Are There Processes of Occlusion and Recanalization in the Esophageal Development?

Jantima Roongruangchai, D.D.S., Ph.D.*, Sataporn Menbangphung, M.S.* Kasem Kuedpuch, B.Sc.*, Nachaya Amonmettajit, B.Sc.*, Pae Pochanasomboon, B.Sc.*, Kosol Roongruangchai, M.S., M.D.**

**Department of Anatomy, ***Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

ABSTRACT

Background: Esophageal atresia (EA) or stenosis is well known among the congenital abnormalities of the esophagus which may result from the failure or incomplete recanalization after solid stage during the 8th week of human development or deviation of the tracheo-esophageal septum. The occlusion and recanalization theory of gut development cannot explain some types of esophageal atresia.

Objective: To study the serial section of continuous stages of esophageal development of chick embryos from related human Carnegie stages 14-23, observe histologically whether there are occlusion and recanalization processes, at which stages and to calculate the mitotic and apoptotic indices of the epithelial lining of the esophageal lumen as well as measuring the lumen areas of each stage of esophageal development.

Methods: The fertilized Leghorn hen eggs (*Gallus domesticus*) were incubated at different time intervals to gain five embryos for each developing stage 14-23. They were processed histologically into serial sections after the total bodies were photographed. The serial sections were observed at the esophageal area by one skipped five sections technique. The length and lumen area were measured, total epithelial cell count, mitotic and apoptotic cell counts were performed and the mitotic and apoptotic indices were calculated.

Results: The lengths of the esophagus increased with advanced age from 126.80 ± 3.03 µm at stages 14 to 7, 234.80 ± 396.62 µm at stage 23. The lumen areas decreased from stages 14-16 and increased from stages 17-23. There was no evidence of occlusion at any stage. At the stages of increasing the lumen area there was also no evidence of vacuole formation. The mitotic indices decreased from stages 15-20 and were constant from stage 21-23, which showed no correlation with the lumen area. The apoptotic index was highest at stage 16 which also showed no correlation with the process of recanalization.

Conclusion: By studying in the continuous stages of esophageal development, we could not demonstrate histologically the occluded esophageal lumens, or vacuolation of the lumens at any stage. In the same way, the mitotic and apoptotic indices show no correlation with the lumen area. These results lead to the conclusion that processes of occlusion and recanalization never occur in the normal esophageal development.

Keywords: Esophageal atresia, stenosis, occlusion, recanalization

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INTRODUCTION

The esophagus develops from the foregut, lies caudally to the pharynx and partitions from the trachea by the tracheoesophageal septum. This septum separates the foregut into esophagus dorsally and respiratory primordium ventrally. The development of various histological parts of the esophagus wall requires coordination of a variety of genes and mediators. Initially the esophagus is short but later elongates rapidly to keep up with the growth of heart and lungs. The esophagus is lined by the ciliated columnar epithelium by the 10th week and is replaced by stratified squamous epithelium by the 4th month. About the 7th week, the epithelial lining proliferates and partly or completely occludes the lumen until at the end of the embryonic period, the lumen is recanalized. Failure of this recanalization process may lead to esophageal atresia or stenosis.[1]

Esophageal atresia (EA) is a condition in which the esophagus is completely obstructed which is divided

Correspondence to: Kosol Roongruangchai
E-mail: parasite52@hotmail.com
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into 2 types, they are esophageal atresia with and without tracheo-esophageal fistula. EA usually results from abnormal division of the tracheo-esophageal septum. EA with tracheoesophageal fistula occurs in 90% of cases. The fetus is unable to swallow which results in polyhydramnios because the amniotic fluid cannot pass into the intestines for returning to the maternal blood.[1-3]

The misplaced or ectopic location of notochord may also lead to the disruption in sonic hedgehog (Shh) signaling and result in the EA condition. Rat models which are followed by Adriamycin administration leads to pathogenesis of EA followed by the abnormal location of notochord with the lack of down-regulation of the Shh activity at the site of the tracheo-esophageal separation in embryonic day 12.[4] EA occurs in 1:3,000-4,500 live births and about one third are born immaturely. Half of cases of these EA are associated with VACTERL (vertebral, anal, cardiac, trachea, esophagus, renal and limb defect).[2,3]

