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Transportation of liquid crystal and $CaCO_3$ vaterite crystal in chicken embryo and early postnatal development

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ABSTRACT

During chick embryo development, the liquid crystals appear in more than 20 organs at certain developmental stages, and vanish in early post-natal stage. In this study, we found a complex in embryo, the YS-J/I tunnel system, in charge of transporting the LCs and CCVCs preserved from yolk sac into the embryo. This transportation initiates from yolk sac connecting to central transportation region/reservoir via a main tunnel, spreads radically to various parts of digestive system via sub-tunnels. Our data indicate that the liquid crystals could be the best form of nutrition which is necessary in embryo development and early post-natal development.

KEYWORDS

Embryonic development; Yolk sac liquid crystal; Yolk sac-Jejunum/Ileum (YS-J/I) tunnel system; CaCO3 crystallization

Introduction

Historic research of liquid crystal has always been closely related to biology [1]. In 1854, Virchow described an image of myelin (lipid-water system), and it is identified liquid crystal structure. In 1988, Keinitzer reported their findings on liquid crystal in the process of the preparation of a cholesterol ester, cholesteryl benzoate. Subsequently Lehmann, Friedel, Rinne and Bernal and et al. described the physical and chemical properties of liquid crystal [2] and then the term of liquid crystal was accepted. In Brown GH and Wolken JJ systematically summarized entire research proceedings on liquid crystalline in biological organism in 1978 and 1979. Meanwhile, He and Wu reported their findings of liquid crystal during chicken development. Their work revealed that liquid crystalline exists massively in liver, yolk sac, blood, and other 20 developing tissues and organs during chicken embryogenesis [3, 4]. Later they reported that liquid crystal configuration can also be observed in fish development [5, 6]. Chao and Li then reported $CaCO_3$ vaterite existence within liquid crystal yolk fluid [7, 8]. This result revealed that spherical calcified structures found in 1979 is one of three isoforms of calcium carbonate [9].

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/gmcl.
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Recently we have demonstrated that this crystallization is associated with liquid crystal in yolk sac. Subsequently, by study of chick embryo development, we find that in form of Maltese crosses birefringence bright out in more than 20 organs and tissues at sequential stage of development, and vanished around early stage of post-natal developmental stage [10]. Among these organs/tissues, liver and yolk sac are two major embryonic tissues with massive Maltese crosses birefringence. The studies characterized that hepatic liquid crystal in embryonic liver experiences phase transition between liquid crystal, isotropic droplet and crystal *in vivo* system indicating that their function in development could be related with these physical features [11, 12]. In chicken yolk sac, we revealed that the liquid crystal functions in the crystallization of calcium carbonate vaterite crystals (CCVCs). The centric lamellar structure of the liquid crystal could provide the possible base for the CCVCs crystallization in two approaches i.e. the liquid crystal – crystal transition mechanism of inside-out and outside-in [13].

However, how the massive yolk sac liquid crystal and the CCVCs are utilized completely in 6 post-natal days by new-born chicken remains unknown. In this study, we perceived that size of yolk sac full of CCVCs dramatically decreased from 5–7 cm in diameter to completely be absorbed eventually in 6–8 days of postnatal development. And we found that in new-born chicken, the liquid crystal and calcium carbonate fully preserved within yolk sac, were transported from the yolk sac to a transition zone by a special tunnel system. We termed this tunnel as the yolk sac-Jejunum/Ileum tunnel system (YS-J/I tunnel system) and characterized the phase transition of the liquid crystal within this system. The yolk sac-Jejunum/Ileum tunnel system formed with the tunnel complex plus vessels employed by the embryo is in charge of transportation of the liquid crystals from yolk sac. Our data indicate that the liquid crystals could be the best form of nutrition which is necessary in embryo development and early post-natal development.

Experimental materials and methods

Animals and materials

All animals care and experiments were performed in accordance with the protocol approved by the Animal Care and Use Committee of Shaanxi Normal University. All procedures were conducted in chicks care facility in the university.

The chickens and chicken embryos are HY-LINE chicken and were purchased from Hu county chicken farm of Xi'an. The age of embryos was documented as day (D) and postnatal age of chicks was documented as Postnatal (P). 50 chickens were used totally for experiments.

Sample preparation and frozen section

Sample preparation included two procedures, smear-slide preparation and cryosection. For smear-slide preparation, yolk sac were dissected from embryos at different stages. Sample was smeared on a slide and mounted with a cover slip within 30% of glycerol in PBS (pH7.4). Then, polarization microscope observation was executed immediately. To determine the yolk sac-Jejunum/Ileum tunnel system, the samples were obtained from P5 embryo. Injection of Chicago Blue (CB) were employed to check tunnel flow on the system. Cryosections were performed to observe the detailed structure of the system. The dissected samples were set into cryomatrix embedding agent (OTC) and placed in an aluminum foil basket. Samples were frozen by setting the foil basket into liquid nitrogen and then located on a flat surface

immediately. In the cryostat microtome section, samples were cut at a thickness of 10–20 μm. The sections for Hematoxylin and Eosin (H&E) staining were cut at a thickness of 10 μm and 10–20 μm for polarization microscopy.

