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VITAMIN A INDUCED TERATOGENICITY OF HEART IN ALBINO MICE

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ABSTRACT

The current study was designed to determine in vivo the embryotoxic potential of retinoic acid during organogenic period of mouse embryo. Retinoic acid (60 mg/kg), was administered orally to pregnant mice on 7th, 8th and 9th days of gestation. Animals were sacrificed on the 8th day. Fetuses, whose dams had received retinoic acid showed, growth retardation and cardiac malformations. The decrease in mean fetal weight and their CRL was statistically significant. Effects on the heart included enlargement and change in its shape; the myocardium showed myofibrillar disarray, apoptosis and hypoplastic compact zone. Retinoic acid, which is an effective therapy for cystic acne and other dermatological disorders, is highly teratogenic during the period of organogenesis and therefore, should be avoided in early pregnancy.

Key words: *Vitamin A, Teratogen, Heart.*

INTRODUCTION

Vitamin A is essential for life, yet its influence is particularly critical during periods when cells are proliferating and differentiating, such as during pregnancy and early childhood.[1] A complicated set of inter related processes are involved in tissue organization from blastomere to final development and differentiation.[2] Retinoic acid is an active metabolite of vitamin A, which exists in two forms Trans (Trans- RA) and Cis retinoic acid (Cis- RA).[3]

Trans-RA and Cis-RA bind to the cellular retinoic acid-binding proteins (CRABPs) in the cytoplasm and are transported to the nucleus of the cell. Within the nucleus, RA binds to retinoic acid receptor proteins. Binding of Trans-RA and Cis-RA to ratinoid acid receptors (RAR) and retinoic acid X receptors (RXR) respectively allows the complex to regulate the rate of gene transcription thereby, influencing the synthesis of certain proteins used throughout the body.[4, 5] Both excess and deficiency of vitamin A are known to have similar patterns of birth defects.[6,7] Embryonic exposure to retinoic acid causes a wide spectrum of severe malformations in the offspring of humans and other animals like rodents and chickens.[8] These birth defects are termed retinoic acid embryopathy (RAE) and consist of craniofacial, cardiac, thymic and neural tube defects.[3]

The developmental stage of the embryo reflects critical time period for specific malformations. Administration of RA on day 9 of gestation is reported to induce dysmorphogenesis of the inner ear in mice.[8] Abnormalities of appendicular skeleton were induced when RA was administered to pregnant mice on day 11 of gestation.[9]

A lot of work has been done on teratogenic effects of retinoic acid on nervous system and other organs of the body but there is hardly any experimental work on the effect of RA on the developing heart; the present study was, therefore, designed to investigate teratogenic effect of RA on heart, using mouse as an experimental model.

MATERIALS AND METHODS

Sixteen albino mice (Twelve females and four males) 6-8 weeks old, weighing 25-30 gm were procured from the National Institute of Health, Islamabad. All the animals were examined thoroughly and weighed before the commencement of the experiment. The mice were housed in the Experimental Research Laboratory of University of Health Sciences, Lahore under controlled conditions of temperature, (22±0.5°C), humidity (50 ± 10%) and light and dark cycles of 12 hours each. The animals were fed on standard mouse diet and water ad libitum. Mice were randomly divided into two groups each containing eight animals, six female and two males; the animals were put in a cage with a ratio of 1 male to 3 females mice. Mice were left overnight for mating, the pregnancy was confirmed the following morning by the presence of vaginal plug and this was considered as gestational day 0 (zero).[1, 10]

Group A served as a control which received 0.1ml of olive oil orally on 7th, 8th and 9th days of pregnancy. Animals of group B, received orally 60 mg/kg/day of retinoic acid dissolved in 0.1ml of olive oil on 7th, 8th and 9th days of pregnancy. Pregnant mice were sacrificed and dissected on the 18th day of gestation to obtain the fetuses. The fetuses

were killed under anaesthesia and dissected using dissecting stereo microscope before their hearts were removed.