Several embryogenesis theories on the causes of EA previously explained could not answer the whole anomaly spectrum. The abnormal recanalization after solid stage theory cannot explain the incidence of two esophageal pouches connected by a fibromuscular band while this theory can answer only the rare esophageal web, mucosal fold and plate. Does Tandler's theory[3] about solid stage and recanalization stage of the esophageal development really exist? Therefore we planned to study the histological changes of the epithelium, mitotic and apoptotic epithelial cell counts, morphometric analyses of the length and lumen area of the esophagus of the continuous stages of chick embryos from related human Carnegie stages 14-23. The stages correspond to human embryos of the 5th to 8th week. We intended to use related human Carnegie stages in chick embryos to relate the chick and human developments following the Atlas of staging mammalian and chick embryos by Butter H and Jurr link BHJ in 1987. We expect that the mitotic index should increase in the solid stage and decrease in the recanalization stage, while the apoptotic index should increase in the recanalization period. About the lumen areas, they were expected to decrease in the solid stage and increase in the recanalization stage.[4,5]

MATERIALS AND METHODS

The fertilized Leghorn hen eggs (*Gallus domesticus*) were purchased from the Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok. They were stored at 12ºC with the air space (blunt end) upward. The eggs from this cool store were kept in room temperature for 8 hours and transferred to the incubator at 37.5-38ºC for 72 hours and every 3 hours later to day 10 until we got 5 embryos for each stage, which followed the description of Butler H and Jurrlink BHJ in 1987[4] as follows: Human Carnegie stages 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23 can be related to the incubation times 3, 3.25, 3.75, 4.75, 5.5, 6.25, 7.25, 7.75, 8.5 and 10 days respectively. All embryos were fixed in Bouin's solution for 30 minutes and post fixed in 70% alcohol until the yellow embryos turned white. Photographs were taken by the stereomicroscope and the digital camera. The crown-rump length of each embryo was measured from photographs by Motic images plus version 2.0 ML for the embryos less than 10 mm, and for those larger than 10 mm, the lengths were measured by vernier caliper. All embryos were then dehydrated and cleared with xylene, infiltrated and embedded with paraplast. The serial sections were made at the thickness of 5 µm. Fifty embryos were used in this study and 10 development stages were divided, which were Carnegie stages 14 to 23. The stained serial sections were observed by one skipped five sections technique and photographed by the digital camera attached to the light microscope at the area of the development of the esophagus. All photographs were then quantitatively studied by measuring the lumen area, the total epithelial cells. The mitotic and apoptotic cells were counted and the mitotic and apoptotic indices were calculated. These measurements were done by UTHSCSA image tool version 3.0.

RESULTS

50 chick embryos were divided into 10 stages, which were related to human Carnegie stages 14-23, five of each stage, which corresponded to human embryo of the 5th to 8th week. The serial sections of each embryo were observed by light microscopically at the developing esophageal region, aimed at the esophageal lumen and the epithelial cell to determine whether there was any area of lumen occlusion and epithelial vacuolation or the process of recanalization.

In the Canegie stages 14-23 chick embryow, the length of the esophagus were 126.8 ± 3.03, 454.60 ± 7.16, 874.60 ± 13.79, 1017.80 ± 49.87, 1498.60 ± 10.67, 1889.40 ± 21.85, 3,768.00 ± 21.85, 4451.80 ± 129.78, 5649.40 ± 161.30 and 7234.80 ± 369.62 µm (Table 1). The epithelial lining was pseudostratified columnar type at stages 14-21 while the epithelial cells at the luminal surface of stages 22 and 23 were cuboid. The mitotic cells in every phase were mostly found in the apical cell layer (Fig 2,3). The apoptotic cells which appeared in the light microscopy could be divided into 4 types, darkly stained nucleus, cytoplasm increased eosiophilic, nuclear materials broken into fragments and lastly apoptotic cell forms membrane bound fragments as apoptotic bodies (Fig 4). The apoptotic cells were mostly found in the basal layer of the epithelium. There were no occlusion and vacuolation in all stages observation. Even tracing the serial section from the beginning to the end of the esophageal length, there was no lumen obstruction by cell proliferation and also there was no sign of cell degeneration (Fig 2,3).

