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MORPHOLOGICAL AND HISTOLOGICAL STUDY OF THE CHORIOALLANTOIC MEMBRANE OF CHICK EMBRYO

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Abstract: In present study, morphological and histological structure of the chorioallantoic membrane (CAM) of domestic chick (Gallus gallus) were assessed between days 4 and 11 of embryonic development. Natural CAM at day 4 showed normal morphology with clear directional pattern of blood vessels; continued with day 6 where major and minor CAM vessels were observed with dendritic branching pattern formed by process of vasculogenesis (derived from angioblasts) and angiogenesis (sprouting of existing vessels). Vessels were designated as primary, secondary, and tertiary vessels. The vascular density was increased with tortuosity at 11 days of incubation. Histologically the CAM showed three distinct layers; the ectoderm with numerous small capillaries, the stromal mesoderm with sparsely arranged fibrous tissue and the endoderm with normal architecture. CAM at early stage of development could be used for cardiovascular and toxicity studies.

Key words: Chorioallantoic membrane, *Gallus gallus*,

INTRODUCTION

Chicken eggs in the early phase of breeding may provide an immunodeficient, vascularized test environment [1]. The chorioallantoic membrane (CAM) of the chicken embryo is one of the most important extra-embryonic membranes formed on the fourth day of incubation by the fusion of two extra-embryonic membranes: chorion and allantois [2,3]. It is attached with the inner shell membrane and provides barrier between the watery environment of the embryo and the inner space. It serves as a gas exchange surface [3]. Its respiratory function is provided through an extensive capillary network [4]. It is a highly vascularized membrane which lies inside the surface of egg shell and is relatively thin and transparent. It facilitates oxygen, calcium and nutrient transport to the embryo [5,6].

The early developmental stages of chick embryo are useful because the initial formation of the capillary plexus occurs at this time [7-9]. This is a period of rapid neovascularization indicating normal development of blood vessels and capillaries [10,11]. CAM area provide a bed for vasculature generation and sprouting in addition to its nutritional and waste management significance.

The in vivo CAM assay is well established and widely used as reliable biomedical assay system for the study of complex physiologic processes such as vasculogenesis, angiogenesis or metastasis. The extent of the angiogenesis or vascularization in the chorioallantoic membrane can be quantified by morphometric measurement directly by counting the number of "vessel endpoints" with or without the assistance of a computerized image analysis system [12]. In present study, the CAM with vascular development were assessed at day 4, 6 and 11 of all normal embryos. The morphology of pre- and post-

capillary vessels in the chick CAM were studied by focusing on morphometric and microscopic parameters that quantify vascular complexity and density with vascular branching pattern and tortuousity.

MATERIALS AND METHODS

The embryos used in this study were natural embryos. Fertilized eggs of Gallus gallus (0 hrs) were obtained from the Assistant commissioner of animal husbandry, central hatchery, Kolhapur. The shells of fertilized eggs were disinfected and incubated at 37.50C temperature with relative humidity of 70-75% in aseptic conditions. The eggs were incubated for different periods of time to obtain CAM of different embryonic ages i.e. 4, 6 and 11 days. Infertile and dead embryos were identified by candling on 96 hrs of incubation and were removed. Absence or fading of prominent chorioallantoic blood vessels or lack of embryo movement were taken to indicate infertility or death of the embryo. The development was continued until completion of different experimental days.

On completion of scheduled incubation periods, phosphate buffer saline (PBS) was used to wash the surface of the CAMs, following which shells were removed and CAM was carefully separated from the inner shell membrane in the glass plate with PBS containing glycerin. Photographs of the developing CAM with vasculature was obtained with a digital camera and exported to a computer for image analysis. Visual assessment of the vessels and surface of the CAM with the naked eye was performed as described in our previous work [13,14]. The same was confirmed with direct stereomicroscopic observations of well spread embryonic plates on glass slides of appropriate dimensions.

Histological preparation: For histological evaluation, the CAM was surgically removed, fixed in 10% buffered formaldehyde for 8 to 12 hrs and was processed for light microscopy. The membrane was immersed on increasing concentrations of ethanol and infiltered with paraffin (melting point 58-600C). Serial sections (5µm) were cut in a plane parallel to the surface of the CAM and further processed for stained preparation of haematoxylin-eosin which was observed under a Leitz-Dialux 20 light photomicroscope (Leitz, Wetzlar, Germany).

RESULTS AND DISCUSSION

In the present work, normal growth of CAM was assessed from day 4 to day 11. It is a period of rapid vascular development that accompanies rapid overall growth of the CAM. In chick CAM primary vascular network is formed by vasculogenesis attributed de novo formation of a primitive vascular network by differentiation of precursor cells (angioblasts) into endothelial cells (ECs). While new blood vessels are formed by spurting angiogenesis from pre-existing blood vessels.