The samples collected employing these two procedures were mounted with 30% of glycerol in PBS (pH7.4) before proceeding to further analysis.

Gravity separation of yolk sac components

Yolk sac fluid obtained from D21 days chicken embryos were washed by ddH_2O. Then yolk sac precipitates were divided into four layers by gravity. The four layer's precipitates were collected separately, and their size and integrated density were detected.

Samples from each layer were smeared on a slide and mounted within 30% of glycerol in PBS (pH7.4). Polarization microscopy were conducted instantaneously following sample smear preparations. Samples were set as three groups, the control group, the temperature sensitive phase transition group, and the pressure applied and release group.

Histology and polarization microscopic analysis

H&E staining was carried out on frozen section as previous described [14–16]. After the staining, slides were dehydrated in gradient ethanol and were treated for transparent in xylene, then the samples were permanent preservation with glass cover slips within cryo-crystal. Observations were performed under Carl Zeiss Microscope.

Frozen sections for polarization observation were mounted with 30% of glycerol in PBS (pH7.4). Optical activity was documented under polarizer and analyzer. Experiments were carried out in three groups as mentioned above i.e. the control group, the temperature sensitive phase transition group, and the pressure applied and release group.

Microscopy and statistics

The images from conventional and polarization microscopy were documented with the Inverted microscopy (Carl Zeiss Microscopy GmbH) and manufacturer software ZEN. Quantification analysis of birefringent intensity of the LC and CCVC particles from yolk sac were performed with the image analysis software Image 1.50d (NIH, Bethesda, MD). The statistical analysis was conducted by SPSS.

Results

In our experiment, through histology and polarization microscopy analysis, abundant LC and CCVC were observed in chicken embryonic yolk sac. We reported the novel finding of the yolk sac-Jejunum/Ileum tunnel system (YS-J/I tunnel system), which was utilized as a specific nutrition network for transporting the birefringent LCs and CCVC particles to other developing tissues and organs until the LCs and CCVCs were utilized completely.

Distribution of birefringent particles in YS-J/I tunnel complex

By anatomic observation, we found that yolk sac were connected with intestine through two tunnels as indicated by arrows in Figure 1A, in which the birefringence LCs and CCVCs particles in the tunnels were observed (Figure 1B c and f). One tunnel straightly connected to

