ORIGINAL ARTICLE

Quantitative volumetric analysis of a retinoic acid induced hypoplastic model of chick thymus, using Image-J

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Abstract

Objective: To assess the total volume change in a retinoic acid-induced, hypoplastic model of a chick thymus using Image-J.

Methods: This experimental study was carried out at the anatomy department of College of Physicians and Surgeons, Islamabad, Pakistan, from February 2009 to February 2010, and comprised fertilised chicken eggs. The eggs were divided into experimental group A and control group C. Group A was injected with 0.3µg of retinoic acid via yolk sac to induce a defective model of a thymus with hypoplasia. The chicks were sacrificed on embryonic day 15 and at hatching. The thymus of each animal was processed, serially sectioned and stained. The total area of each section of thymus was calculated using Image-J. This total area was summed and multiplied with the thickness of each section to obtain volume.

Results: Of the 120 eggs, there were 60(50%) in each group. Image analysis revealed a highly significant decrease in the volume of the chick thymus in the experimental group A than its matched control at the time of hatching (p=0.001). Moreover, volumetric depletion progressed with time, being substantially pronounced at hatching compared to the embryonic stage.

Conclusion: The volume changes were significant and were effectively quantified using Image-J.

Keywords: Image-J, Volumetric, Chick thymus, Hypoplasia. (JPMA 67: 1357; 2017)

Introduction

The thymus is derived from the third pharyngeal pouch and develops under the inductive effect of neural crest cells.[1] Defective development of the thymus is one of the main and consistent features encountered in congenital syndromes, including retinoic acid induced embryopathy.[2] Retinoic acid (RA) is a potent teratogen which primarily targets normal neural crest cell migration, affecting the development of neural crest derived structures.[3] Impaired neural crest contribution leads to the deranged development of the thymus gland in a manner similar to that observed in congenital conditions, such as DiGeorge syndrome.[2,4] This defect predominantly manifests itself in the form of thymic hypoplasia or aplasia,[4] therefore, the total volume of thymus can serve as a key parameter to assess structural deformation. The chick embryo, being an oviparous organism, is expected to respond directly to an external intervention like RA. Moreover, the chick thymus in particular has been described to be similar to that of mammals;[5] therefore, any effects of experimental intervention may well be applicable to humans.

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Although chick thymus has been a popular model for developmental and toxicological studies, with a number of scientists characterising its cellular population,[6,7] data defining total volume is scarce. Image-J is a popular java-based image processing and analysis programme that was developed at the National Institutes of Health by Rasband.[8] It provides an effective means to calculate the area of a user's defined image selections, which can then be used to determine the exact volume. It holds a unique position because it not only is in the public domain but also runs on any computer system. Due to this ease of utility and availability, Image-J has been extensively employed for research purposes.[9,10] The current study was designed to assess the efficacy of Image-J as a useful volume analysing tool. The total volume of the chick thymus was determined using Image-J and then compared with the volume of an experimentally created, retinoic acid-induced, hypoplastic model of the chick thymus.

Materials and Methods

This experimental study was carried out at the Department of Anatomy, Regional Centre, College of Physicians and Surgeons, Islamabad, Pakistan, from February 2009 to February 2010, and comprised fertilised, Egyptian Fayoumi chicken eggs. The eggs were randomly selected and were counted and marked with numbers starting from 001 to the total count of the eggs. Using

