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Methods of *In Ovo* and *Ex Ovo* Ostrich Embryo Culture with Observations on the Development and Maturation of the Chorioallantoic Membrane

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Abstract

Culture of shell-free and windowed eggs for drug testing and other experiments has been perfected for smaller eggs such as those of chickens, where the developing blood vessels of the chorioallantoic membrane (CAM) become accessible for manipulative studies. However, due to the thickness and hardness of the ostrich egg shell, such techniques are not applicable. Using a tork craft mini rotary and a drill bit, we established windowed egg, in-shell-membrane windowed egg, and in-shell-membrane shell–free methods in the ostrich egg, depending on whether the shell membranes were retained or not. Concomitant study of the developing CAM revealed that at embryonic day 16 (E16), the three layers of the CAM were clearly delineated and at E25, the chorionic capillaries had fused with the epithelium while the CAM at E37 had reached maturity and the chorion and the allantois were both 3–4 times thicker and villous cavity (VC) and capillary-covering cells were well delineated. Both intussusceptive and sprouting angiogenesis were found to be the predominant modes of vascular growth in the ostrich CAM. Development and maturation of the ostrich CAM are similar to those of the well-studied chicken egg, albeit its incubation time being twice in duration.

Key words: angiogenesis, chorioallantoic membrane, ostrich, shell-free culture

Introduction

The unique accessibility of the avian embryo and the chorioallantoic membrane (CAM) both in terms of acquisition of eggs, the ease at which both can be visualized by simply opening the shell, have made bird eggs an instructive model system for the study of development (Asai et al., 2021; Willoughby, 2014; Willoughby et al., 2016). The two most prominent avian species currently used to study development are the domestic chicken (*Gallus gallus* variant *domesticus*) and the Japanese quail (*Cortonix japonicum*), mainly due to their ease of breeding and availability the year round. For smaller eggs such as those of chickens, ducks, and quails, the egg shell is quite thin (less than 1 mm) and easy to crack using a scalpel blade (Makanya et al., 2015; Willoughby et al., 2016). In contrast, the ostrich egg shell is much thicker with measurements of up to 2.5 mm (Willoughby, 2014). As such, it is not possible to crack with a scalpel and the ordinary techniques used in those other species are not applicable. Therefore, it is imperative that better techniques for opening the eggs need to be devised, so that either a shell-free or windowed egg culture method can be achieved with minimal disturbance to the CAM or embryo. Previously, imaging of *in ovo* ostrich embryos has been accomplished using positron emission tomography (PET) combined with computed tomography (Freesmeyer et al., 2018). In the latter study, a small window to allow access to the blood vessels was made using a dremel rotating cutter. Conversely, Willoughby and colleagues (Willoughby et al., 2016) used a hacksaw to open ostrich embryonated eggs to study the developing embryo.

In the avian embryo, the CAM is a vascular membrane that results from the fusion of the mesodermal layers of two developmental structures: the allantois and the chorion (Fáncsi & Fehér, 1979). For much of the incubation period, the CAM is the sole source of gaseous exchange, with the situation changing only after internal pipping when the lungs become involved. Once the allantois and the chorion have fused, blood capillaries and sinuses invade the chorionic layer and become lodged in between the epithelial cells and in so doing gain close proximity with the ambient air (Fáncsi & Fehér, 1979). Very little is known regarding the development of the ostrich CAM. A recent preliminary investigation shows that the size of the CAM increases with the incubation period (Maina, 2017). In a recent study of the chicken embryo, it was shown that the chorion expands by recruiting cells from the mesenchymal layer and that after embryonic day 18 (E18), the CAM starts degenerating through apoptosis of the epithelial cells of the chorion (Makanya et al., 2016). Even in the absence of the shell and shell membranes, the various cell types of the chorion differentiate well, although they may be a bit delayed (Narbaitz & Jande, 1978). Presence in and transportation of calcium from the shell are necessary for proper differentiation of CAM (Dunn & Fitzharris, 1979).

The most commonly used eggs (quail, domestic fowl, duck, and turkey) pose no manipulation challenges but offer a limited surface area for xenograft implantation or multiple testing. An extensive morphometric investigation has shown rapid extension of the CAM surface area from 6 cm^2 at day 6 to 65 cm^2 at day 14 in the chicken (DeFouw et al., 1989)

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while in the ostrich, it expands from 104 cm^2 at E14 to 336 cm^2 by E28 (Willoughby et al., 2016). As such, the ostrich CAM potentially offers a remarkably greater surface area for investigative studies compared to the chicken egg.

Study of various angiogenic and antiangiogenic factors as well as tumor cell inoculation and drug testing requires that either the area vasculosa or the CAM be accessed in a shell-free culture or windowed egg method (Ribatti, 2008; Storgard et al., 2005). In the chicken embryo, the CAM by E8 covers 75% of the inner shell surface rising to 100% by E12 (Leeson & Leeson, 1963). Close to hatching, signs of degeneration have been reported in the cells of the chorionic layer (Makanya et al., 2016).

