THE TERATOGENIC EFFECTS OF RETINOIC ACID ON EPITHELIORETICULAR CELLS AND HASSALL'S CORPUSCLES IN THE MEDULLA OF THE CHICK THYMUS

Ayesha Haque, Muhammad Yunus Khan*

Universidad Westhill, Santa Fe Cuajimalpa México, *College of Physicians and Surgeons Pakistan (CPSP) Islamabad Pakistan

ABSTRACT

Objective: To examine the effects of prenatal administration of retinoic acid on the proliferation of epithelioreticular cells and Hassall's corpuscles in the medulla of chick thymus.

Study Design: Experimental study.

Place and Duration of Study: Anatomy Department, College of Physicians and Surgeons Pakistan (CPSP) Regional centre, Islamabad, from February 2009 to February 2010.

Materials and Methods: A total of 120, fertilized chicken eggs were divided into an experimental group A, and its control group B. Group A was injected with retinoic acid via yolk sac on day zero of incubation. Group B was sham injected with saline. Subgroup A1 and B1 were incubated till embryonic day 15 and subgroups A2 and B2 were incubated till hatching. The number of epithelioreticular cells and Hassall's corpuscles were counted. Diameter of Hassall's corpuscles was measured. The parameters were compared statistically between the experimental and control groups.

Results: The number of epithelioreticular cells was significantly less than the control group at embryonic stage (p=0.009) as well as at hatching (p=0.001). The number of Hassall's corpuscles was not different between the embryonic groups but was considerably more than the control in the experimental group at hatching (p=0.01). The diameter of the Hassall's corpuscles belonging to the experimental groups was significantly different than the age matched control groups at the embryonic stage (p=0.004) as well as at hatching (p=0.008).

Conclusion: Prenatal exposure to retinoic acid significantly reduced the number of epithelioreticular cells and increased the size and the number of the Hassall's corpuscles.

Keywords: Cell proliferation, Epithelial cells, Retinoic acid, Thymus.

INTRODUCTION

Thymus is a primary lymphoid organ derived from the third pharyngeal pouch under the inductive effect of neural crest cells. The interaction between the endoderm of the third pharyngeal pouch and neural crest derived mesenchyme from the third and fourth pharyngeal pouches is crucial to thymic development[1]. Responsible to generate T lymphocytes, thymus is critical for the immune system. The lymphopoiesis in thymus is complex and requires intricate interactions of thymocytes with specialized thymic microenvironment[2]. The composite structure of thymic microenvironment is a three dimensional network mainly composed of epithelioreticular cells (ERC's) interspersed with non-epithelial cells, and divided into a cortex and medulla[3]. The ERC's present in the cortex and medulla arise from the endoderm and possess distinct specialized functions. Cortical ERC's acting as the antigen presenting cells are involved with the positive selection of thymocytes while the medullary ERC's, through negative selection establish a self tolerant repertoire of T cells[4].

Retinoic acid (RA) is a known teratogen with over the counter availability and wide medical applications[5]. Because of its continued use, teratogenic potential of RA, remains a constant threat[6]. The teratogenecity of RA is manifested primarily by arresting the migration of cranial neural crest cells (CNCC), to their destined tissues, including to the pharyngeal arches. Since CNCC contributes to thymus organogenesis[7], it

Correspondence: Dr Ayesha Haque, House # 255, St # 62, I-8/3 Islamabad Pakistan (*Email: windoc786@yahoo.com)*
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is one of the constant targets of retinoic embryopathy[8]. Thymus has been a subject of intense research, and a number of scientists have focused on its involvement in neurocristopathies and the role of the signalling molecules involved in mesenchymal-lymphocytic interaction in thymic organogenesis is well described[9,10]. However knowledge regarding the detrimental effects of defective CNCC migration on thymic microenvironment is limited. Since medullary ERC's constitute a crucial subset of this microenvironment and are indispensable for central T cells tolerance[11-13], any deleterious effects on their development can have serious implications on immune system.

Keeping all this in view, our study was designed to investigate the teratogenic impact of retinoic acid on the quantity of ERC's in the medulla of chick thymus. A number of studies suggest that ERC's contribute to the formation of Hassall's corpuscles[14], therefore any effect on ERC'S might affect Hassall's corpuscles. To investigate this possibility, the number and length of Hassall's corpuscles was also determined.

Avian embryos are suitable experimental animals due to con-cording data between chick and mammals[15] therefore any effect manifested, can well be expected in humans.