Each of heart was fixed in 10% formalin for 48 hours; the tissues were processed for routine histology in a usual way using automatic tissue processor and were dehydrated through graded series of ethanol, cleared in xylol, infiltrated with molten paraffinwax and embedded in it to make blocks using Leuckhart's brass moulds. Blocks were trimmed to the size, keeping their upper and lower edges parallel, before sectioning. Longitudinal sections of heart, 5µ thick, were obtained using Leica rotatory microtome (RM 2125); the sections were then transferred to water bath at 55°C, the folds of section were removed using fine pointed probe or camelhair brush; these were then placed on albumenised glass slides and dried on the slide warmer. The sections were stained using Haematoxylin and Eosin and were examined under light microscope (Leica DM 1000).

Micrometry

The sections of ventricular wall were randomly selected from every slide and selected from 6 different places wall thickness of left and right ventricles[11] and the heart size were calculated after calibrating the ocular with the stage linear micrometer.

Statistical Analysis

The data was entered an analysed using SPSS 15.0. Mean ± SD is given for quantitative variables. Frequencies and percentages are given for quailitative variables. Two independent sample t test was applied to observe group mean differences. Fishere exact test was applied to associations between qualitative variables. The difference was considered statistically significant if the 'P' value was < 0.05.

Table 2: *Comparison of various parameters of fetuses from groups A and B.*

Parameter	Group A Mean S.D	Group B Mean S.D	p-value
CRL	2.79 ± 0.02	23 ± 0.04	0.000*
Weight of fetuses	1.3 ± 0.02	1.49 ± 0.01	0.000*
Heart weight	11.1 ± 0.1	12.7 ± 0.2	0.000*
Heart size	1691 ± 21	1899± 80	0.000*
Lt. ventricular wall thickness	361 ± .6.6	274 ± 10	0.000*

* p value < 0.05 is statistically significant.

RESULTS

Litter size

The litter size in the control group A was 50; it was 41 in group B. The comparison of dead and abnormal fetuses between group A & B was found to be statistically significant (p<0.05; Table 1).

Gross appearance

The heart appeared ellipsoid ("rugby ball" shape) and was situated on the left side in all fetuses of the group A, whereas it appeared globular in group B with no dextrocardia. The increase in the weight of the organ in group B compared with the group A was found to be statistically significant (p<0.05; Table 2). In some fetuses from group B, the heart size was discernibly reduced, possible due to structural defects and malformation of myocardium.

Table 1: *Effects of retinoic acid on pregnancy outcome:*

Parameter	Group (A) n (50)	Group (B) n(41)	P value
Dead Fetuses	00	11[a]	0.001*
Abnormal fetuses	00	10[a]	0.022*
Inter ventricular Septal defects	00	9	0.000*

Figures in parenthesis indicate total number of fetuses.
* p value < 0.05 is statistically significant.

Histological Features:

The longitudinal section through the heart of a normal embryo at 18 day post coitum (dpc) showed aorta with its valves emerging from left ventricle, separated from the right by inter-ventricular septum; it also showed sectional profile of both the atria (Fig. 1). The ventricular wall consisted of three layers: (1) epicardium; (2) myocardium; and (3) endocardium.

The epicardium was smooth and consisted of a single layer of mesothelial cells resting on basement membrane and a thin layer of connective tissue on the outer surface of the heart. The blood vessels supplying the heart were also evident in the epicardium.

The epicardium, in group B, lost its smooth texture, and became uneven and undulating in most of the sections. There was epicardial detachment with an increase in sub-epicardial space (Fig.2).

The size of the ventricles increased in the treated group; their measurements are given in Table 2. The increase in the

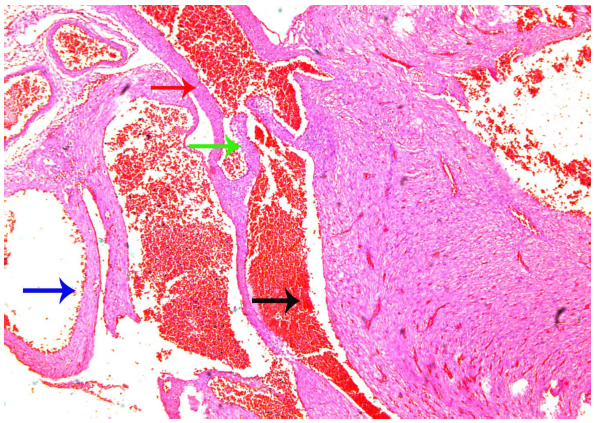

Fig. 1: Photomicrograph of longitudinal section of the heart taken from the fetus of a control group; it shows aorta (red arrow) emerging from left ventricle, aortic valve (green arrow), ventricular cavity is filled with RBCs (black arrow) and left atrium (blue arrow). H & E stain X 50.