The physiological process through which new blood vessels form from pre-existing ones is known as angiogenesis (Risau, 1997): it encompasses two broad processes, namely, sprouting angiogenesis (SA) and intussusceptive angiogenesis (IA) (Makanya et al., 2009; Makanya & Djonov, 2009). The initial capillary plexuses expand through both SA and IA, but subsequent growth and remodeling are achieved through IA and the two processes may vary temporospatially (Makanya et al., 2005). The quintessence of IA is pillar formation, which is accomplished within a short time (4–5 h) after stimulation and occurs in the virtual absence of endothelial cell proliferation (Djonov et al., 2002). In contrast, SA is energy expedient, requires massive endothelial cell proliferation and migration (Makanya et al., 2009), and takes several hours to be completed. Both IA and SA have different phenotypes and any proangiogenesis or antiangiogenesis assay targeting either of the two needs to focus on their respective archetypical characteristics. In the chicken embryo CAM, both IA and SA have been shown to participate in the crafting and remodeling of the vasculature (Wilting et al., 1993) but no information exists on the preponderant processes in the ostrich CAM.

This study was motivated by the need to utilize the enormous CAM surface area that the ostrich potentially offers for manipulative investigations, considering that the ostrich egg is about 50 times larger than the size of the chicken egg. Additionally, study of the development and maturation of the ostrich CAM gives salient time points to allow informed decisions when doing manipulative studies using this model, especially when doing angiogenesis-related investigations. Furthermore, the relatively larger structures of the developing ostrich egg CAM may allow better interpretation of the morphological changes.

Materials and Methods

This study was approved by the Animal Ethics and Control Committee of the University of Johannesburg (RP 0785211-2015).

Animal Provenance

Embryonated ostrich eggs from South African Black Ostrich (*Struthio camelus australis*) were obtained from Klein Karoo International in the Oudtshoorn area of the Little Karoo province of South Africa. The eggs were carefully packed in paper cartons and cushioned with shredded paper to minimize shock and breakages and transported to the Department of Zoology, University of Johannesburg, where further incubation and experimentation were done. Eggs that were broken or had abnormal sizes (ranges outside 1100–1950 g, see Kokoszyński, (2017)) were discarded as well as those whose embryos were dead. The eggs were incubated and subjected to various procedures as described below. The numbers of eggs used per procedure are detailed in Table 1 below.

Incubation of Eggs to Obtain CAM

The incubation period of the ostrich egg at 42 days is twice that of the chickens. It has also been demonstrated that the lung development process is slower by a factor of two in the ostrich compared to the chicken (Makanya et al., 2012). To study the CAM, eggs were incubated in an Inco Therm incubator maintained at 36.5°C and 65% humidity.

Windowing of Eggs

Windowing of eggs allows access to the CAM while the embryo remains in-shell. To capture the developing CAM, eggs were windowed on E8. Windowing was done on the broad side with the air cell (top windowing). Air cell identification was done by candling (Kjelland et al., 2012).

A tork craft mini rotary drill fitted with a fine bit (Fig. 1a) was ideal for cutting free the top part of the egg shell to expose the CAM. A plastic cylinder 8 cm in diameter was used to outline the level at which the shell could be cut. This should be about 2 cm from the margin air cell (Fig. 1b). The egg was placed on a Styrofoam egg holder (Fig. 1c). Using the mouth cylinder, a circular line was drawn just above the lower margin air cell. A hole was drilled 2 cm below the margin air cell and 120 mL albumen siphoned out. The top-part egg shell was cut out using the rotary drill along the previously marked line.

The egg may be left as an in-shell membrane windowed egg culture method, in which case the shell membrane is left intact (Fig. 2a). Alternatively, the shell membrane is removed so that the method now is shell membrane–free windowed egg, the shell covering the air cell is deflected to reveal the CAM (Fig. 2b).

Rubber rings of different colors may be placed on the CAM to delineate areas for drug application (Fig. 2c). The air cell cover can easily be reattached using duct tape and the cover secured over the air cell to re-establish local conditions for CAM/embryo development (Fig. 2d).

Table 1. Number of Animals Used per Each Technique.

S/no.	Technique	Number of animals	Remarks	Total
1	Windowed in-shell membrane	3		3
2	Windowed shell membrane free	3		3
3	*Ex ovo* shell membrane-free	4		4
4	*Ex ovo* in-shell membrane	3		3
5	Partial *ex ovo* in-shell membrane	3	This group had a piece of shell left intact	3
6	LM/TEM/SEM	3 animals for each stage	E16, E25, E29, E36, and E37	15
7	Corrosion casting	2 animals for each stage	E16, E25, E29, E36, and E37	10