MATERIAL AND METHODS

This experimental study was undertaken from February 2009 to February 2010, at the Department of Anatomy, College of Physicians and Surgeons of Pakistan, Regional centre, Islamabad. Freshly laid fertilized eggs, belonging to "Egyptian Fayoumi" breed of Gallus domesticus were collected from the "Poultry Research Institute", Punjab, Rawalpindi. Eggs which were cracked or stored for more than 02 days were excluded from the study. To divide them into groups, eggs were randomly selected. For this purpose, after rapid swabbing with 70% alcohol, eggs were marked with numbers, starting from 001 to the total count of 120 eggs. Using random number tables, the specimens were allocated to the experimental group A and the control group B. A volume of 50µl containing 0.3µl of RA (Sigma) in saline was administered via yolk sac to the group A on day 0 of incubation[16]. To inject, two apertures were created by gentle scraping of the shell by a sterile mini saw, until membrane was visible. First aperture was created on the surface of the wider pole of egg above the airspace to allow the air to escape. Second aperture was scraped 1.5 cm, above the lower pole to inject the drug through intact membrane. Same procedure was used to sham inject the control group B with same volume of saline and the apertures were sealed with melted wax. The eggs were further subdivided into four subgroups depending on the day of harvesting. Subgroups A1 and its control B1, were incubated till the embryonic day (ED)[15] and subgroups A2 and B2 were incubated till the day of hatching or day 22 of incubation (which ever was earlier). The day of placement of eggs in the incubator was considered as day 0. Dead embryos or chicks were excluded at the time of sampling.

Embryos (ED-15) and chicks (hatched) were harvested and fixed in 10% neutral buffered formalin solution for 24 hours. After fixation, using a hand lens, lobes of thymus were removed along with the surrounding connective tissue. All lobes belonging to one animal were processed together in a sealed permeable packet till embedding. Transverse sections were cut at 7µm using a rotary microtome and stained for histological analysis.

Hassall's corpuscles were identified by their characteristic swirly appearance. Their number was counted at the magnification of 40x in three randomly selected different fields of the same section using grid.

Similarly the diameter of Hassall's corpuscles was measured along its long axis through 100x objective in three randomly selected different fields. Micro-metric measurement was done using an ocular micrometer fitted in the

eyepiece. This ocular-micrometer was already calibrated using a stage micrometer.

Epithelioreticular cells were recognized by their lighter staining and bigger nuclei. Number of the nuclei of epithelioreticular cells was counted in the medulla of thymus under oil immersion lens using grid in a unit area at three randomly selected fields of a section. The arithmetic means of these measurements were calculated.

Statistical Analysis

The data were analysed using Statistical Package for Social Sciences (SPSS) version 10. Independent samples t-test was used to detect any significant difference between the experimental and control groups by comparing mean numbers of epithelioreticular cells and the size and number of Hassall's corpuscles in the medulla of thymus. A *p*-value of <0.05 were considered significant.

RESULTS

Hundred and twenty eggs were divided into an experimental and a control groups, which were further subdivided into embryonic and hatched subgroups each comprising 30 eggs. Due to teratogenic effect of retinoic acid, five out of thirty embryos belonging to the subgroup A1, and six embryos belonging to the experimental subgroup A2 did not survive. In the embryonic experimental subgroup, A1 the number of ERC's in medulla/unit area was significantly less than the age matched control group B1 (*p*=0.009). Although, no considerable difference was encountered between the experimental and control groups regarding the number of Hassall's corpuscles, their diameter was considerably larger in the group exposed to retinoic acid (*p*=0.004) (table).

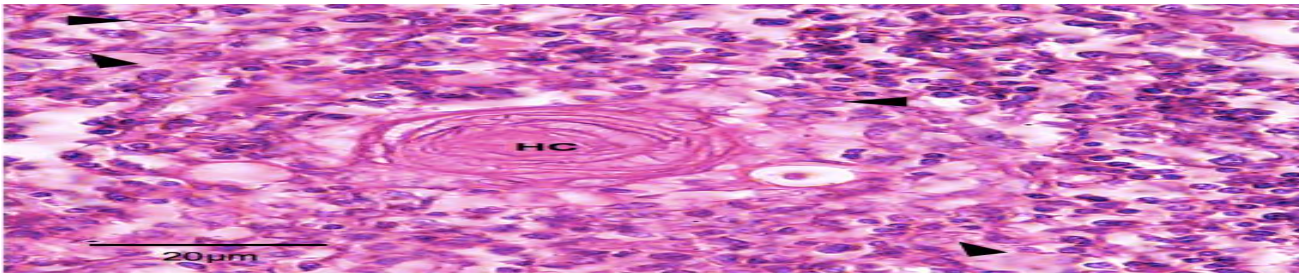

Figure-1: Photomicrograph of Thymic section from the control group (B2) showing normally developed, pale staining, epithelioreticular cells (arrow heads) and Hassall's corpuscles (HC). Stained by haemotoxylin and eosin.