The length and lumen area per cross section of the esophagus of stages 14-23 have been shown in Table 1. The length of the esophagus increased with advanced ages while the lumen area decreased from stages 14-16 and increased from stages 17-23. The total cell count per cross section increased from stages 14-23 while mitotic and apoptotic cell count per cross section alternatively increased and decreased. The calculated mitotic index was highest at stage 15 while the apoptotic index was highest at stage 16 (Fig 1).

TABLE 1. The table reveals the length, lumen area, total cell count, apoptotic cell count, mitotic cell count, mitotic index and apoptotic index of the epithelial cell of the esophagus of chick embryos of continuous stages 14-23.

Carnegie stage	Length (µm)	Lumen area (µm^2)	Total cell count	Mitotic cell count	Apoptotic cell count	Mitotic Index	Apoptotic Index
14	126.8 ± 3.03	31.75 ± 3.33	78.24 ± 3.97	6.59 ± 1.44	0.98 ± 0.10	0.08 ± 0.02	0.01
15	454.60 ± 7.16	29.50 ± 1.97	108.85 ± 5.38	10.87 ± 1.06	0.97 ± 0.07	0.10 ± 0.01	0.01
16	874.60 ± 13.79	18.24 ± 0.80	115.69 ± 9.55	10.66 ± 1.44	5.79 ± 0.94	0.09 ± 0.01	0.05
17	1017.80 ± 49.87	20.84 ± 1.90	140.16 ± 12.05	10.25 ± 1.25	4.46 ± 0.35	0.01	0.03
18	1498.60 ± 10.67	28.63 ± 3.78	143.60 ± 6.69	5.08 ± 0.48	3.00 ± 0.10	0.04	0.02
19	1889.40 ± 21.85	63.02 ± 3.85	145.38 ± 18.57	5.17 ± 0.32	2.93 ± 0.14	0.03	0.02
20	3768.00 ± 21.85	735.33 ± 27.21	169.74 ± 3.40	4.82 ± 0.26	2.57 ± 0.11	0.03	0.02
21	4451.80 ± 129.78	882.32 ± 48.33	289.91 ± 5.55	2.49 ± 0.40	2.02 ± 0.13	0.01	0.01
22	5649.40 ± 161.30	2471.71 ± 265.35	352.90 ± 25.34	2.49 ± 0.28	2.26 ± 0.41	0.01	0.01
23	7234.80 ± 369.62	2973.10 ± 158.29	369.46 ± 9.07	2.42 ± 0.14	1.92 ± 0.07	0.01	0.01

DISCUSSION

Previous studies found the occlusion and recanalization processes occur in a craniocaudal direction which included decrease in the caliber and area of the lumen and occlusion of the lumen which was referred to as the solid stage, while vacuoles were found in the epithelium which was referred to as the recanalization stage and finally there were villi formation in the intestines.[2] The occlusion process was firstly mentioned by solid stage theory in the study of Tandler[3] who described the occlusion of the duodenum as a normal process of gut tube development. This theory, also had later been thought to be the cause of esophageal atresia and intestinal anomalies.[7,8,9] Because of anomalies in humans and animals, this theory was widely accepted.[10,11] Nowadays we have all agreed with the concept of the solid stage theory which begins with narrowing or occlusion process by the epithelial cell proliferation which is followed by recanalization process which results from epithelial cell vacuolation.

The esophagus is occluded in the 7th week, which corresponds to Carnegie stage 20 and recanalized in the 8th week or stage 23.[12] However some authors such as Johns BAE[13] found no epithelial occlusion at any stages studied (stages 17,18). Smith EI[14] found only narrowing of the lumen. Grand RJ[8] also found no total occlusion of the esophagus and no vacuolation at stage 20 and Tanaka O[15] found the esophageal lumen of human embryos of Carnegie stages 17-18 were narrowed.