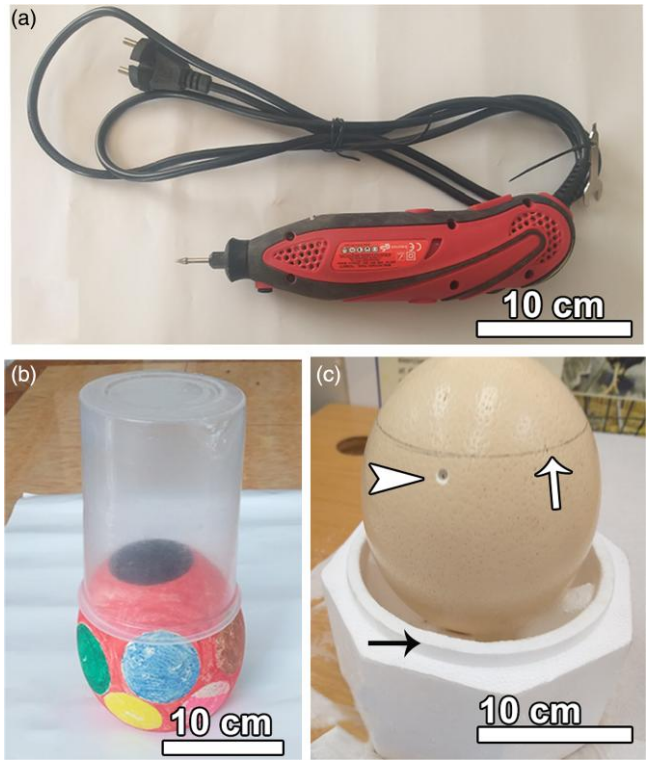

Fig. 1. Photograph showing the tools necessary in making a windowed ostrich egg. (**a**) The tork craft mini rotary and drill bit are ideal for cutting free the top part of the egg shell to expose the area vasculosa/CAM. (**b**) A plastic cylinder of diameter 8 cm is used to outline the level at which the shell should be cut. This should be about 2 cm from the margin of the air cell. This egg shell was painted as part of ornamental use of ostrich products (courtesy—Lydia Ndegwa). (**c**) The egg is placed on a Styrofoam egg holder (black arrow). A hole (white arrowhead) is drilled 1 cm below the margin of the air cell and 120 mL of the albumen siphoned. The top part of the egg shell is cut out using the rotary drill along the previously marked latitude (white arrow). The hole should be sealed with duct tape soon after withdrawal of the albumen.

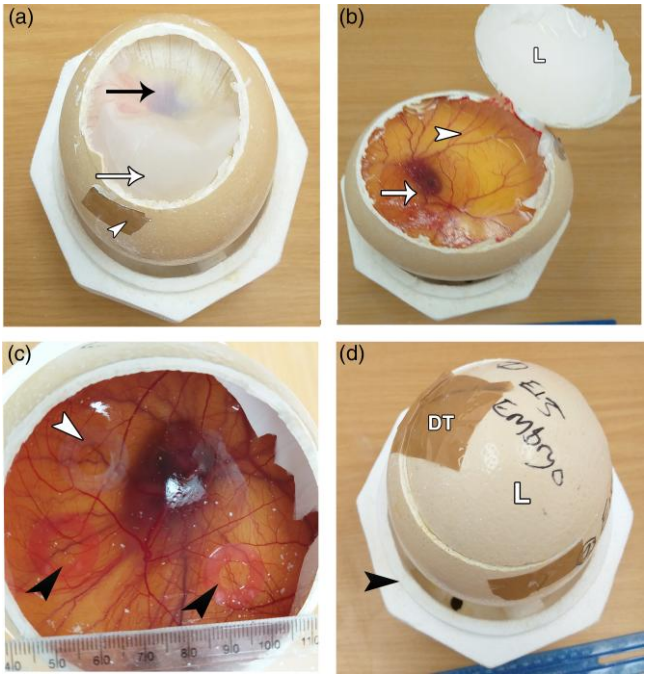

Fig. 2. Photographs showing the steps in making a windowed ostrich egg. (**a**) This photograph shows in-shell membrane windowed egg culture method. Notice that in this case, the shell membrane (white arrow) is left intact. The embryo appears as a dark shadow (black arrow) below the shell membrane. The hole through which albumen is withdrawn is sealed with duct tape (arrowhead) after the fact. Blood vessels of the CAM and the embryo are faintly visible below the inner shell membrane. (**b**) Alternatively, the shell membrane is removed so that the method now is the windowed egg method. The shell (**L**) covering the air cell is deflected, and the inner shell membrane is removed so that the CAM (arrowhead) and embryo (arrow) can be revealed. (**c**) Rubber rings of different colors (arrowheads) may be used to delineate areas for drug application. (**d**) The air cell cover (**L**) can easily be reattached using duct tape (DT), and the cover can be secured over the air cell to recover local conditions for CAM/embryo development.

Shell-Free Culture Method

While opening of the incubated ostrich eggs, we took into consideration the fact that the incubation period is twice that of the chicken egg. Here, to study the CAM, eggs were incubated for 6 days, the egg to be opened was put in a vertical position for about 1 min (egg holder), and the upper part was marked with a pencil as described above. The egg was then opened using the rotary drill cutter along the marked line. The contents were then released onto the top of a previously prepared 10-L plastic bucket with a cling film cover to support the embryo and the membranes (Fig. 3a) (for details, see also Makanya et al., 2015) (Makanya *et al*, 2015). The culture was returned to the incubator, maintained at 36.5°C and 65% humidity. The buckets served as plastic cups were used elsewhere (Makanya et al., 2015). In case of the shell-free shell membrane–free method, the embryos were found to die rather quickly either due to breakage of the yolk or of the CAM blood vessels, and as such, the method was discontinued. The ostrich outer egg shell membrane is quite strong and is capable of holding the contents intact as shown in Figure 3. The preferable method was to remove the hard shell and leave the shell membranes intact (Fig. 3a). Shell dust may be placed on the shell membrane as a source of calcium (Fig. 3b) necessary for growth of the skeleton (Makanya et al., 2015).The dome from the wrap paper (Fig. 3c) should be big enough to allow embryo movements. Alternatively, a component of the shell may be left intact as a source of calcium (Fig. 3d) while the rest of the embryo-CAM complex is covered by the shell membranes.

