\text{- oxoglutarate} + \text{L- aspartate} \xrightarrow{\text{GOT}} \text{L- glutamate} + \text{Oxaloacetate}$$

ALP was assayed using Randox AST kit following the manufacturer's instruction according to the method of Reitman & Frankel (1957).

$$\text{P-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{Phosphate} + \text{P-nitrophenol}$$

RESULTS

Limb and Craniofacial parameters: Gross morphological observations of the pups of all groups did not show observable anomalies as viewed by normal vision and hand lenses (*see table* 1).

Liver Function Tests (LFTs): There were significant differences in the serum level of the examined liver function enzymes – Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). All the experimental groups B, C and D showed significant increase in the level of serum AST compared to those of the control Group (A). There was a significant increase in serum levels of ALT in experimental groups C and D but not B compared to the control. The increase in serum ALP levels did not show any statistical significance except in experimental group C as compared to the control (*see Table* 2).

Histological Findings: As shown in the test plates (1B, C and D), there were no deviation from the normal cytoarchitecture of the liver when compared with features in slide 1A (control).

DISCUSSION

This study investigated if maternal vitamin A consumption will affect (and to what degree) the development liver and gross morphological features of the pups. The results showed that an overdose of vitamin A can be toxic, leading to threatening health conditions like spontaneous abortion, general body weakness, inactivity, ulcerations and death. These were the observation as the rats experienced these signs in a dose dependent manner (that is, the effects increased as the dose of consumption increased). This is in agreement with the report of Holder and Hill, (1991). All the rats in the experimental groups (A, B, C) lost their pregnancies and their level of activity decreased by the day. This was manifested as the rats became smallish as though shrunk, weak and waif. Food as well as water consumption reduced drastically and the rats showed several degrees of ulceration in their mouth as well as body regions. This is in agreement with the report from Ross et al., (2000) which showed that retinoid prescribed to treat skin disease used during pregnancy could cause spontaneous abortions and congenital malformations including limb defects. However, the control group remained healthy, and increased in size as gestational age increased. They also delivered their litters between 21 and 23 days of gestation.

All the deaths recorded took place between the 12th and 14th day of administration. After stopping the administration, it took the surviving rats between 26 to 30 days to bring forth their litters. Based on this report, calculating from the normal 21 days gestation of rats, it took the rats between 5 to 9 days of discontinuation of the drug to reconceive.

Table 1: Effects of maternal Vitamin A consumption on the gross morphological features of the pups of Wistar rats.