When the serial sections of 10 continuous stages of embryos were studied in the reasonable sample size which were composed of 5 specimens per stage, we found that the lumen area of the esophagus tended to decrease from Carnegie stages 14 to 16 which was the narrowest, but there was no evidence of occlusion. The lumen area was then increased at Carnegie stage 23. When the lumen area of the esophagus was decreased from Carnegie stage 14 to 16, if this resulted from epithelial cell proliferation, the mitotic index should increase from Carnegie stage 14 to 16 and should peaked at Carnegie stage 16. Contrary in this study, the mitotic index peaked at Carnegie stage 15 and decreased from Carnegie stage 16. This also indicated that the mitosis did not result in narrowing of the lumen. The lumen of the esophagus increased from Carnegie stage 16 and was widest in Carnegie stage 23 with no evidence of vacuolation in the whole length of the developing esophagus. If increasing lumen area resulted from degeneration or apoptosis, the apoptotic index should increase from Carnegie stage 16 to 23. Contrary in this study, the apoptosis did not correspond to the widening of the lumen.

Studying in an animal model is more advantageous, because we can follow the continuous stages of development and moreover the serial sections give us the real histological evidence in the whole length of the developing esophagus. We have clearly seen in the whole length at continuous stages that there was no solid stage or occlusion stage and there was also no evidence of vacuolation or recanalization process. These findings suggest

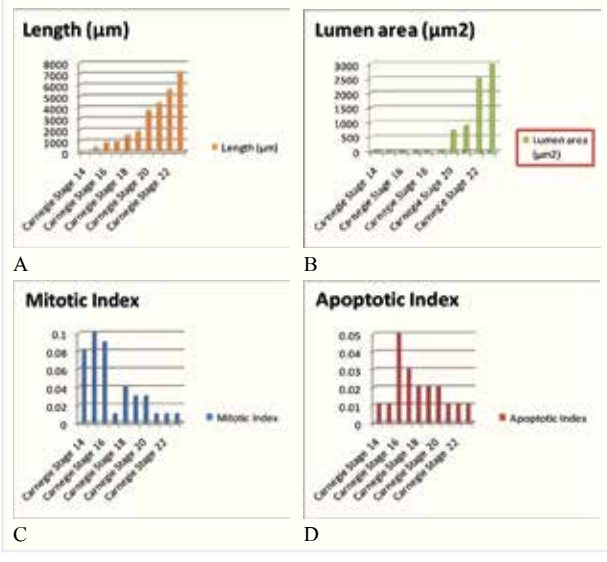

Fig 1. A is the graph plotting between each stage of chick embryo and its length.

B is the graph plotting between each stage of chick embryo and its lumen area.

C is the graph plotting between each stage of chick embryo and its mitotic index.

D is the graph plotting between each stage of chick embryo and its apoptotic index.