Microscopy

At selected time points, embryonated eggs were opened and the embryo together with all the membranes and their contents carefully separated from the shell but maintained within the shell membrane. Embryos at 26 days or older were anaesthetized by intraperitoneal injection of sodium pentobarbitone. The CAM was carefully separated from the embryo and yolk sac and washed in phosphate-buffered saline, immersed in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 350 mOsmol/kgH2O). As an alternative method for older embryos (over 26 days), the entire embryo and CAM were first flushed with heparinized physiological saline through the vitelline artery (vitelline vein opened) and then fixed by perfusion through the same artery.

The embryo mass was obtained, and after at least 4 h of fixation, the CAMs were examined under a dissecting microscope and any remnants of shell membranes, albumin, or yolk were carefully cleaned off with fine forceps. The total volume of CAM was obtained by the Scherle method of volume

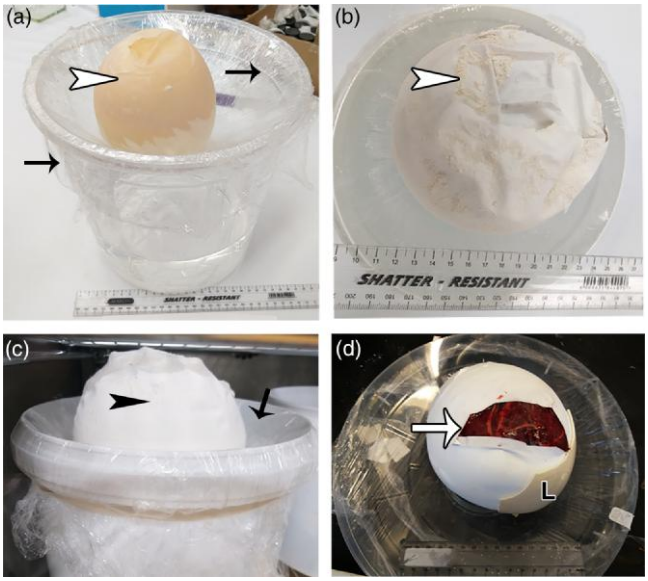

Fig. 3. Photographs demonstrating shell-free in-shell membrane culture methods. (**a**) This bucket method is analogous to the cup method for the chicken egg. The ostrich egg shell membrane (white arrowhead) is quite strong and is capable of holding the contents intact as shown in this photograph. This is an unfertilized egg 12 days post hatching. Three liters of distilled water are placed in a 10-L transparent plastic bucket. A cling film cover (black arrows) is placed at the opening and pressed to make a dome that support the shell-less egg. (**b**) The in-shell membrane culture method at E16 on a tray. Removal of the egg shell is done as described before, and then, the egg is placed on a large plastic tray. Use of the plastic tray is analogous to the petri dish culture method described elsewhere (Makanya et al., 2015). Shell dust (arrowhead) is placed on the shell membrane as a source of calcium. (**c**) The in-shell membrane culture method at E16 using the bucket method. This method is similar to the one described in (**a**) above. The egg in this case had been fertilized and incubated for 16 days. The shell membranes in the fertilized eggs looked whiter (arrowhead), probably due to deposition of calcium. The dome (arrow) creates more space for possible embryo movements. (**d**) Partial shell-free in-shell membrane culture method. A component of the shell (L) is left intact as a source of calcium. Notice a window (arrow) opened on the shell membrane to access the CAM. The shell membrane at this stage is tougher, probably due to incorporation of calcium.

displacement (Makanya et al., 2016; Scherle, 1970), and data were stored for future investigations.

Fixed CAM was placed on wax plates and diced into quadrats measuring 5 cm × 5 cm. Samples to be processed further were picked by systematic random sampling (Cruz-Orive & Weibel, 1981). The selected slice was divided into two halves, one of which was processed for light microscopy (LM, paraffin embedding) while the other one was processed for transmission electron microscopy.

Light and Transmission Electron Microscopy

For LM and TEM, CAMs were cut into smaller slices and postfixed in osmium tetroxide, block stained using uranyl acetate, dehydrated through ascending concentrations of ethanol, and embedded in epoxy resin. Semithin sections were obtained at a nominal thickness of 1 μm, stained with toluidine blue, and viewed under a digital light microscope. Ultrathin sections were obtained at 90 nm, counterstained with lead citrate, and viewed on a transmission electron microscope.

Scanning Electron Microscopy

For SEM, samples were dehydrated with ethanol and exposed to two changes of hexamethyldisilazane (HMDS) (Sigma-Aldrich, St. Louis, MO, U.S.A). The samples were mounted on aluminum stubs and sputtered with a gold–palladium complex. Care was taken to note the chorionic and allantoic surfaces of the CAM while mounting on stubs. The samples were viewed on a TESCAN®VEGA3 scanning electron microscope (Brno, Czech Republic) at an accelerating voltage of 8 kV.