Macroscopic observation of natural CAM on day 4 showed less number of vitelline veins but normal pattern formation of blood vessel formation was observed (Fig.1A). On day 6, normal pattern of blood vessel formation with normal CAM development was noted (Figs. 1B,1C). It showed normal architecture of major and minor vessels observed with dendritic pattern with hierarchical sizes of blood vessels. It was also showing normal neovascularization with uninterrupted and clear directional patterns of the blood vessel development. Vessels were designated as primary, secondary, and tertiary vessels. The major mechanism for the network of blood vessel formation is sprouting of pre-existing vessels i.e. angiogenesis. The spaces within the plexus is subdivided by intussusceptive angiogenesis [15]. It occurs by formation of transcapillary pillar [16-18].

The CAM of 11 days development showed improved CAM area on which primary veins were extended. Alongwith primary vitelline veins, secondary and tertiary blood vessels were also clearly recognized with increased tortuosity and random blood vessel orientation. It showed presence of numerous large and small blood vessels with radial orientation of the secondary and tertiary blood vessels and no disturbance of CAM structure (Fig. 1D).

Light microscopy of the chorioallantoic membrane of the chick after various days of incubation was also carried out. Histological analysis of haematoxylin-eosin stained sections of 144 hrs natural CAM showed three distinct layers, the ectoderm, the stromal mesoderm and the endoderm. The ectoderm and endoderm layer were flattened and single-layered. The ectoderm showed normal development of numerous small capillaries. The mesoderm showed sparsely arranged fibrous tissue with few scattered blood vessels, which developed in normal patterns. Red blood cells were also observed inside blood vessels. There were numerous ectodermal sparse cells around capillaries that are distributed in the regular manner.