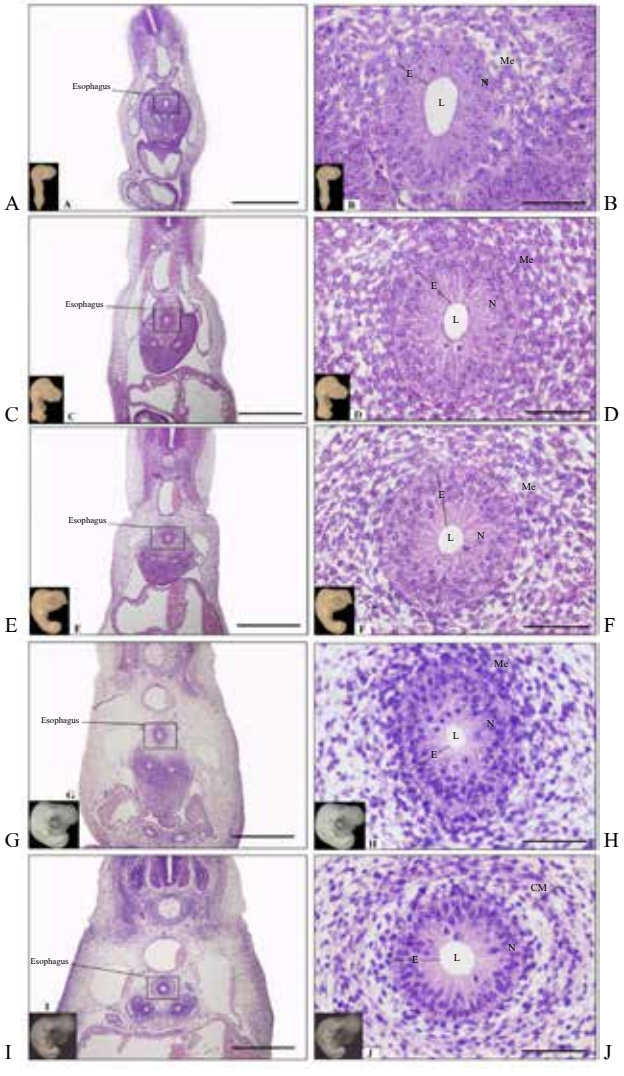

Fig 2. A. Chick embryo stage 14, total feature and low magnification of esophagus (x40).

B. Chick embryo stage 14, higher magnification of esophagus in rectangle of A (x400) (L = lumen, E = epithelium, N = nucleus, Me = mesoderm).

C. Chick embryo stage 15, total feature and low magnification of esophagus (x40).

D. Chick embryo stage 15, higher magnification of esophagus in rectangle of C (x400).

E. Chick embryo stage 16, total feature and low magnification of esophagus (x40).

F. Chick embryo stage 16, higher magnification of esophagus in rectangle of E (x400).

G. Chick embryo stage 17, total feature and low magnification of esophagus (x40).

H. Chick embryo stage 17, higher magnification of esophagus in rectangle of G (x400).

I. Chick embryo stage 18, total feature and low magnification of esophagus (x40).

J. Chick embryo stage 18, higher magnification of esophagus in rectangle of I (x400).

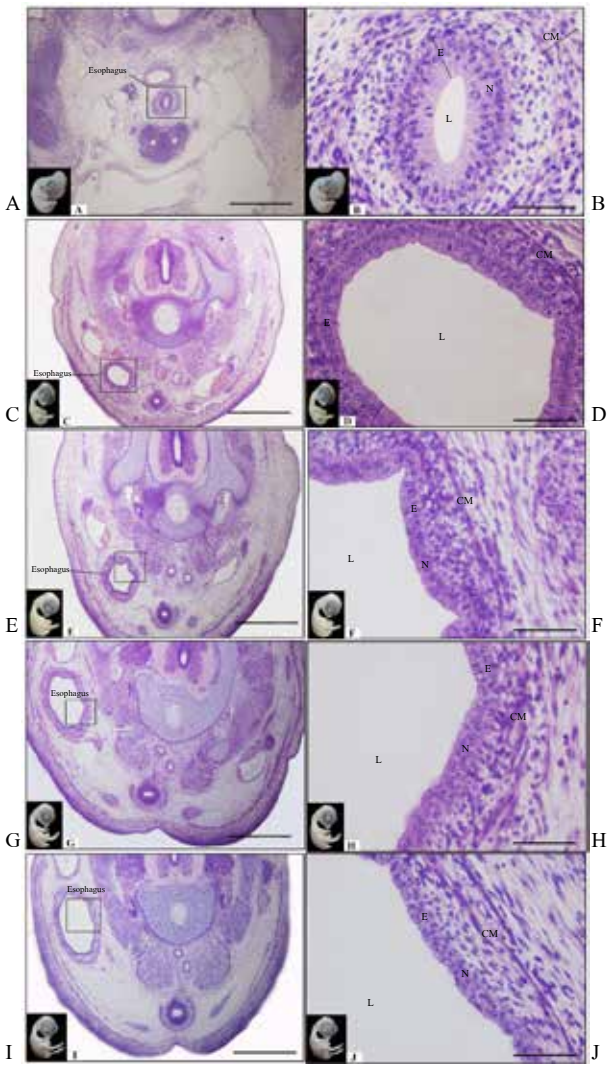

Fig 3. A. Chick embryo stage 19, total feature and low magnification of esophagus (x40).