Corrosion Casting

To study sprouting and IA, the CAMs was perfused with a solution of 0.9% sodium chloride containing 1% heparin through the vitelline artery. The vasculature was then filled with polyurethane containing 0.1 mL accelerator per every 5 mL of the resin. One hour after perfusion, the CAM was immersed in Ringer's solution for at least 2 h and subsequently transferred to a 15% potassium hydroxide solution for 2 weeks. After dissolution of the tissues, casts were washed, dehydrated in ascending concentrations of ethanol, and dried in a desiccator. Samples were dehydrated with ethanol and exposed to two changes of HMDS (Sigma-Aldrich, St. Louis, MO, U.S.A). The samples were sputtered with gold–palladium and viewed on a TESCAN®VEGA3 scanning electron microscope (Brno, Czech Republic) at an accelerating voltage of 8 kV.

Results

The various methods described above resulted in several CAM culture models defined by the presence or absence of shells or shell membranes, or parts thereof. The results of the various approaches and the CAM general developmental process are described below.

Chick CAM and Embryo Culture Models

Windowed Egg Model

The ostrich egg shell is quite thick and hard, and drilling out the window required careful manipulation. Withdrawal of 120 mL of albumen creates enough space for manipulation at the air cell level and reduces the chances of rapturing the shell membranes. Windowed eggs were of two types: the in-shell membrane windowed egg method resulted in a model whereby the shell membrane is left intact (Fig. 2a) or the shell membrane may be removed, i.e. the shell membrane–free windowed egg model (Fig. 2b). In the former case, localization of specific parts for drug application can be difficult since the CAM is not directly accessible. The shell membrane is porous, and test substances in liquid form can be applied directly. The shell membrane–free culture model allows localization of application sites using sterile rubber rings (Fig. 2c). The window cover can be retained with duct tape to secure homogeneous conditions after drug application (Fig. 2c).

Shell-Free Culture Models

In this category, three approaches were designed. The bucket method was analogous to the cup culture method used in the chicken CAM (Makanya et al., 2015) whereby a clean, sterilized 10-L transparent bucket was used (Fig. 3a). The bucket was half-filled with distilled water, and the opening was covered with kitchen wrap polythene paper and pressed down to create a shallow dome.

Shell-Free In-Shell Membrane Culture Model. In this case, the egg shell was opened as described before but the entire shell was carefully broken out with forceps, leaving the embryo and albumen enclosed by the shell membranes (Fig. 3). Shell dust was applied on the shell membrane to provide the calcium necessary for the development of the embryo (Fig. 3b).

Partial Shell-Free In-Shell Membrane Culture Model. Alternatively, a portion of the shell may be left intact for the purpose of availing some calcium for resorption, what aids in bone development in the embryo (Fig. 3c). Embryo movements were easily observable in the shell-free in-shell membrane culture method (data not shown), and as such, the dome formed on the bucket provided the space for such movements (Fig. 3d). The in-shell membrane shell–free method is another possibility of raising the ostrich CAM. A small window is made on the air cell side of the egg to allow for direct drug applications (Fig. 3c). While the latter method potentially allows localization of drug application sites, embryo movements may be limited since the egg would have to be firmed in an upright position.

Complete Shell-Free Shell Membrane-Free Culture Model. This method is the one normally used for the eggs of the smaller avian species. The eggs were opened, and the contents were carefully and slowly poured out onto the plastic dome of a previously prepared 10-L plastic bucket as described above. In most cases, the yolk would break either during the pouring out process or soon after the contents settled on the polythene dome. The method was thus discontinued.

Development of the CAM

At E16, the three layers of the CAM, namely, the chorion (Fig. 4), the mesenchyme, and the allantois, were clearly delineated. The E16 chorion comprised a single layer of low cuboidal epithelial cells beneath which developing chorionic capillaries approached the epithelium to form the thin blood–gas barrier (BGB) portions. The allantois at this stage had a single layer of low cuboidal cells (Fig. 4).

At E25, the chorionic capillaries had fused with the epithelium, the mesoderm had both supplying and draining vessels but the allantois still had a single layer of cuboidal cells. The CAM at E37 had reached maturity and the chorion and the allantois were both 3–4 times thicker. Distribution of cells in the mesenchymal layer was almost uniform.

At transmission electron microscopic level, areas of the thin BGB were discernible even at E16 where other areas of the chorion had a bilayered epithelium with the exchange capillaries developing beneath. The allantois at E16 was a single layer of broadened low cuboidal cells with several vacuoles and short microvilli. At E25, there were more portions of thin BGB and the exchange capillaries were more superficial.

The chorion was well differentiated at E29 (Fig. 5) with clearly delineated basal cells (BC); well-developed villous cavity (VC) cells and the chorionic capillaries were superficially positioned with portions of thin BGB. The capillary covering (CC) cells had their nuclei shifted to the inner side of the capillaries, away from the thin BGB. The allantois already had two well-developed cell layers and an inner developing one (Fig. 6). At E37, both mature portions of the CAM and others with degenerating cells were encountered. The allantois had three well-developed layers of cells with the outermost layer-bearing numerous short microvilli. The VC cells had numerous microvilli and a superficial/apical cytoplasmic process that spread out and anastomosed with those of their neighboring cognates (Fig. 6).