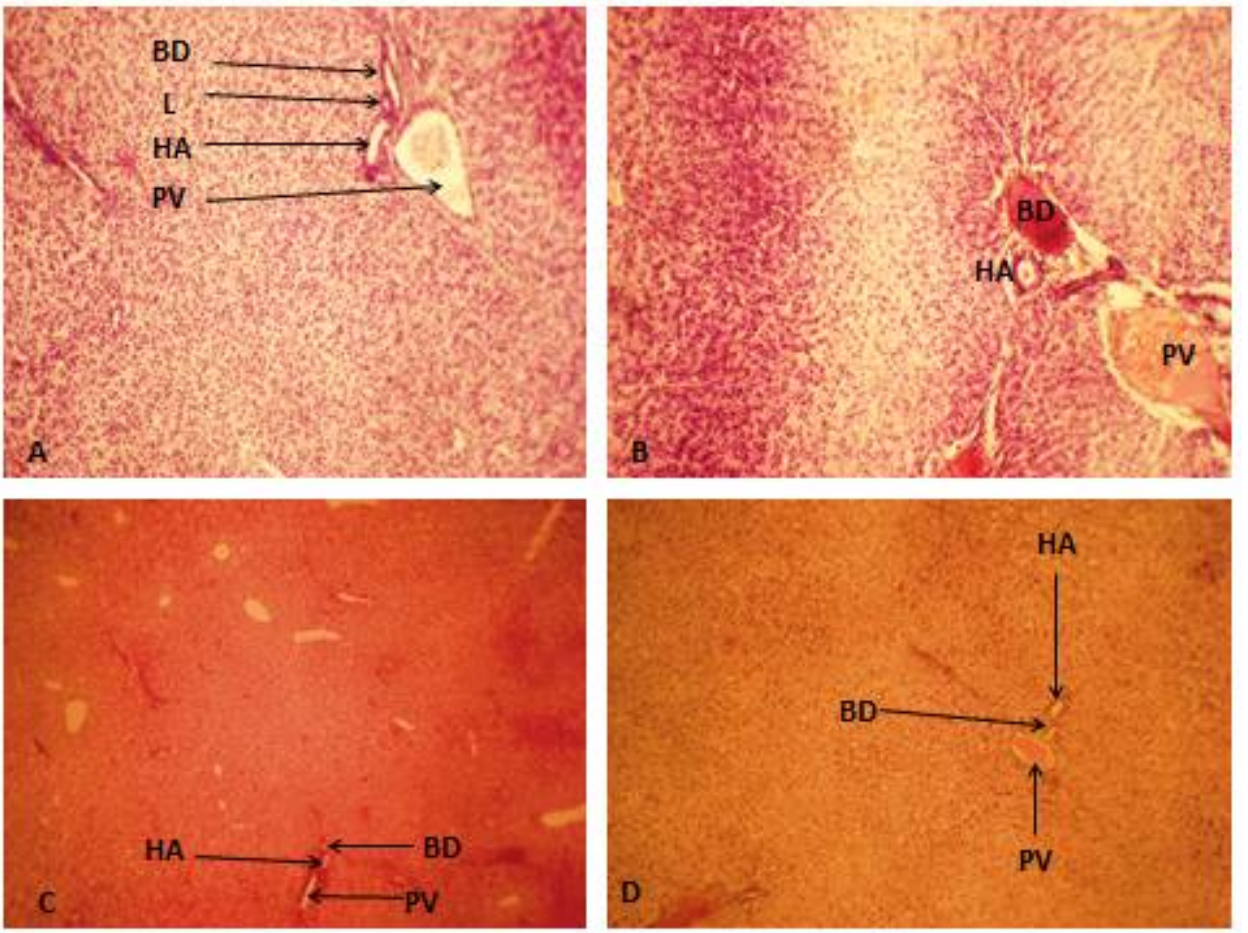
Defect	Group A Days			Group B Days			Group C Days			Group D Days		
	1	7	16	1	7	16	1	7	16	1	7	16
Upper Limb Defects												
Polydactyly	-	-	-	-	-	-	-	-	-	-	-	-
Pre-axial	-	-	-	-	-	-	-	-	-	-	-	-
Post-axial	-	-	-	-	-	-	-	-	-	-	-	-
Syndactyly	-	-	-	-	-	-	-	-	-	-	-	-
Amelia	-	-	-	-	-	-	-	-	-	-	-	-
Meromelia	-	-	-	-	-	-	-	-	-	-	-	-
Synostosis	-	-	-	-	-	-	-	-	-	-	-	-
Hemimelia	-	-	-	-	-	-	-	-	-	-	-	-
Ecrodactyly	-	-	-	-	-	-	-	-	-	-	-	-
Adactyly	-	-	-	-	-	-	-	-	-	-	-	-
Brachydactyly	-	-	-	-	-	-	-	-	-	-	-	-
Lower Limb Defects												
Polydactyly	-	-	-	-	-	-	-	-	-	-	-	-
pre-axial	-	-	-	-	-	-	-	-	-	-	-	-
post-axial	-	-	-	-	-	-	-	-	-	-	-	-
Syndactyly	-	-	-	-	-	-	-	-	-	-	-	-
Amelia	-	-	-	-	-	-	-	-	-	-	-	-
Meromelia	-	-	-	-	-	-	-	-	-	-	-	-
Synostosis	-	-	-	-	-	-	-	-	-	-	-	-
Hemimelia	-	-	-	-	-	-	-	-	-	-	-	-
Ecrodactyly	-	-	-	-	-	-	-	-	-	-	-	-
Adactyly	-	-	-	-	-	-	-	-	-	-	-	-
Brachydactyly	-	-	-	-	-	-	-	-	-	-	-	-
Craniofacial Defects												
Eye defects	-	-	-	-	-	-	-	-	-	-	-	-
Facial defects	-	-	-	-	-	-	--	-	-	-	-	-
Cranial defects	-	-	-	-	-	-	-	-	-	-	-	-
Exencephaly	-	-	-	-	-	-	-	-	-	-	-	-
Spina bifida	-	-	-	-	-	-	-	-	-	-	-	-
Abdominal wall Defects	-	-	-	-	-	-	-	-	-	-	-	-

Keys: √ =Present; **-** = absent

Table 2: Effects of maternal Vitamin A consumption on the Liver enzymes of the pups of Wistar rats.

GROUP	ALT	AST	ALP
A	40.8±1.53	26.33±1.53	100.67±8.08
B	45±4.36*	27.5±2.38	107.67±8.08
C	42.75±0.50*	31.33±1.15*	122±5*
D	42±1.83*	30.5±1.91*	108.5±8.66

Values are expressed as mean ± standard deviation of 5 animals in each group.* Statistical significance at $p< 0.05$.



