DOI: https://doi.org/10.53350/pjmhs2023171160

ORIGINAL ARTICLE

Differentiation of Sclera in Chick Embryo at Various Stages of its Development- A Histological Study

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ABSTRACT

Background: The sclera is the outer strong opaque covering of the eye that functions as protective layer and provides site for attachment of extra ocular muscles.
Aim: To study the microscopic anatomy of sclera in chick at various stages of embryological development keeping in mind the practical application of this information in understanding human development and disease processes.
Methodology: It was an experimental study. The study was carried out at department of Anatomy, CPSP Regional Centre, Islamabad. Eyeballs were collected from group A, B and C (n=30 each) on day 10, 12 and 15 of incubation respectively. Dissected out tissues were processed and stained with Hematoxylin & Eosin and Alizarin red. The prepared specimens for group A and C were analysed under light microscope while the specimens from group B were observed via stereomicroscope. Data was evaluated by using SPSS version 23. Independent t-test was applied with p-value of less than 0.05 was considered significant.
Results: As the chondrocytes in cartilaginous layer of sclera became mature, they change their shape from flattened to more rounded cells. The proteoglycan content in the cartilaginous matrix increased. The mean number of chondrocytes increased from 17.4 ±1.4/unit area on day 10 to 10.0 ±0.8/unit area on day 15. The mean thickness of cartilaginous layer increased from 44 ±2.4µm on day 10 to 77 ±8.3 µm on day 15 of development. Calcification of scleral ossicles became obvious at about 12 days of incubation when seen under stereomicroscope.
Practical Implication: This study highlighted that understanding for the development of scleral elements (understudied tissue of the eye) can be critical in research involving autoimmune diseases and neural crest defects. Considering similar developmental stages among humans as in chick embryo.
Conclusion: It was concluded that growth of sclera in eye of chick has been indicated by increase in thickness of its layers and increase in the number of chondrocytes/unit area and progressing calcification of ossicles in its bony part.
Keywords: Chick Embryo, Chondrocytes, Hyaline Cartilage and Ossicular Ring.

INTRODUCTION

The sclera is the outer strong opaque covering of the eye that functions as protective layer and provides site for attachment of extra ocular muscles. In fish, reptiles and birds, this part of eye is also reinforced by cartilage or bone[1]. However, in humans, the sclera lacks the bones.

The sclera in humans is not a cartilaginous tissue, but it shows chondrogenic potential. The cartilage-associated genes have been expressed on cultured human infant scleral cells[2]. Additionally, a connection between cartilage diseases and sclera issues has been discovered. Rheumatoid arthritis, an autoimmune disease that causes swollen joints and scleral inflammation, or scleritis, frequently targets both of these structures[3]. It is also interesting to note that cartilage formation can be induced by stress and during disease processes[4] as evident in mice sclera when they were exposed to stress[5].

Chick has been described as an excellent model for ocular research and has been used for a number of studies that led biomedical developments. Sclera in birds presents unique features like bones and cartilages encircling the sclera. Ordinarily, these elements are missing in humans however, under certain pathological conditions like cancer or following trauma, atavistic ossification can occur in mammalian sclera[4].

Although sclera of chickens differs from mammals in the presence of skeletal elements, interventional studies could contribute to understand the disease processes regarding cartilage development in humans[4,6].

An important cell population, the neural crest cells, contributes in the developmental process of the scleral cartilage and ossicles[7]. The scleral ring develops through neural crest induced epithelial-mesenchymal interactions.

Received on 13-08-2022
Accepted on 24-12-2022

Neural crest cell population is a focus of many studies due to their potential to induce congenital anomalies. Employing chick sclera as an experimental model can enhance our understanding of this dynamic population of migratory cells.

Chick has been used as an experimental model extensively because of easy availability and rapid development. Moreover, eyes of the chick are quite big, and this provides the advantage to collect large amount of tissue with ease[6].

Although considerable research has been conducted on many tissues of the eye including cornea, lens and retina, studies examining sclera are deficient. In fact, the literature suggested that it is one of the most understudied tissue of the eye.[8] Understanding the development of these scleral elements can be critical in research involving autoimmune diseases and neural crest defects.

The objective of the study was that the microscopic anatomy of sclera in chick at various stages of embryological development keeping in mind the practical application of this information in understanding human development and disease processes.

METHODOLOGY

This study was experimental and was conducted at the Department of Anatomy, College of Physicians and Surgeons, Islamabad. Fertilized eggs of "Egyptian Fayoumi" breed of *Gallus domesticus* were provided by Poultry Research Institute, Rawalpindi. After excluding eggs with any visible cracks or deformities, a total of 90 eggs were randomly assigned to three groups A, B, C and placed in an incubator. The day of placing the egg in the incubator was considered as day 1 of development.[9] Embryos from groups A, B and C were allowed to develop for 10, 12 and 15 days respectively. Incubation was carried out under standard conditions of temperature (38±0.5^{0}C) and humidity (60-70%)[9]. Eggs were rotated half turn twice daily to distribute nutrition

to the embryo adequately and this also prevents formation of adhesions between developing embryo and egg shell membrane[10].