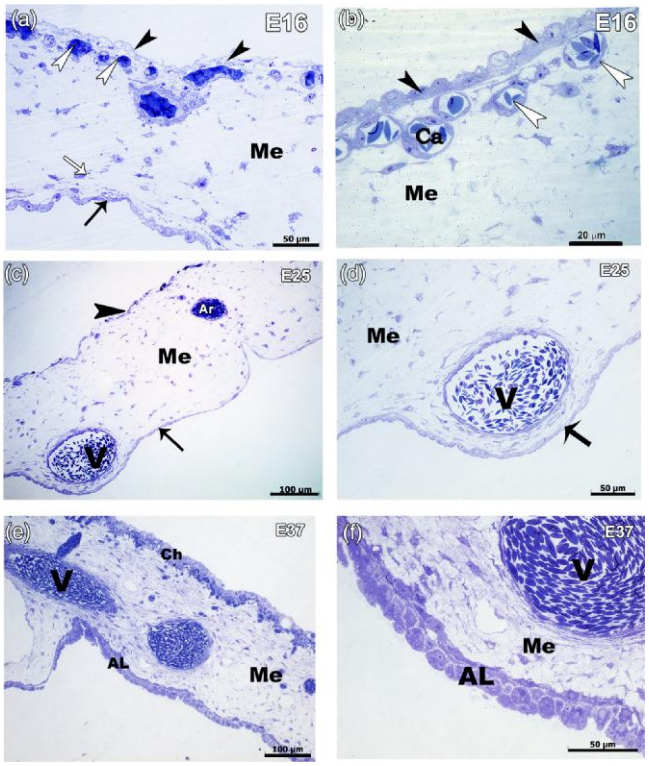

Fig. 4. Semithin micrographs showing the changes in the developing CAM between days 16 and 37. (**a, b**) At E16, the three layers of the CAM, namely, the chorion (black arrowheads in **a**), the mesenchyme (Me), and the allantois (black arrow in **a**), are clearly delineated. Note that the chorion has a single layer of low cuboidal epithelial cells (arrowheads in **b**) beneath which chorionic capillaries are developing (ca in **b**). (**c, d**) At E25, the chorionic capillaries have fused with the epithelium and the mesoderm (Me) has both supplying (Ar) and draining (V) vessels. Notice that the allantois (arrows) still has a single layer of cuboidal cells. (**e, f**) At E37, the CAM has reached maturity and the chorion (Ch) and the allantois (AL) are both much thicker as a result of differentiation of VC cells in the chorion and increase in the number of cell layers in the allantois. Distribution of cells in the mesenchymal layer (Me) is almost uniform. The large draining vessels (V) are also indicated.

At the scanning electron microscopic level, the chorion (Fig. 7) had well-delineated CC cells, and where they had peeled off, the gas exchange capillaries were clearly visible. In such areas, the VC cells and their superficial interconnecting processes were also evident. Surface view of the allantois also revealed the broadened cells with the short microvilli and rather prominent intercellular junctions (Fig. 7). The three-dimensional pattern of the developing CAM vasculature as visualized by corrosion casting (Fig. 8) revealed the various categories of blood vessels as well as the preponderant methods of angiogenesis. The mature CAM at E37 comprised of large supplying arteries and even larger draining veins and in between the two was the meshwork comprising the arterioles, capillaries, and the venules. The arterial system started at the umbilical artery and the venous system drained into the umbilical vein (data not shown). The preponderant modes of angiogenesis at E29 were IA represented by pillars and SA evidenced by capillary sprouts. The tissue pillars had expanded to tissue meshes by E37, and capillary sprouts were no longer present at this stage, showing capillary maturation.

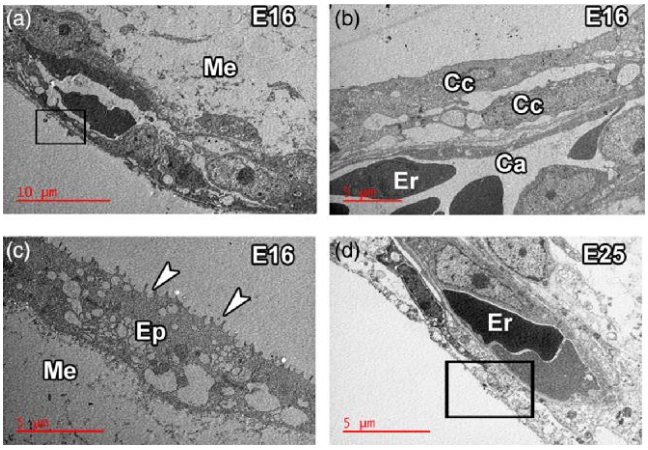

Fig. 5. Transmission electron micrographs (TEM) showing the changes in the developing CAM at E16 and E25. (**a, b**) At E16, the chorion has some thin portions of the blood–gas barrier (rectangle in **a**) and one to two layers of CC cells (CC in **b**) are present in some areas. The developing chorionic capillaries (ca in **b**) with large erythrocytes (Er) are closely associated with the CC cells. (**c, d**) At E16, the allantois has a single epithelium (Ep) of low cuboidal cells with large vacuoles and numerous short microvilli (arrowheads). Next to the allantois is the mesoderm (Me) with abundant mesenchyme. (**d**) At E25, the chorionic capillaries have fused with the epithelium, forming a much thinner blood–gas barrier (rectangle). Er denotes the nucleus of an erythrocyte.