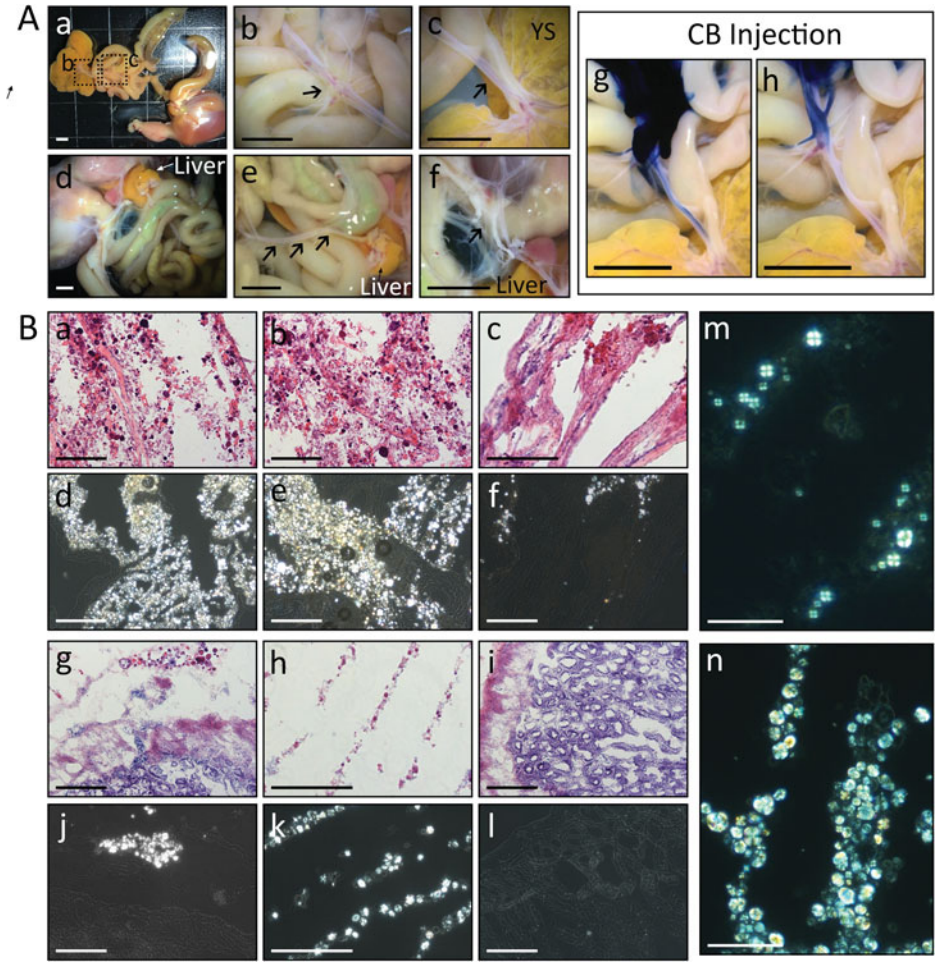

Figure 1. The yolk sac-Jejunum/Ileum tunnel complex of chicken embryo. This net-work complex is composed of yolk sac (YS), transportation region/reservoir and radical sub-tunnels (TZ/R and RSC). Panel A a shows the whole digestive system and yolk sac of chicken embryo, and b-f are the enlarged image of the complex. In A g-h, the injection of Chicago Blue (CB) displays a feature of one-way flow. The CB dye moved backward into the center region/reservoir of the complex. Panel B a-n present the histological and polarization results of chicken embryo yolk complex. In panel B, a-b and d-e are the different region of yolk sac, c and f are the connecting tunnel of yolk sac to ileum. Panel B g-h, j-k and m-n are the transportation region/reservoir and radical sub-tunnels. Panel B i and l show the Jejunum intestines of chicken embryo. Scale bars in A a-h, 4 mm; in B a-l, 200 μm; in B m-n, 100 μm).

ileum (Figure 1A c). The other one spread radially to jejunum (Figure 1A b) and may link to liver (Figure 1A e and f). When Chicago Blue (CB) injected from its radial center, the blue dye could go through the YS-J/I Tunnel Complex and could be observed in the tunnel (Figure 1A g). Due to a pressure from yolk sac, the dye could be pushed backward into the center region (Figure 1A h). This phenomenon suggested that the tunnel-flow from yolk sac to the center region of YS-J/I Tunnel Complex could be a one-way tunnel, which just allow the birefringence LCs and CCVCs particles flow into the center. This one-way flow was the quick way for embryo to absorb nutrition from the yolk sac. While accomplishment of the transportation for the birefringence LCs and CCVCs, the yolk sac will be absorbed by embryo and the YS-J/I

Tunnel Complex could become vestigial organ and function as a support structure mesenterium of adult intestine with artery system.

The further histological and polarizing microscopic analysis showed that the birefringence LCs and CCVCs particles were largely distributed in yolk sac (Figure 1B a-b, d-e) and partly dispersed in transportation center region and radical sub-tunnels (Figure 1B c, f, g-h, j-k and m-n). The birefringence LCs and CCVCs particles could not detected in intestine of Jejunum/Ileum but only trace amount of tiny small size of birefringent residues spread on the basal region of the intestine (Figure 1B i, l). This results demonstrated that the YS-J/I tunnel complex are certainly function in transportation of these birefringent particles from yolk sac to other organs.

Temperature dependent phase transitions of the LCs in the YS-J/I tunnel system

Previous studies confirmed that the birefringent particles in yolk sac contain both LCs and CCVCs, and we demonstrated that both of the LCs and CCVCs were existed in the YS-J/I tunnel system. The LCs are characterized by typical temperature-dependent phase transition [17]. As our experiment results shown here, with temperature alternation within a range of 23°C to 60°C, the CCVC particles underwent no phase transitions in the system, while LCs exhibited three states i.e. Maltese cross LC, isotropic state and its resumed LCs. The phase transition process of yolk sac birefringent particles from LCs (anisotropic state) to isotropic state with temperature increase were documented in Figure 2A-B, and the transition from the isotropic state to the Maltese crosses LCs was recorded in Figure 2B and C. The enlarged images of this temperature dependent phase transition shows more detailed in Figure 2D-F. The intensity comparison were calculated and exhibited in both gray value line graphs (Figure 2G-I) and 3-dimensional pixel graph (Figure 2J-L) and these results indicates that the phase transitions occurred massively in the yolk sac.

The above temperature dependent phase transitions can be detected in the YS-J/I tunnel system as well. Within the transportation zone of the system, two sets of experiments documented the phase transitions with the tunnel of the YS-J/I. The isotropic states displays in Figure 2M and P after temperature increase, and the Maltese cross liquid crystals recovered in Figure 2N-O and Q-R after temperature decrease.

Characteristics of the yolk sac mixture of the liquid crystals and the CCVCs

The birefringent precipitates were mainly collected from the chicken yolk sac and were graded to four layers in water gravity approach. Four layers were identified from top to bottom of the precipitation. Images of each layer using polarization microscopy were documented in figure 3A-D (top layer), E-H (second layer), I-L (third layer), M-P (bottom layer). And the corresponding size and integrated density (ID) displayed gradual increase of the particle numbers from top layer to bottom layer (Figure 3Q-U). These data suggest that the grade increase of crystallization displays a trend from the top to the bottom in four layers precipitates.