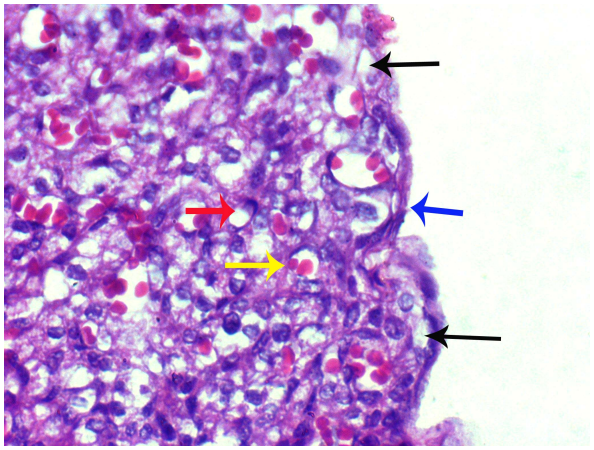

Fig. 2: Photomicrograph of fetal heart from group B: Ventricular myocardium shows detached epicardium with sub-epicardial spaces (black arrows) resulting in rough epicardium (blue arrow); it is also exhibiting large intercellular spaces and vacuoles (red arrow) with large number of vessels of variable size (Yellow arrow). H & E stain X 400.

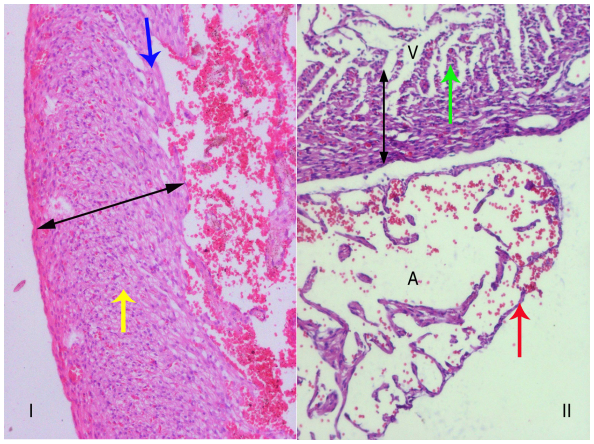

Fig. 3: (I); Photomicrograph of fetal heart from group A: ventricle showing compact myocardium (yellow arrow); the trabeculae (blue arrow) springing from the myocardium are evident and are apparently responsible to increase its thickness (black arrow).
(II); Photomicrograph of fetal heart from group B: showing atrium (A) flanking ventriclular wall (V) there is marked loss of compaction (green arrow) resulting in thinning of myocardium (red arrow). H & E stain X 100.

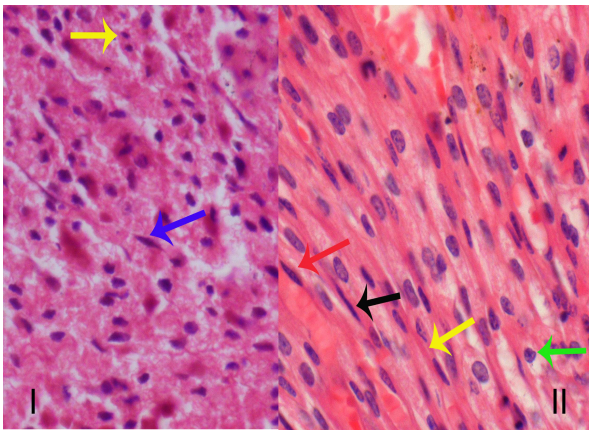

Fig. 4: (I) Photomicrograph of fetal heart from group B; showing cardiac muscle cells with pyknotic (yellow arrow) crescentic nuclei (blue arrow); branching pattern is not discernable. H & E stain X 400.
(II) Photomicrograph of longitudinal section of fetal heart from group A showing cardiac muscle; Myocardial cells appear to branch and intermingle to form a meshwork (Yellow arrow), most of the nuclei are oval in shape and centrally located with perinuclear clearing (green arrow). It also shows interspersed spindle shaped nuclei of fibroblasts (black arrow). H & E stain X 400).