Discussion

The chick CAM shell-free culture method is a well-established model for study of angiogenesis, tumorigenesis, and development (Kundeková et al., 2021). The most commonly used eggs (quail, domestic fowl, duck, and turkey) pose no manipulation challenges but offer a limited surface area for xenograft implantation or multiple testing. Opening of the ostrich egg is, however, not easy because the shell is thick (~1.83 mm on average) and hard (Willoughby et al., 2016), and thus cutting inevitably results in vibrations and subsequent breakage of the CAM capillaries. Kjelland et al. (2017) have indicated that of 36 windowed ostrich eggs, 1 embryo survived and hatched and the efficiency of the windowing and embryo transfers to produce chicks was low and further refinements of the methods are needed. (Kjelland et al., 2017). It is for this reason that we endeavored to investigate possibilities of doing windowed ostrich eggs as well as shell-free CAM culture. Minimizing vibrations during cutting of the shell was seen to be important in the survival of the embryos as this reduced breakage of the CAM blood capillaries. Two methods of windowed egg were recommended, namely, the in-shell membrane windowed and the shell membrane–free windowed. For the shell-free method, three categories were described: the in-shell membrane shell free, the in-shell membrane semi-shell–free, and the shell-free shell membrane free. In the latter case, it was extremely difficult to keep the yolk intact, both during preparation and even after preparation due to the enormous size of the yolk. It was possible to maintain the in-shell membrane shell-free embryos but it was necessary to spray them with sterilized physiological saline on the shell membrane to prevent desiccation and dehydration. The extent of survival or hatchability was, however, not examined, but an E16 embryo was maintained until E21. The shell-free in-shell membrane culture method also allowed creation of a window for access to the CAM blood vessels. This latter method was

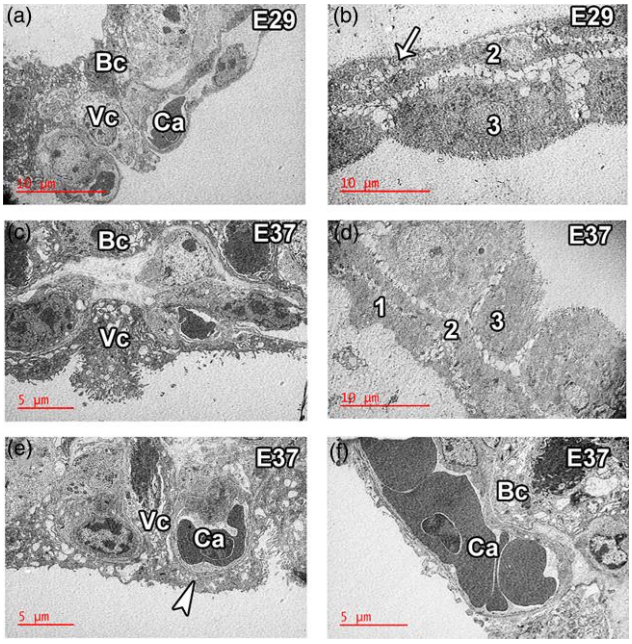

Fig. 6. TEM micrographs showing the changes in the developing CAM at E29 and E37. (**a, b**) At E29, the BC, the VC cells, and the chorionic capillaries are well developed. The allantois already has two well-developed cell layers (2 and 3) and an inner developing layer (white arrow). (**c, d**) At E37, the chorion has well-developed BC and VC cells and the allantois has three well-developed layers of cells (1, 2, and 3). (**e, f**) In some instances, at E37, some cells of the chorion (VC in **e** and BC in **f**) show signs of degeneration. Notice the cytoplasmic extensions from the VC cells (arrowhead in **e**) and the prominent superficial chorionic capillary (Ca in **f**).

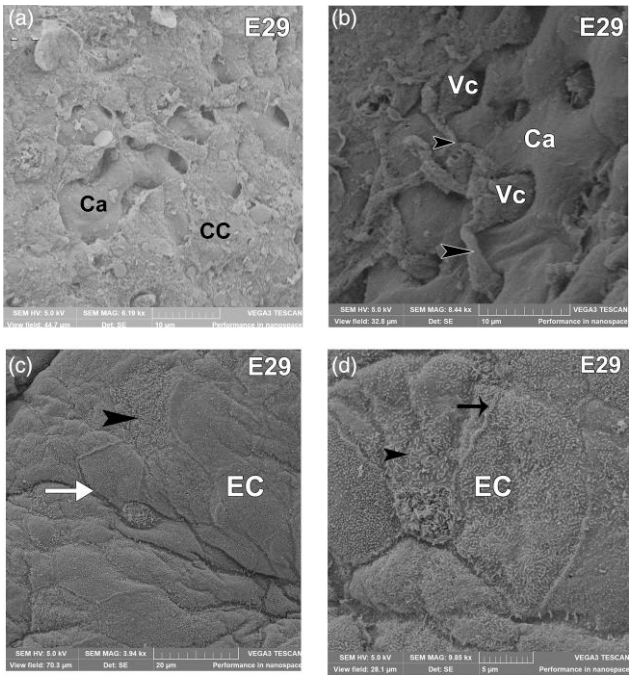

Fig. 7. Low magnification surface view of the chorion (**a, b**) and the allantois (**c, d**) studied using the scanning electron microscope (SEM). (**a, b**) At E29, the exchange capillaries (Ca) of the chorion are visible where the CC cells have peeled off. At a higher magnification, (**b**) both the VC cells and the exchange Ca are visible. Notice that the VC cells send processes that join those of neighboring cognates (arrowheads). (**c, d**) At E29, the outermost cells of the allantois (EC) are broad and are studded with short microvilli (arrowheads) and are delineated by very prominent apical cell junctions (arrows).