The results above showed that the yolk sack contains both the LCs and CCVCs massively. However, in the samples isolated with the water gravity approach, the precipitates from four layers displayed no phase transition (Figure 4A-F, top layer; G-L, second layer; M-R, third layer; S-X, bottom layer) with temperature increase applied. This data demonstrated that the basic component of these precipitates were the CCVCs instead of the mixture of the LCs and CCVCs. These CCVCs exhibited intense multicolor birefringent activity under polarization microscope. Furthermore, in the pressure-recovery approach, these multicolor birefringent

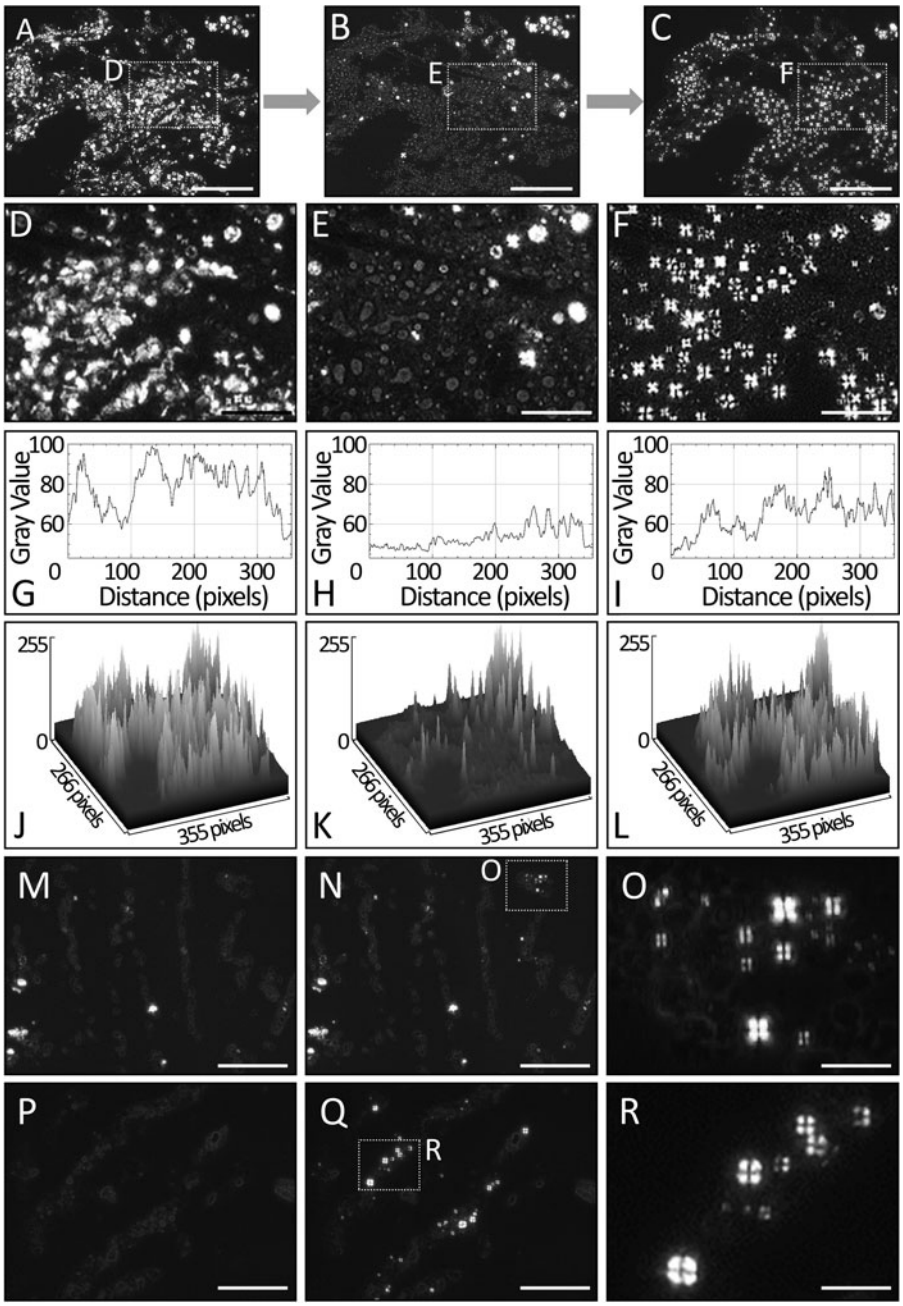

Figure 2. Phase transitions of LCs in the yolk sac-Jejunum/Ileum tunnel complex under polarized microscope. Unlike CCVCs, LCs in the embryonic complex experiences phase transition between liquid crystal, isotropic droplet and crystal. In panel A-C respectively, the birefringence patterns of before, after temperature increase and later 4 °C recovery phase transition in yolk sac are shown. Accordingly, the intensities of birefringence changed between the phase transition were analyzed, and shown in both gray value line graphs (G-I) and 3-dimensional pixel graph (J-L). The excerpt views of panel A-C are enlarged to visibly exhibit the birefringence change before (D), after (E) temperature increase and later recovery transition (F). Panel M-N and P-Q present the birefringence changes of transportation zone after temperature increased (M, P) and then decreased (N, Q) sequentially. And the dotted boxes in panel N and Q are enlarged and presented in panel O and R for the detailed changes. Scale bars in A-C and M-N, 200 μm; in P-Q, 100 μm; in D-F, 67 μm; in O and R, 22 μm.