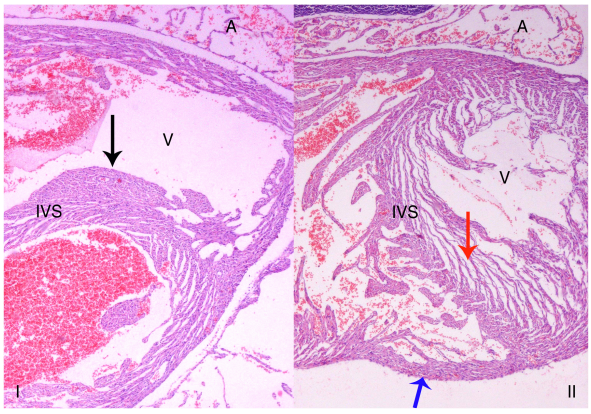

Fig. 5.1: Photomicrographs from group B, (I) showing atrium (A), ventricle (V); (I); ventricular septal defect is seen in the membranous part of IVS (black arrow);
(II); fetal heart, globular in shape (blue arrow) and ventricular septum show non-compaction of myocardium (red arrow). H & E stain X 40.

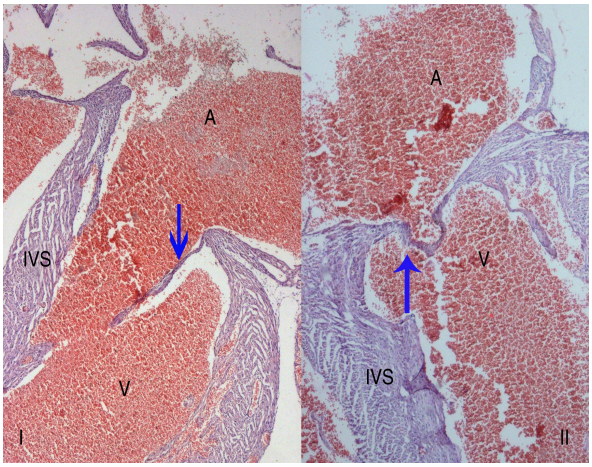

Fig. 6: *Photomicrograph of fetal hearts from group B, showing atrium (A), ventricular cavity (V) and atrio-ventricular valves; (I) there is thinning of its leaflets (blue arrow). (II) The valve with fused leaflets (blue arrow). H & E stain X 40.*

size of the ventricles in group B compared with that in group A, was statistically significant ($p< 0.05$). Mice in group B also showed a marked but variable decrease in wall thickness of both ventricles when compared separately with the control (Table 2).

Myocardium

The fetal heart from the group A showed well formed multilayered compact ventricular myocardium; the trabeculae springing from it further added to myocardial thickness (Fig. 3.1). In group B, the myocardium was thin apparently on account of loss of process of compaction (Fig.3.2).

Examination of Haematoxylin and Eosin stained sections revealed shreds of developing, ill formed myocardium with multiple confluent loci of degenerated cells accompanied with severe myofibril disarray. These changes were observed consistently through out the right and left ventricles of group B (Fig. 3.II).

The control group had normal well-organized myocardial architecture. Cardiac muscle fibers were characteristically long, cylindrical with one or two centrally located nuclei, possessing well stained nucleoli (Fig.4.II).

The myocardium in group B showed multifocal myocardial apoptosis in various stages; its myocytes exhibited multiple nuclei with great variation in size showing degenerative changes. The nuclei showed dense chromatin indicative of pyknosis; in other myocardial fibers, they were crescentic in shape and fragmented (Fig.4.I).

Septum

In group B, fetuses showed malformations of inter ventricular septum (IVS) (Fig.5), which were more pronounced in the upper part (membranous part) of the septum.

Atria

Fetal heart from group B, showed grossly dilated atria with few cardiomyocytes in their walls. There was significant reduction in the atrial wall thickness and at places endocardium apposed epicardium.