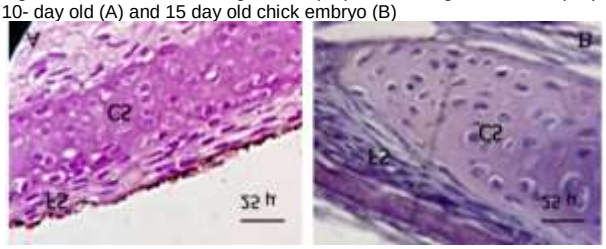
On the days designated for sacrifice, eggs were removed from the incubator, the embryos were carefully removed after the eggshell had been broken, and they were fixed in 10% neutral buffered formalin for 24 hours. The anterior half of the eyes from group A and group C embryos were then processed, sectioned, stained, and examined under a light microscope after the eyeballs had been removed. Using light green and alcian blue, the amount of proteoglycans in cartilaginous sclera was measured. With the help of Alizarin red, the sclera's ossicles could be seen. In HandE-stained paraffin sections, the morphology of the various scleral layers was investigated and the thickness of the layers was determined. In both age groups, it was counted and compared how many chondrocytes there were per unit of surface area. Scleral ossicles from 12-day-old group B embryos' anterior halves were stained with Alizarin red as a whole, and they were examined under a stereomicroscope.

Statistical analysis: Data will be entered and analyzed in SPSS version 23.0. Mean ± SD was calculated for quantitative variables like thickness of cartilaginous sclera and number of chondrocytes. Independent t-test was applied with p-value of less than 0.05 was considered significant.

RESULTS

The histological features of the chick embryo at day 10 of development showed that the sclera comprised of a fibrous layer outside lined by a cartilaginous layer from inside. The outer fibrous layer was thin and mainly was constituted by tightly packed collagen fibers arranged longitudinally (Figure-1). The histology of the sclera at day 15 of development shows advancing characteristics in form of visibly larger chondrocytes with clearly marked territoreal and interterritoreal matrix. The nuclei of central chondrocytes were seen to be rounded as compared to those at the periphery of the cartilage at this stage of development also (Fig.1-B).

Figure-1. H&E stain showing fibrous (FS) and cartilaginous sclera (CS) in a 10- day old (A) and 15 day old chick embryo (B)



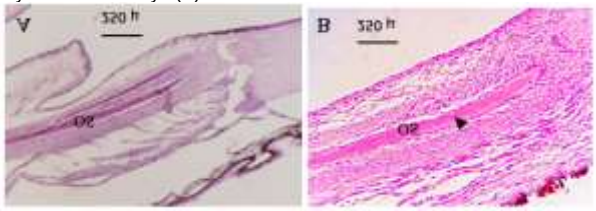
The inner layer made up of hyaline cartilage was thicker and was populated by chondrocytes present inside the lacunae. The chondrocytes near the periphery were flat as compared to those near the centre of the cartilage. There was generalized basophilia with no clear demarcation of matrix. The mean thickness of cartilaginous sclera at this stage was measured in micrometres (Table-1). The mean thickness of cartilaginous sclera was more than that at day 10 of development (Table-I). The number of chondrocytes per unit area was also considerably more than that at 10 days of development (Table-I).

Table 1: Histological comparison of Group A and C

Parameter	Day 10	Day 15
	n=28	n=25
	Mean±SE	Mean±SE
Thickness of cartilaginous sclera(μm)	43.546±0.463	77.485 ±1.573
Number of chondrocytes in sclera/UA	17.411± 0.352	10.021±0.12

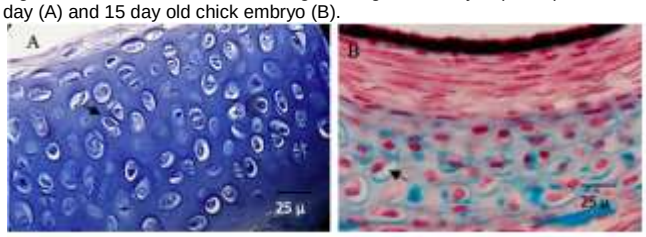
The bony part of sclera appeared as preossicular plate at sclero-corneal junction (Figure-2A).

Figure-2. H&E stain showing Ossicle in sclera (OS) in a 10 day (A) and 15 day old chick embryo (B).