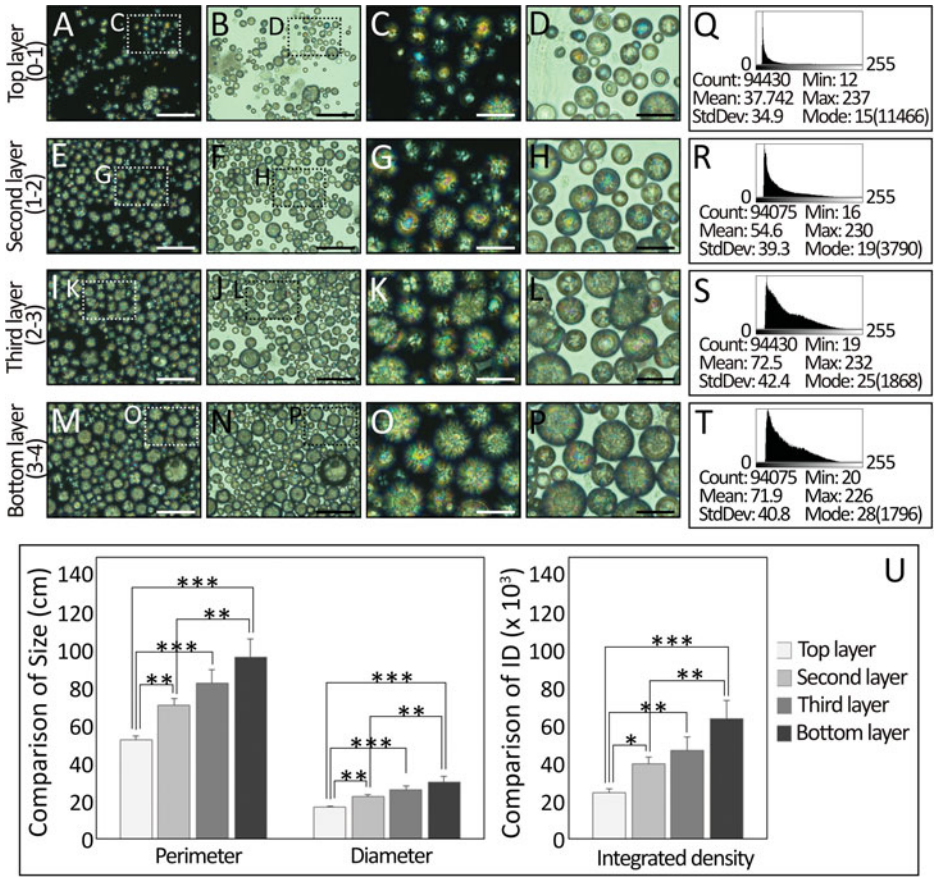

Figure 3. Differences on size and integrated density of the yolk sac precipitates with gravity gradient approach. The integrated density (ID) value of the particles of the yolk sac precipitates is gradually increased from top layer to bottom layer (pane Q-U) along with the precipitates size in panel U. And the size and ID of the particles in top layer precipitates (pane A-D) show significant difference compared to that of the other three layers showed in panel U. The comparison between the second layer and bottom layer led to the same significance (E-H and M-P) in panel U. The differences between the third layer (I-L) to the second or the bottom layers are not statistically significant ($P > 0.5$). In panel U, * indicates p-value < 0.05, ** indicates p-value < 0.01 and *** indicates P-value < 0.001. Scale bars in A-B, E-F, I-J, and M-N are 100 μm; in C-D, G-H, K-L, O-P, 33 μm.

particles could not bear the pressure applied and fractured into an outside ring (Figure 4Y, panel a-b), or sectorial parts (Figure 4Y, panel c-d) and some smaller fragments (Figure 4Y, panel e-f).

Previous researches proved that the liquid crystal functions in the crystallization of the CCVCs with mechanisms of inside-out and outside-in precipitation, the liquid crystals were considered as the precursor of the CCVC crystals. The increasing ratio of the CCVCs vs the LCs during chicken embryonic development was reported as well [18]. Therefore, in this study, we speculated that the LCs washed away from the yolk sac precipitates and these assumption are confirmed by the later experiments.