Valves

The cardiac valves showed a central core of dense fibrous connective tissue and lined on both sides by endothelial layers. The AV-valves of fetal heart from group B showed various malformations like, thinning and fusion of the leaflets (Fig.6).

DISCUSSION

Retinoids, powerful modulators of cell proliferation, differentiation and apoptosis, are widely used for various dermatologic diseases including psoriasis and acne.[12] Retinoids are found to be highly teratogenic in humans even in therapeutic range of dosage i.e. 0.5 to 1.5 mg/kg/day.[13] Some teratogenic effects of retinoids may be due to their ability to induce apoptosis.[8] Migrating cranial neural crest cells show high affinity for RA and are necessary for normal septation of the heart and development of the cardiac valves.[13]

Cardiovascular system is first to develop and is essential for providing nutrients to the developing embryo. In mice it begins on 7th day of gestation with the migration of precardiac cells from epiblast lying lateral to primitive streak.[14] This was the basis for determining 7th, 8th and 9th day of gestation for retinoic acid treatment. Moreover, Padamanabhan and Ahmed pointed out that most striking fetal teratogenic effect of RA depends on its concentration on 8th day of pregnancy.[15] It is also stated that mouse embryos begin to synthesize RA from retinol at approximately 7.5dpc. This increased susceptibility of heart as well as other derivatives of neural crest may be correlated with high levels of CRABP which act as a moderator of RA fluctuations, in regions where cranial neural crest cells are known to migrate.[1,7,16,17]

Micrometry of fetal hearts in histological sections were carried out at the level of its maximum transverse diameter below atria; it revealed cardiac enlargement since globular form of the heart (Fig. 4) could result from its biventricular enlargement; this was presumably the cause leading to heart failure and increased mortality of the fetuses in the experimental group. Similar changes were observed by Emily Dyson[18] in an experimental animal model using mice in which receptors for retinoic acid (RXR) were blocked. The results were produced on account of deficiency of RA which apparently contradicted our observations; however, it had been reported in the past that both excess and deficiency of RA produced comparable effects.[19-21]

On 18th day of gestation the fetuses of the control group showed well developed four chambered heart with fully differentiated cardiomyocytes and regular branching pattern. In group B, there was thinning of myocardium on account of failure in expansion of the compact zone. Our findings are consistent with previous studies in which RXR α - gene knockout mice showed severe cardiac muscle defects with the deficiency in expansion of compact zone.[12] Myocardial fibers in group B also showed scanty cytoplasm, pyknotic and fragmented nuclei in developing myocardium, indicative of apoptosis; this could possibly be explained on basis of teratogenic effects of retinoids and their ability to induce apoptosis. RA- induced

cell death with the characteristics of apoptosis has been observed during blastocyst studies by Huang et al (2003). They found apoptosis in the mouse blastocysts in vitro when transiently exposed to excess RA.[23]

In our study histological analysis of embryos in group B, showed malformations of inter-ventricular septum, it was present in 9 out of 41 fetuses. The VSDs were exclusively present in the upper part (prospective membranous part) of the septum, which is neural crest derivative. The role of cardiac neural crest cells in heart development was given by Waldo (1999), who found disorganized myofibrils in cardiac neural crest ablated chick embryos[24]. similar to our finding in RA treated group. Our work indicating signs of abnormal myocardial, septal and valvular development and normal endocardium could imply that cardiac neural crest cells failed to migrate; therefore, its influence on normal differentiation and function of the myocardium was lacking. This corroborates similar findings reported earlier that the neural crest cells influence the cardiac remodeling, normal septation and the development of the cardiac valves.[24]

Two principal foci for cell death are in the cushions of out flow tract and AV regions.[25,26] In our study atrioventricular valves also showed thinning which was presumably due to apoptosis of cells; there was, however, no change in the endocardium, implying that retinoic acid is a proapoptotic agent leading to excessive cellular apoptosis followed by thinning of valves. These findings support those reported earlier indicating decrease in number of mesenchymal cells in endocardial cushion in developing heart of mice after giving retinoic acid.[27]

It is, therefore, **concluded** that RA induced cardiac malformations might be on account of changes in genetic regulation of cardiac development or on account of direct deleterious effect of RA on the cardiac morphogenesis; additional study is required to pinpoint the factors responsible for cardiac malformations after RA treatment.

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