Plates 1A, B, C and D: Photomicrographs of liver of rats (H&E x240) showing hepatic artery (HA) with thick walls and smaller lumen compared to the pulmonary vein (PV) having thinner walls with wider lumen. A small lymphatic vessel (L) is visible. The lumen of the bile duct (BD) is smaller and the wall is not as thick as the HA.

Histological analysis carried out on the liver did not reveal significant differences in the histology of the liver in the experimental groups compared to those of the control. However, the result of the liver function tests (LFTs) presented in Table 2 shows that there were significant differences in the values of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) for the pups from the experimental groups A (400mg/kg), B (500mg/kg) and C (600mg/kg of vitamin A and 500mg/kg of vitamin E) compared with those of the control. Liver enzymes ALT and AST are present in high concentrations in the normal hepatocytes. These enzymes however, leak out into the circulation when hepatocytes or their cell membranes are damaged as reported by Charles (2012). Palmer (2004) also reported that ALT is a more specific indicator for liver damage compared to AST as elevations of ALT lasts longer than those of AST. Our results show elevated levels of ALT across the experimental groups compared to the control. Increase in ALT is a specific characteristic of hepatocellular necrosis, which ultimately leads to hepatic damage as reported by Charles (2012).

It appears from this report that the alterations seen in these pups were at the molecular level which could not be revealed by light microscopy. This is glaring, as the significant differences seen in the biochemical studies were not obvious under the light microscope. Gross morphological examination was carried out on all the pups on days 1,7 and 16 to ensure that all observable organs have attained functional status. The result of gross morphological examination done on all the litters of the experimental groups as well as the control to examine limb deformities, craniofacial and any other physically observable anomalies shows that there was neither any limb abnormality nor any observable craniofacial or other gross

morphological anomalies seen. This is contrary to the results of Rutledge et al., (1994) that reported limb abnormalities and limb duplications in mice after receiving single doses of Vitamin A -68mg/kg, 53mg/kg and 41mg/kg on days 4.5,5 and 5.5 post-fertilization respectively. Our result is also contrary to this same report which shows that mice which received 21.5mg/kg and 15mg/kg on days 6 and 7 post-fertilization respectively had craniofacial abnormalities. The variations in these reports could be due to the fact that these were targeted administration that took effect on particular days of development in which the observed features were supposed to develop. Also, our work was carried out on Wistar rats and not mice and the administered doses are different. It is obvious from the report of these studies that the mere fact that a new born does not show any physical anomalies at birth does not mean that all is well. Apart from phenotypic manifestations of abnormalities, there are possibilities of underlying metabolic as well as biochemical anomalies which may still manifest later. To this end, women of child bearing age must be very careful in taking medications and this should only be by the prescription of a qualified medical practitioner.

CONCLUSION

Vitamin A is very important for optimum body and developmental functions. High doses of vitamin A are very toxic and can lead to the manifestation of various kinds of toxicity symptoms, including death. Women of child bearing age who consume high doses of vitamin A are likely to lose the pregnancy and when they don't, the baby may come down with various kinds of abnormalities including limb abnormalities as well as central nervous system defects. However, to remove these effects, the best solution is to first discontinue the administration. This report has shown that in rats recovery may be attained within 5 to 9 days. This work also shows that the damaging effect of over-dose of vitamin A increases as the days of consumption increases. No rat died from days 1 to 11 of receiving the over-dose of vitamin A (even up to the highest doses), although other signs of toxicity were already present, including increased inactivity, loss of appetite for food and water and spontaneous abortion. This implies that probably none of the rats would have died if we stopped administration of vitamin A at day 10. Days 13 and 14 were the most critical and at day 14 we thought all the rats will die before the next morning. This further buttresses the fact that withdrawal is an essential first step in terminating the toxic effects of overdose of vitamin A. However, this does not in any way mean that high doses can be consumed for few days during pregnancy without any effect. Death may not occur, but damage may be done to the offspring. Previous works by Rutledge et al., (1994) has shown that consumption of doses as low as a single dose of 12.5mg/kg produced birth defects including exencephaly, eye defects as well as facial anomalies. Therefore, women of child bearing age must not take vitamin A (or any other drug) in excess of the recommended dose.

RECOMMENDATION

We did not investigate the tissues and different maternal parameters to ascertain the level of damage done to them and the rate of recovery recorded with time. This is a fresh ground for further research. We observed that biochemical studies revealed better details which were not appreciated with light microscopy. Therefore, further studies can be done, utilizing electron microscopy.

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AUTHORS CONTRIBUTIONS

The corresponding author (Aguwa U.S.) did the administration and laboratory analysis with the assistance of Ukoba O. Olu S.I assisted with data analysis and the work was supervised and given professional criticism by Olatunde O.