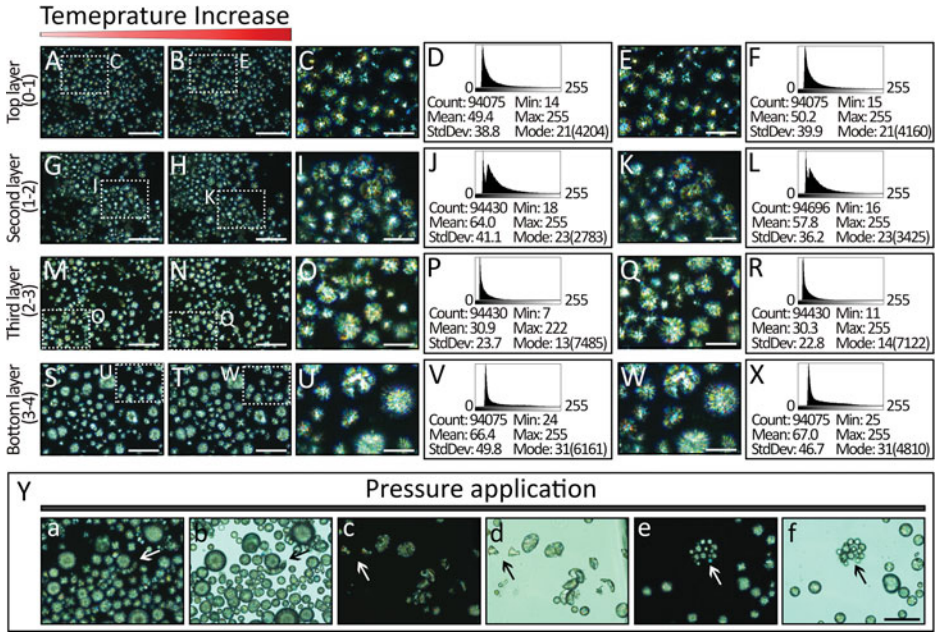

Figure 4. The birefringence characteristic of the embryonic yolk sac precipitates. With the temperature increase, the birefringence activity of the four layers precipitates (A-X) have no basically change (panel D and F, J and L, P and R, V and X). And after external pressure applied, the crystal particles were fractured from outside ring (panel Y a-b, arrows), or into sectorial parts (panel Y c-d, arrows), or some small fragments (panel Y e-f, arrows). Scale bars in A-B, G-H, M-N, S-T, and Y (a-f) are 100 μm; in C, E, I, K, O, Q, U, and W, 33 μm.

Fluidity of the LCs in the YS-J/I tunnel system

Fluidity is a distinctive feature between LCs and CCVCs. In this paper, the pressure-recovery experiment was conducted on yolk sac smear samples to identify the characteristics of the LCs. We also found an existence of a metaphase particle, and this particle presented as a medium configuration of both the liquid crystal and the CCVC crystals.

We applied pressure with rubber applicator evenly on cover-glass and observed the effect of pressure to LCs under polarization microscopy. The results showed that some LCs distorted into birefringent elliptical shapes and these structures resumed to their original birefringent Maltese Crosses along with the removal of pressure. This process was captured under polarization microscope in Figure 5A. The published researches revealed that the flexibility of birefringent elliptical liquid crystal could present as various shape-lifting including twists and helical complexes [18]. Our experiments exhibited a twisting pendulum recovery movement in Figure 5A. Furthermore, a defect recovery were exhibited that the part birefringence of a Maltese cross resumed eventually and formed a complete perfected liquid crystal droplets (Figure 5C a, d, g and j; Figure 5C b, e, h and k; Figure 5C c, f, I and l). These documentations suggested that the LCs in yolk sac dispose of typical fluidity.

Fusion of the LCs from the yolk sac supernatants were documented after pressure applied, which confirmed the characteristics of the liquid crystal fluidity. Some LC droplets performed fusion and form a large birefringent droplet (Figure 5 B a-d) or a birefringent bush (Figure 5B e-h). One possible mechanisms of the CCVCs formation may caught that the CCVC crystallization could form from one side of the LC droplets (Figure 6), indicating the diversity of