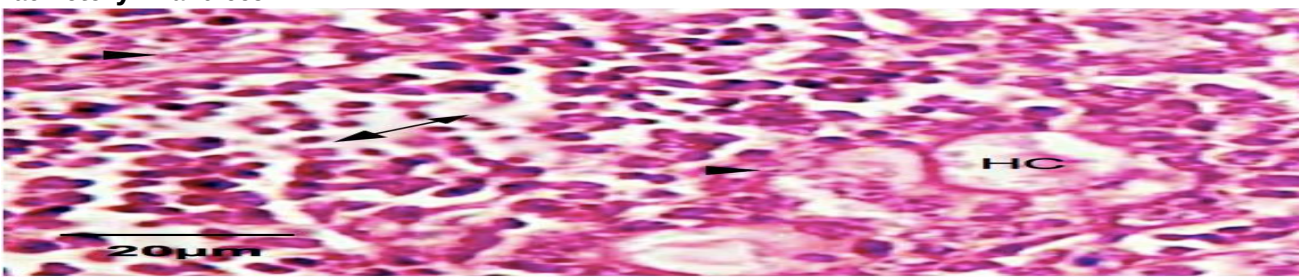

Figure-2: Photomicrograph of Thymic section from the retinoic acid exposed experimental group (A2) showing epithelioreticular cells (arrow heads), areas of cellular atrophy (double headed arrow) and Hassall's corpuscles (HC). Stained by haemotoxylin and eosin.

Regarding the fully hatched group, there was significant reduction in the number of ERC's in the medulla/ unit area in the retinoic acid exposed experimental subgroup A2, as compared to the age matched control group B2. The length and the size of Hassall's corpuscles were also significantly different in the experimental subgroup A2 and control subgroup B2 (table-I).

Histologically the thymic section belonging to the experimental group A2, showed cellular

atrophy (fig-II) as compared to the section belonging to the control B2 (fig-1) EPC's were larger with prominent nuclei and stained lightly eosinophilic. The Hassall's corpuscles showed typical swirly appearance and stained strongly eosinophilic (fig-1 & 2).

DISCUSSION

The functional role of neural crest derived mesenchyme in early development of thymus is indisputable and it has been shown that extirpation of neural crest derived mesenchyme leads to a deranged and a hypoplastic thymus[17]. In the present study, significant reduction of the mesenchyme by providing FGF's, which induce the expansion of thymic epithelial cells in thymus[21]. Since mRNA for FGF-7 and FGF-10 are predominantly found in neural crest derived mesenchyme[21], any neurocristopathy will directly affect their expression[19].

In our study we developed a retinoic acid induced, neural crest defective model of chick thymus. Since the neural crest migration and subsequent infiltration of pharyngeal mesenchyme was arrested, probably, less than optimum levels FGF-7 and FGF-10 failed to induce the proliferation of ERC's in thymus.

Table: Comparison between the experimental and control groups regarding the number of ERC's, number and diameter of Hassall's corpuscles.

Parameter	Subgroup		*p*-value	Subgroup		*p*-value
	A1 n=25	B1 n=30		A2 n=24	B2 n=30	
	Mean ± SE	Mean ± SE		Mean ± SE	Mean ± SE	
Number of ERC's in medulla / UA	9.16 ± 0.21	9.93 ± 0.20	0.009*	57.08 ± 0.57	61.90 ± 0.52	<0.001*
Number of Hassall's corpuscles / UA	1.000 ± 0.00	1.000 ± 0.00		3.0417 ± 0.09	2.666 ± 0.11	0.01*
Diameter of Hassall's corpuscles (µm)	7.461 ± 0.05	7.071 ± 0.01	0.004*	17.500 ± 0.25	16.58 ± 0.22	0.008*

Significant, n=Number of specimens, UA=Unit area, SE=Standard error of the mean.

epithelial cellular proliferation was encountered at embryonic stage as well as at hatching. These findings are in accordance to and reinforces previously documented work[18,19].

The probable mechanism for the reduced number of ERC's is through inhibition of the expression of fibroblast growth factors in neural crest cells. It has been suggested by a number of studies that mesenchymal expression of fibroblast growth factors (FGF) is essential for mitogenic activity in ERC's. These fibroblast growth factors conduct mainly as nutritional factors and promote the proliferation of ERC's[20]. Therefore, lack of FGF-7 and FGF-10 has consistently led to retarded epithelial growth in thymus[19]. Scientists describe functional role of neural crest derived

Additionally, ERC's are also dependent on interaction between the developing lymphocytes and thymic epithelium[3]. Since in our study retinoic acid teratogenesis led to failure of lymphocytic proliferation, their deficient interaction with epithelial cells might have aggravated the defect.

There was an increase in the diameter as well as the number of Hassall's corpuscles in the retinoic acid exposed experimental subgroups. Previous studies by a number of scientists have established that ERC's contribute to the formation of Hassall's bodies[22]. Moreover, the increase in size of the corpuscles represent involution and is regarded as an evidence of degeneration of thymus[14]. Additionally, atrophy of thymic

medulla has been linked with infiltration of Hassall's corpuscles by a variety of other cells[23]. In the present study, similar mechanism might be responsible for these changes. Significant reduction in number of ERC's constituting Hassall's bodies might have been compensated by a variety of other cells infiltrating and leading to increase in the size of the corpuscles. Furthermore, origin of the cells constituting Hassall's corpuscles has been challenged previously[24] and as a future prospective, it would be interesting to elucidate the cellular lineage and multicellular population of this dynamic and unique component of thymic microenvironment[14].

CONCLUSION

Prenatal exposure to retinoic acid significantly reduced the number of epithelioreticular cells and increased the size and the number of the Hassall's corpuscles.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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