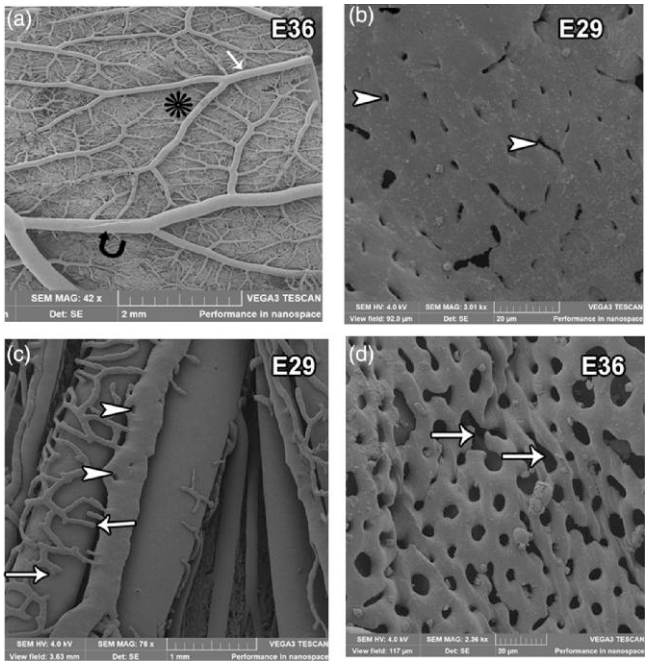

Fig. 8. Intravascular casts of the developing ostrich CAM viewed under SEM. (**a, b**) At E36, a well-developed vascular network with large supplying vessels (white arrow) and even larger draining vessels (curved arrow). These vessels give branches that form a dense capillary network (asterisk). (**b**) At E29, the early pillars that delineate the capillary meshwork (white arrowheads). (**c**) In addition to the pillars (arrowheads), capillary sprouting is also an important process of anagenesis in the ostrich CAM at E29. Notice the early sprouts (arrows) and the tiny pillars (arrowheads). (**d**) By E36, most pillars have expended to form tissue meshes (arrows) showing maturation of the capillary network.

found to be convenient because it could be achieved at any time of incubation as well in non-embryonated eggs, and the entire surface of the shell membrane was available for manipulation. Opening of the eggs at later stages of incubation may prove advantageous since the shell becomes progressively thinner. The egg shell thickness varies with incubation from 1.92 mm at day 0 to 1.8 mm at day 42 (Amer, 2012), and this is probably due to calcium resorption by the embryo across the circulatory system. However, care should be taken to avoid opening too close to hatching. In the ostrich, the sequence of the average hatching process is for the chick to pip first into the air cell on day 40 (internal pipping), then pip through the outer shell during day 41 (external pipping), and complete hatching by day 42. This, of course, is subject to normal biological variations (Berry, 1996).

In addition to establishing the culture methods, growth of the ostrich CAM was studied at selected time points between E16 and E37. It was observed that at E16, the CAM resembled that of the chicken embryo at E8 (Makanya et al., 2016) where a few portions of BGB were available and the VC cells were not differentiated. At this time, the incipient exchange capillaries are seen developing close to the formative chorionic epithelium; the latter epithelium forms the CC and the VC cells visible from E25. As in the chicken CAM, both the chorion and the allantois grew plausibly by recruiting stem cells from the mesodermal layer beneath. As the exchange capillaries abutted the CC cells, the nuclei of the latter cells shifted location to lie on the ventral (mesodermal) surface of the capillaries, thus allowing formation of thin portions of BGB. An interesting finding was that neighboring VC cells had a large interconnecting cytoplasmic process, besides the preponderance of large apical microvilli. These cells are known to contain carbonic anhydrase enzyme (Gabrielli et al., 2001; Rieder et al., 1980) that is important in calcium resorption. Indeed, Tuan & Zrike (1978) showed that carbonic anhydrase activity in the CAM increased in an age-dependent fashion during embryonic development and is related to calcium deposition in the embryonic skeleton (Tuan & Zrike, 1978). The large cytoplasmic processes associated with the ostrich VC cells may be important in augmenting calcium resorption, considering that the ostrich egg shell with a thickness of about 1.92 mm (Amer, 2012) is about 5 times thicker than that of the chicken egg estimated at about 0.367 mm (Sun et al., 2012). VC cells differentiate progressively until their large microvilli reach the egg shell membranes and the mamillary processes are eroded as calcium resorption takes place (Halgrain et al., 2022). Further studies on the developing ostrich CAM would elucidate the actual function of the VC cell processes and their relation to calcium resorption.

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Author Contributions Statement

A.N.M. and J.N.M. conceived and designed the experiments; A.N.M. and S.A.J. performed the experiments; A.N.M., S.A.J., and J.N.M. analyzed the data. A.N.M. wrote the paper; S.A.J. and J.N.M. edited the manuscript.

Conflict of Interest

The authors declare that they have no competing interest. The embryonated ostrich eggs were fully paid for at commercial rates.

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