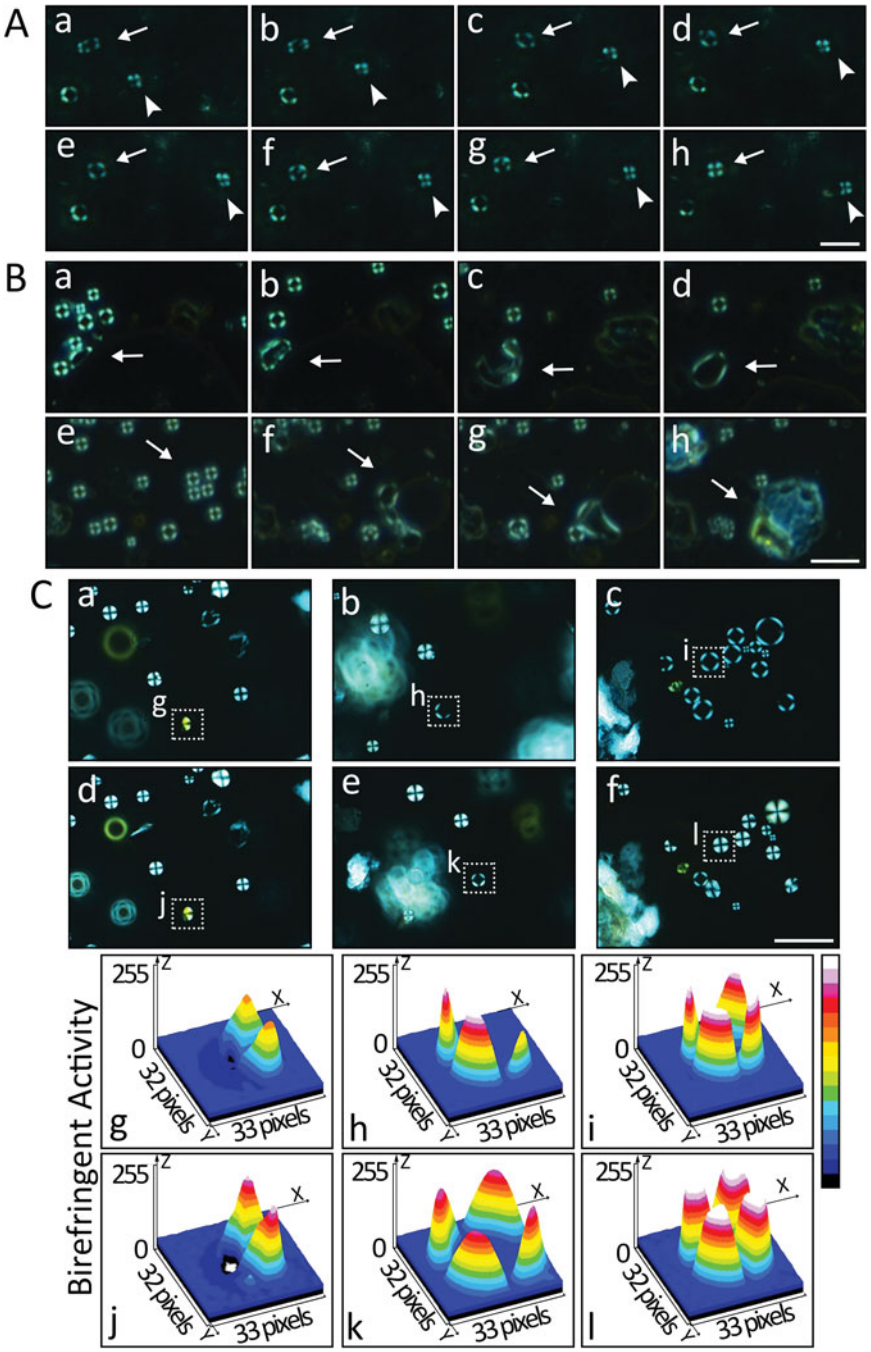

Figure 5. The yolk sac birefringent particles display the fluidity characteristics of liquid crystal. In panel A, the elliptical structures indicated by white arrows were generated by external pressure, and then resumed its original shape, the Maltese Crosses. Defect indicated by arrow head were recovered. A single liquid crystal tubule exhibits its fluidity with twisting pendulum movement. In panel B, fusion of LCs was generated by the pressure. After external pressure was applied, a few adjacent LCs occur fusion (a-d) and then develop to possible sematic liquid crystal (e-h). Scale bars in panel A, 17 μm; in B, 25 μm; and in C a-f, 33 μm.