random number tables, the specimens were randomly allocated to the experimental group A and the control group C. Defective model of chick thymus was induced by injecting 0.3µm in 0.05ml[11] RA (Sigma-R2625) in group A. matched control was sham-injected with saline. The groups were further subdivided. The subgroups differed by the time of their sacrifice. Eggs in subgroups A1 and C1 were to be opened on day 15 of incubation. Subgroups A2 and C2 were opened at hatching or day 22 (whichever was earlier). After a chick was fixed en bloc, lobes of its thymus were exposed and removed along with the surrounding connective tissue (Figure-1). All the lobes from one animal were processed together in a semi-permeable packet until they were embedded. Using a microtome, transverse serial sections were cut at 10µm with 5 sections cut 7µm thick after every 20 sections. All the sections were sequentially spread on glass slides and stained with haematoxylin and eosin (H&E) for routine histology. Additionally, 7µm thick sections were also stained with Mallory's trichrome and Gomori's stain for reticular fibres. Approximate number of slides per animal belonging to groups A1 and C1 was 13 or 14. For group A2 there were 25 or 26 slides while for group C2 the number of slides per animal was 26 to 29. After the slides were stained, photographs were taken, at an objective magnification of 4x. An eyepiece fitted with an ocular micrometer, was used to capture the photographs. The image of the scale was thus visibly superimposed on the image of the section. Photographs of all the individual sections were captured, uploaded and then opened in Image-J. A line parallel to the scale superimposed on the picture was drawn. Total divisions covered by this line on the scale were computed and converted into micrometers. A scale was set in the programme by filling the precise calibrated distance thus measured. By using the option of 'free hand' tool, contours of all the individual sections of thymus were defined manually, thus eliminating the surrounding connective tissue (Figure-2). The number of pixels embodied within the outlined contours on each section was determined automatically by the software and the cross-sectional area of the thymus was procured on a section-by-section basis. The value of the demarcated areas for all the sections belonging to complete thymus from one animal were computed and compounded by the already known thickness of the section, giving total volume of the thymus.

The statistical comparison of differences among groups was evaluated by Student's t-test. $P<0.05$ was considered significant.

Results

Of the 120 eggs, there were 30(25%) in each subgroup, i.e. A1, A2, C1 and C2. The volumetric quantification of the thymus showed that although the RA-exposed group A1 20(20.83%) had a slightly lower volume than the sham-injected control group C1 30(25%) ($p=0.823$).

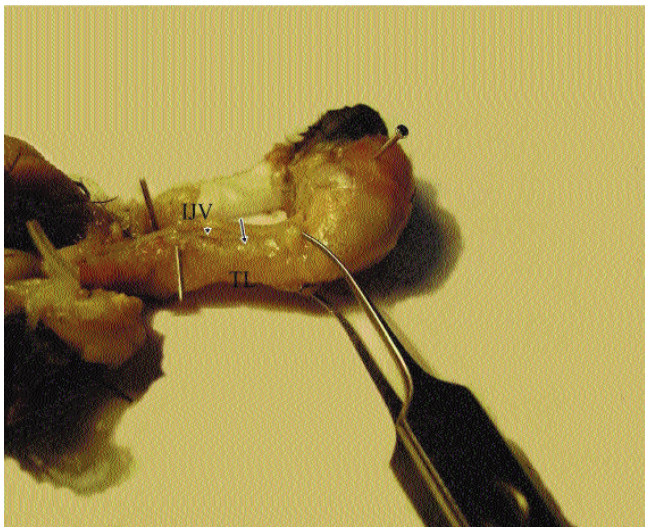

Figure-1: Photograph showing chain of the thymic lobes (arrow), next to internal jugular vein (arrow head) in a 21 day old chick.