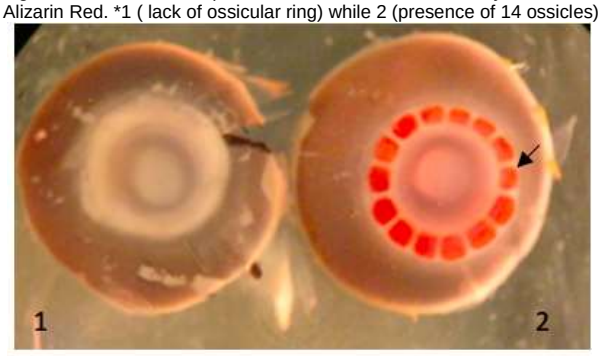
In the Alcian blue stained slides the rounded territorial matrix was moderately stained and formed a prominent ring around the lacunae. The proteoglycans content was evident in cartilaginous sclera as evenly distributed light blue shade in the interterritorial matrix. The interstitisal growth of cartilage was shown by mitotic figures present in the dividing chondrocytes (Figure 3A). Alcian blue stained sections at day 15 of development shows more prominent presence of proteoglycans as compared to that at day 10. The territoreal and interritoreal matrix is intensely stained (Figure-3-B)

Figure-3. Alcian blue stain showing dividing chondrocyte (Arrow) in a 10 day (A) and 15 day old chick embryo (B).



Out of a total of 90 animals, 12 (13.3%) did not show presence of scleral ossicles (Figure.4-1). Scleral ossicles are also more well developed and thicker at day 15. The layer of scleral ossicles overlapped the cartilaginous portion of sclera. The sclera of day 12 chick embryos that were stained with Alizarin red had 14 ossicles lying at the junction of sclera with the cornea. (Figure 4-2). The number and arrangement of the ossicles in the scleral ring of both right and left eyes of the embryos were same.

Figure-4: Stereomicroscopic view of whole anterior half of eyes stained by Alizarin Red. *1 (lack of ossicular ring) while 2 (presence of 14 ossicles)



DISCUSSION

The present study describes differentiation stages of developing sclera of chick embryos**.** Although, the development of sclera has already been mentioned in literature [11,12] this study, expands our knowledge of early developmental processes and its significance.

Sclera of chick constituted of fibrous and cartilaginous layers in all animals of our study. This is in accordance with previous studies which document the presence of an additional cartilaginous layer in chick embryos at all stages of development[13,14].

Our results indicate the developmental landmarks present at day 10, 12 and 15 of incubation. The results show that there is a progression in development of the layers as well as the chondrocytes and connective tissue elements. This correlates with the findings of studies describing scleral development in chicks according to Hamilton Hamburg stages[15].

In our samples, the chondrocytes changed their shape from flattened to more rounded and moved from surface to inwards of the cartilage as they got matured. This differentiation of chondrocytes from periphery towards centre has been shown in previous studies also and the dividing chondrocytes inside the lacunae indicated the interstitial growth that has resulted in increased number of chondrocytes as well as increased thickness of sclera in our sample.[16.]

Higher intensity of staining shown by day 12 and day 15 embryos correlates with increased quantity of proteoglycans present at this stage. The number of collagen fibres increase and increase of proteoglycan content with age complements the findings of similar studies in chick embryos at this stage[17].

The calcification of bone matrix of ossicles also increased with advanced stages of development in our study. Although previous studies have shown ossicles to be calcified at age of day 12 of incubation.[18] In our study out of 90 animals, 12 (13.33%) did not show ossicles at this age of development. This can be due to lack of calcification process indicating that it may be delayed in some animals (Figure 4-1).

With regards to number of ossicles, some studies show that the number of plates may vary by one or two. These studies also report variation in number of ossicles between left and right side.[19, 20] Contrary to these reports in our study, the number of ossicles was 14 in all animals and it was consistent on both sides.

The findings also suggest that chick eye can be easily obtained for experimental studies involving embryonic manipulation or tissue cultures that investigate cartilage and bone development, as well as a model system for researching the function of the neural crest in craniofacial development. Given that the sclera's cartilage and ossicles are made of neural crest cells, understanding how they develop can be used to show how teratogens may affect the growth of tissues or organs made of this significant cell population.

Limitations of study: The limitations included single centre study with limited resources and finance.

CONCLUSION

It was concluded that sclera of chick, was made up of fibrous tissue, hyaline cartilage and a ring of 14 tiny bones at the junction of sclera with the cornea. Augmented thickness of cartilage layer with developmental progress is due to increased number of chondrocytes and more deposition of proteoglycans. The ossicles calcification is also a maturity index seen at day 12 of incubation.

Author's contribution: RSM&SS: Write up and literature review, **HK&SS:** Statistics application, analysis literature review, help in write up. **AM&AQ:** Overall supervision and literature review help in write-up.

Conflict of interest: None

Funding: None

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