B. Chick embryo stage 19, higher magnification of esophagus in rectangle of A (x400) (L = lumen, E = epithelium, N = nucleus, Me = mesoderm).

C. Chick embryo stage 20, total feature and low magnification of esophagus (x40).

D. Chick embryo stage 20, higher magnification of esophagus in rectangle of C (x400).

E. Chick embryo stage 21, total feature and low magnification of esophagus (x40).

F. Chick embryo stage 21, higher magnification of esophagus in rectangle of E (x400).

G. Chick embryo stage 22, total feature and low magnification of esophagus (x40).

H. Chick embryo stage 22, higher magnification of esophagus in rectangle of G (x400).

I. Chick embryo stage 23, total feature and low magnification of esophagus (x40).

J. Chick embryo stage 23, higher magnification of esophagus in rectangle of I (x400).

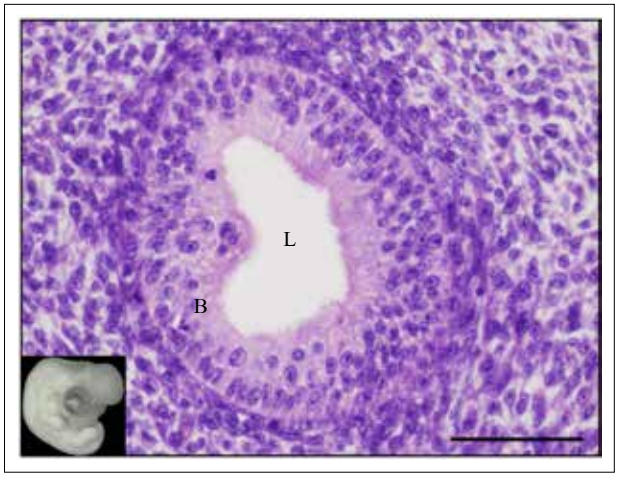

Fig 4. Light micrograph of transverse section of the esophagus of the chick embryo in related Carnegie stage 19 showing the apoptotic body (B) which was found at the basal part of the epithelium.

that changes in diameter or lumen area of the developing esophagus do not correlate with cell proliferation and cell degeneration, but results from gut elongation and dilatation. The distribution timing of activity and intensity of mitosis do not correspond to the narrowing of the lumen. Furthermore, the distribution, timing of activity and intensity of apoptosis also did not correspond to the widening of the lumen.

There are no occlusion and recanalization processes in the developing esophagus of the chick embryo stages 14-23. The esophagus of the chick embryos never occluded, which was different to that which occurred in human embryos explained previously or in the chick study of Ramey BA[16] (1973) and Allenspash AI[17] (1966). Ramey BA[16] treated embryos with radioactive precursor solutions which were iodine and 95% ethanol prior to another period of incubation. Histology finding could show occluded lumen of the esophagus since this was not a normal condition of development. Further studies should be carried out to confirm the solid stage, and also to explain about the congenital abnormalities.

CONCLUSION

Most textbooks stated that in embryogenesis, the lumen of the esophagus is occluded in the 7th week or Carnegie stage 20 and recanalized in the 8th week or Carnegie stage 23. The occlusion process occurs from the proliferation of the epithelial cells and follows by the recanalization process which results from the vacuolation or degeneration of the epithelial cells. When studying the developing esophagus in chick embryos at continuous stages 14-23 with the total number of 5 embryos at each stage, about the length, lumen area, mitotic and apoptotic indices, we found that the lumen area was narrowest at stage 16 and widest at stage 23. We found no evidence of occlusion or recanalization process of the developing esophagus at any stage of any specimen. We found no correlation of mitotic index to the narrowing, and no correlation of the apoptotic index to the widening of the esophageal lumen. Further studies about the solid and recanalization processes should be performed to explain about this congenital anormality.

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