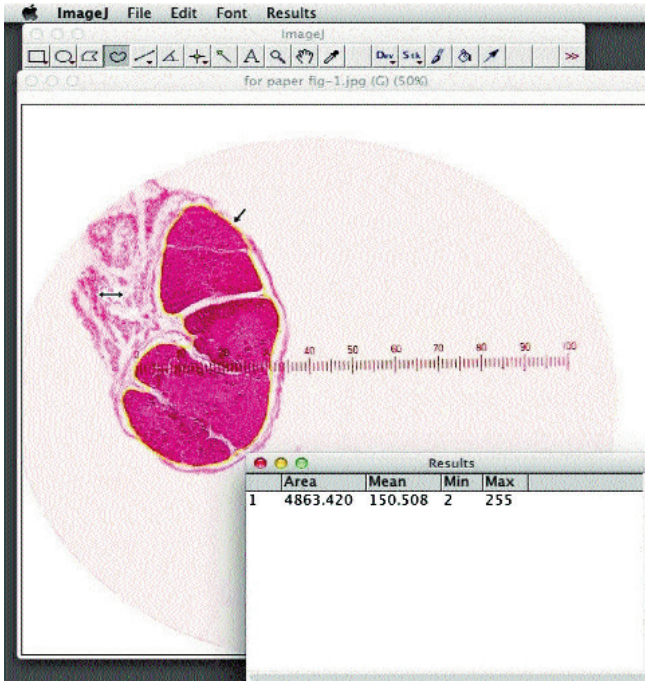

Figure-2: Screenshot of the analysis process, using Image-J.
Image of a section of thymus opened in Image-J showing the area to be measured; manually outlined (arrow) using the free hand tool. Connective tissue (double headed arrow) was excluded from the measured area. Dialogue window on the right shows the calculated area in mm^2.

Compared to the results of embryonic groups, the fully

Table: Comparison of the experimental group A and control C regarding the volume.

Stage	Parameter	GP	N	MEAN±SD	P-Value
ED-15	Volume of Thymus (mm^3)	A1	25	0.5168±0.004	0.82
		C1	30	0.5380±0.003	
Hatched	Volume of Thymus (mm^3)	A2	24	0.8938±0.022	0.001*
		C2	30	1.4407±0.038	

N: Number of chicks
GP: Group
SD: Standard deviation.

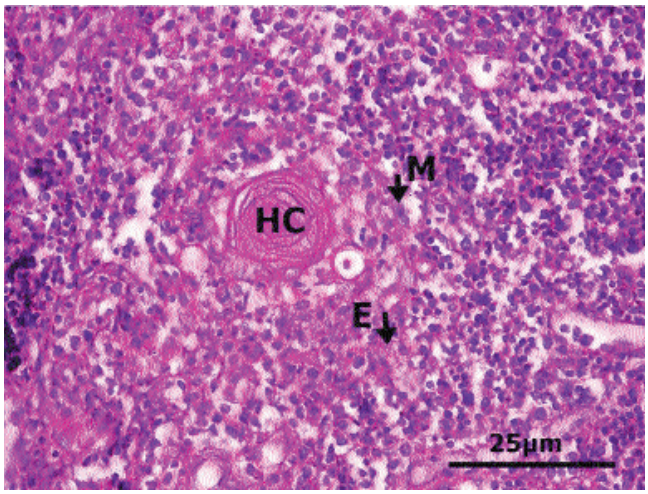

Figure-3: Photomicrograph of thymus belonging to control group C2, showing well developed Hassall's corpuscles (HC), myoid cells (M) and epithelioreticular cells (E). Haematoxylin-eosin staining. Scale bar=25µ.

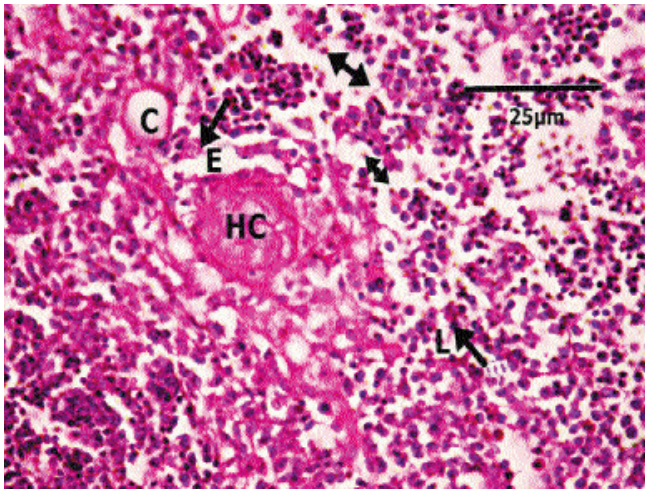

Figure-4: Photomicrograph of thymus, belonging to experimental group A2, with areas of atrophy marked by double headed arrows. Hassall's corpuscles (HC), cyst (C) and epithelioreticular cells (E) can be seen.
Haematoxylin-eosin staining. Scale bar=25µ.