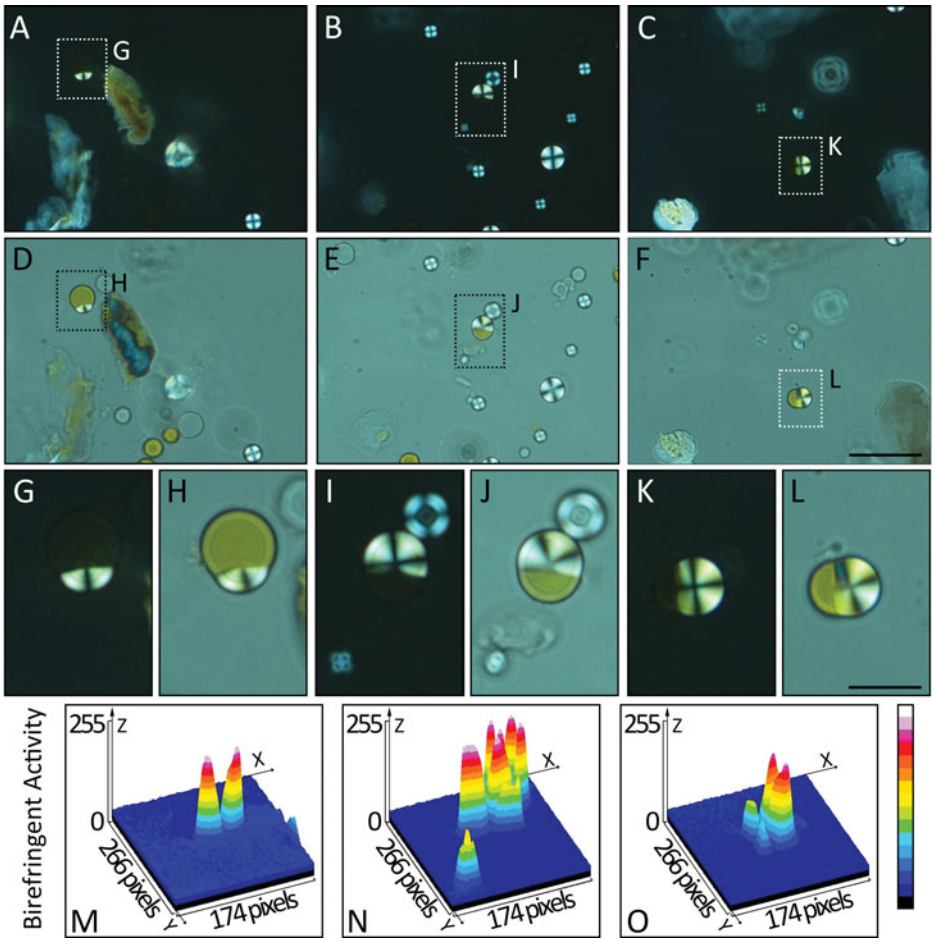

Figure 6. The formation of CCVCs after fusion in pressure applied and release. Panel A-L display three different stages of crystalizing particles. The possible crystallization occurred from one-side of liquid crystal droplets to another. Because of, in vitro, CaCO3 molecules are not enough for this process to accomplish, these crystalizing particles were frozen. The density distribution of crystalizing particles are shown in panels M-O. Scale bars for A-F are 33 μm; for G-L, 11 μm.

crystallization in animal development. Of course, it could also keep stay liquid crystal shell-like droplet as reported structure in vitro [19] and function as micro-reservoir for storing elements for embryonic development. However, these crystal particles would never crystalize completely in vitro and stand in the chimeras in Figure 6 G and H, I and J, K and L.

Discussion

Previous researches demonstrated that the LCs exhibited in multiple organs during chicken embryonic development, including liver, yolk sac, kidney, skin, brain, heart, and so on [20]. Among these, yolk sac is the earliest one that shows LCs appearance and it is absorbed eventually in the early postnatal development [19–21]. In this paper, we found and termed YS-J/I tunnel system, in charge of transporting the LCs and CCVCs preserved from yolk sac into the embryo. This transportation initiates from yolk sac connecting to central transportation region/reservoir via a main tunnel, spreads radically to various parts of digestive system via sub-tunnels. In these parts of the YS-J/I tunnel system, we have found that the birefringent

particles are all distributed as the LCs and CCVCs presented in the results. In liver, we found that the hepatic liquid crystal droplets massively distributed in hepatic cords. These massive liquid crystals could be considered accumulation from embryonic yolk sac via the YS-J/I tunnel system. In this study, we have tracked the distribution of the LCs and CCVCs along the main tunnels, central region, sub-tunnels and joint area of Jejunum and Ileum. In the first three parts, typical LCs and CCVCs were observed in their lumens with the same characters of the yolk sac LCs and CCVCs. However, we did not find that were any typical LCs and CCVCs in the intestine. Instead, we did find that there were some tiny birefringent particles distributing on the basal layer of epithelium. But because these particles are under the limitation of conventional polarization microscope, the characterization on these particles could need to be investigated further.

Before chicken hatching of fertilized eggs, all nutrition absorbed into fetus body with development. When chicken hatching out, the rests of nutrition and calcium absorbed from egg-shell needs to be preserved in yolk sac. We believe that the LCs and CCVCs is the best configuration of these nutritious materials such as Calcium, lipids, and cholesterol preserved temporally, as cholesterol normally could be in liquid crystalline [22, 23]. Within about ten postnatal days, via the YS-J/I tunnel system we reported in this study, the LCs and CCVCs provided the most energetic nutrition to meet the requirement for the first few days of the post-natal development.

Conclusion

Through the use of polarizing microscopy, we demonstrated the presence of the LCs and CCVCs in the yolk sac-Jejunum/Ileum tunnel system. And our data suggested that this system employed by embryo was in charge of transporting yolk sac liquid crystals to other tissues. We assumed that the liquid crystals could be the best form of nutrition in embryo development and early post-natal development.

List of abbreviations

LCs	Liquid Crystals.
CCVCs	calcium carbonate vaterite crystals.
YS-J/I tunnel system	Yolk Sac-Jejunum/Ileum tunnel system.

Competing interests

Guo Ling, Liyang Wang, Feng Rui, Zhongguang Li, Juan Wang, Kexing Ren,Xin Zhou, George Ghartey-Kwansah, MengMeng Xu, Odell Jones, Guifang Yan, Yuexin Pan, Joseph Bryant, Donald Anthony, Jianjie Ma, Williams Isaacs, Xuehong Xu declare that they have no conflict of interest.

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Authors' contributions

XXH conceived of the study. GL, WLY, FR, ZX and GGK developed protocols and collected all data and analysed the data. XXH, GL, WLY, FR, ZX, GGK, MMX, DA, JM and WI prepared the manuscript and all authors edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Availability of data and material

The data set supporting the conclusions of this article are included within the article.

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