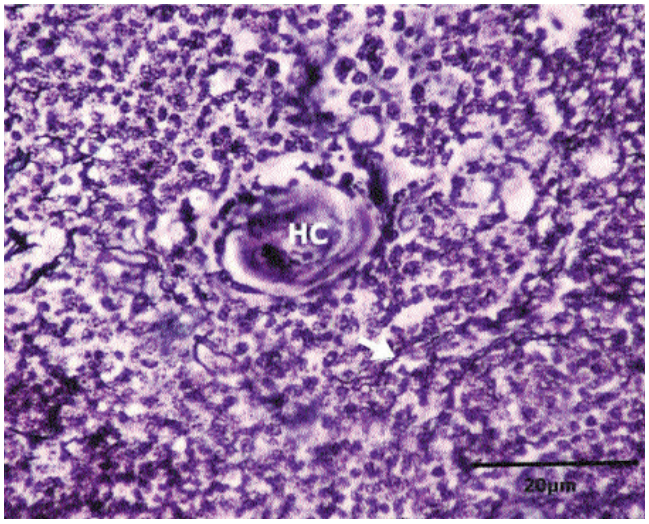

Figure-5: Photomicrograph of thymus from control group C2, showing normal structure with well developed Hassall's corpuscles (HC).
Stained by Gomori's method for reticular fibres. Scale bar = 25µ.

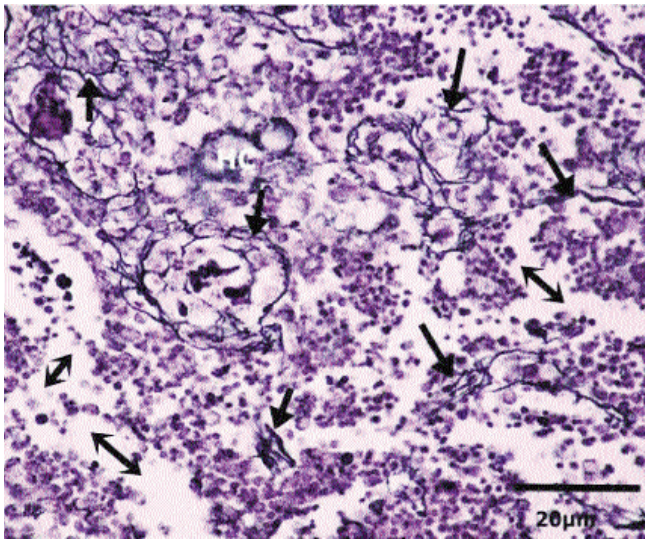

Figure-6: Photomicrograph of thymus belonging to experimental group A2, showing areas of cellular hypoplasia, marked by arrows.
Stained by Gomori's method for reticular fibres.
Scale bar = 25µ.

hatched groups displayed significant difference in their volume. The RA-exposed experimental group A2 had considerably lower volume, i.e. in 24(20%) eggs, than the age-matched control group C2 30(25%) ($p<0.001$) (Table).

The difference in volume was complemented by histological changes. This was evident in the form of areas of cellular depletion visible on photomicrographs of thymus belonging to the group A2 compared to the

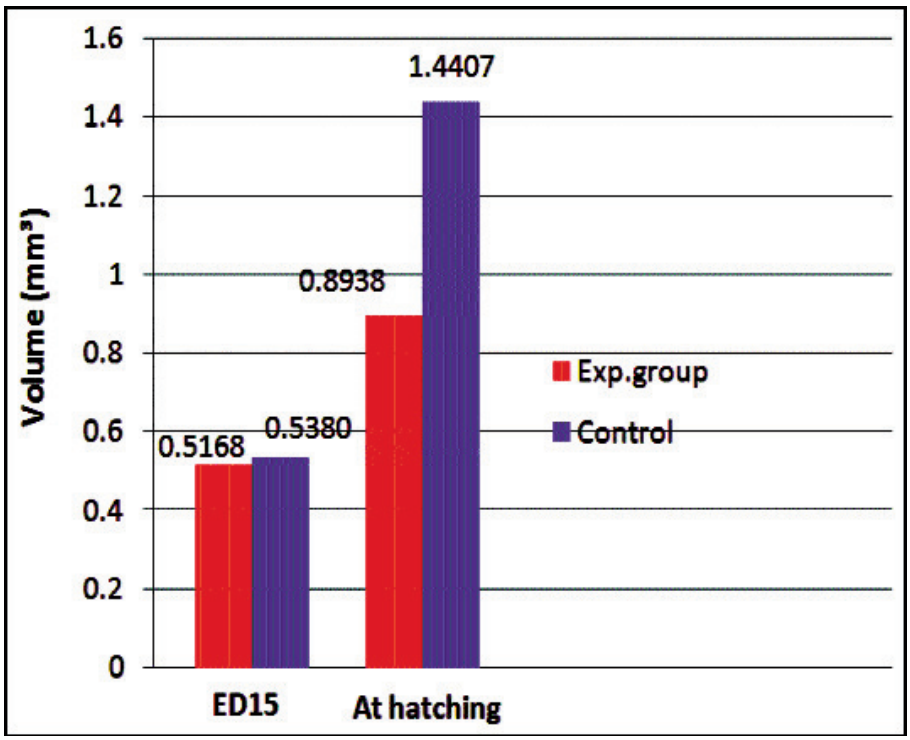

Figure-7: Volumetric comparison of the experimental and control groups.

photomicrographs from group C2 (Figures-3-6). Volumetric depletion progressed with time, being substantially pronounced at hatching compared to the embryonic stage.

Discussion

A constant target of neural crest cell anomalies,[12] the chick thymus has remained a subject of interest for decades.[13,14] Despite being intensively researched, volumetric numerical data on the chick thymus is lacking. Since developmental defects predominantly manifest themselves as hypoplasia and atrophy,[15] volume assessment should be a key parameter to assess such damage.

In this study the total volume change induced by the teratogenic affect of retinoic acid was computed. A defective model of the chick thymus was created by administering RA, which disrupted the signalling molecular cascade involving endothelin-1, essential for normal development of branchial arches.[16] Using Image-J, a simple method was formulated to calculate the volumetric deficiency induced by RA. Our results delineate that the volume of the thymus belonging to the RA administered group, A2 was significantly less than the matched control C2, at hatching ($p<0.001$). These results are in agreement with the previous studies. Scientists have repeatedly shown that the teratogenic potential of retinoic acid is by disrupting the signalling molecular cascade, which is essential for normal development of branchial arches.[15-17] Resulting apoptosis and growth failure of the branchial arches disrupt development of all its derivatives including the thymus.[2] Moreover, RA induces its teratogenesis, via multiple pathways. These include mediation by retinoic acid receptors (RARs) and retinoid X receptors, as well as by increase in the Hox gene expression.[18] Various studies suggest that even small changes in the signalling pattern during early embryogenesis could produce permanent and irreversible effects.[19]

The results also demonstrate that the defective development progressed with time, from a slight difference of volume at embryonic stage ($p=0.823$) to a statistically significant difference at hatching ($p<0.001$). Thus the retinoic acid teratogenesis intensified with increasing age of the embryo (Figure-7).

In this study, using Image-J, a simple method was proposed to accurately calculate and compare total volume, in a control, sham injected and a retinoic acid-induced defective model of chick thymus. Image-J was found to be an accurate, cost-effective and efficient means to analyse volume and it served as an adequate tool to compute the volume differences. Despite being a freeware, it is an objective, reproducible, cost-effective and time-saving method to quantify the volume.

Conclusion

Retinoic acid teratogenicity led to significantly decreased volume of the chick thymus. Image-J can be used as a convenient tool to quantify the volumetric deficiency.

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Conflict of Interest: None.

Source of Funding: None.

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