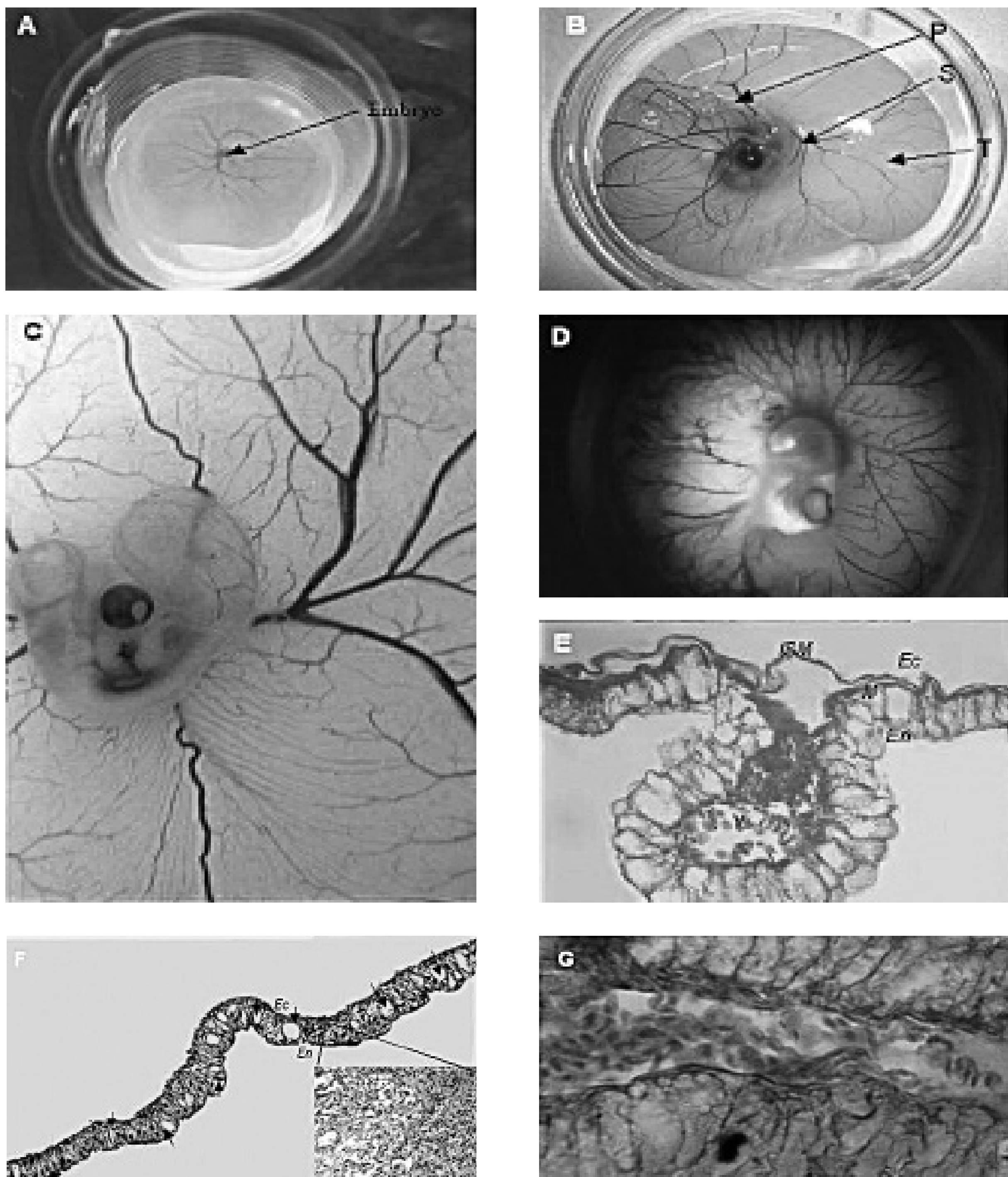

Figure 1. Macroscopic and histological observation of Natural CAMs: A. Natural CAM at day 4, showing less number but clear directional patterns of blood vessels. **B.** The CAM at day 6, showing normal morphology of major and minor vessels with dendritic pattern with hierarchical sizes of blood vessels designated as primary, secondary, and tertiary vessels. **C.** Chick embryo observed with vitelline veins (Major blood vessel). **D.** CAM at day 11, showed increased tortuosity with presence of numerous large and small blood vessels. **E.** Histological structure of CAM at day 6, showed a single, large blood vessel (V) deep in the mesoderm; surrounded by unstained sparse cells and veins. Avian erythrocytes (RBC) were observed inside blood vessel. Thin walled capillaries just beneath the chorionic epithelium. **F.** Histological structure of CAM at day 6, showed three layered CAM i.e. ectoderm, mesoderm and endoderm. Numerous small capillaries with major blood vessels along with small vessels also visible at stroma region. **G.** Section of blood vessels showing endothelial cells and basement membrane with scattered RBCs. (P-Primary vitelline veins, S-Secondary vessels, T-Tertiary vessels, V-major blood vessel, ISM- Inner shell membrane, Ec-Ectoderm, M-Mesoderm, En-Endothelium).

It showed increase in the number and size of blood vessels with nucleated erythrocytes and an increase in fibrous tissue density (Figs. 1E,1F). Unstained veins and lymphatic vessels surround a major artery deep in the mesoderm. Fig. 1G showing lumen of blood vessels with normal endothelial cells and basement membrane which are the fundamental component of blood vessels. They are not just the structural components of vessel walls but also take part in the regulation of angiogenesis by secreting proangiogenic factors and proteases [19].

At day 4 to 6, the capillary network forms and expands. The mesodermal blood vessels migrate closer to the ectoderm and differentiate into a plexus by vasculogenesis, followed by sprouting angiogenesis [11]. They grow very rapidly until day 8; when some vessels differentiate into capillaries and form a layer at the base of the ectoderm. The inner allantoic wall covers the amnion and the yolk sac, and fuses with the amnion at day 7 [3]. The blood vessels from the embryo are connected with the ones from the CAM via the allantoic stalk. From day 7 to 11, intussusceptive microvascular growth, with associated lower endothelial cell proliferation occurs [18]. The CAM extends to line the entire surface of the inner shell membrane and completely surrounds the embryo [20]. At day 14, the capillary plexus is located at the surface of the ectoderm, adjacent to the shell membrane [21]. The respiratory exchange in the CAM occurs by means of an extensive capillary plexus that develops initially adjacent to the chorionic ectoderm and later interdigitates the ectodermal cells of the chorion [7,9]. In the chorioallantois, the veins contain bright red blood as higher O2 contents. After pulmonary respiration begins, the CAM circulation become less active and many vessels turn out to be empty [3].

The structure and function of it is placenta like tissue consisting three distinct layers viz., an ectodermal layer facing the egg cell, a sparsely populated mesodermal layer, and an endodermal layer lining the allantois [22-24]. The structure allows the embryo to harvest the calcium from the shell for bone development. It has also shown that vascular CAM transports essential nutrients and gases to the graft, thereby facilitating differentiation and cartilage formation in the limb. The CAM includes the chorioallantoic fluid into which waste products are delivered. Its two-dimensional vascular structure can be seen entirely with minimal preparation. This is one of the major reasons it has become a popular assay tissue for putative angiogenic and antiangiogenic substances [25]. The CAM is a useful tool to studying angiogenesis because. It is a menable both intravascular and topical administration of study agents. It is a relatively rapid assay and can be adapted very easily to study angiogenesis dependent processes such as wound healing and tumor growth. In our earlier laboratory work we investigated influence of various plant extracts and biomaterials on CAM development, vasculogenesis and angiogenesis [13,14, 26-28].

Angiogenesis and vasculogenesis are accompanied with the development and extension of CAM. Its area influences healthy development of embryo. Extension of all the types of blood vessels and capillaries occurs in the bed of CAM and its development controls both vasculogenesis and angiogenesis influencing the length of vessels. Angiogenesis is powered by several angiogenic and antiangiogenic factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factors (aFGF, bFGF), and transforming growth factor-beta (TGF-β) are the most common. Angiogenesis is influenced by vasculogenesis. Some of the factors that regulate the angiogenesis also regulate the angiogenesis. VEGF and FGF acts as an autocrine or paracrine stimulator of angiogenesis [29]. The basic and probably acidic FGF play an important role in the regulation of the chorioallantoic vascular growth by their angiogenic and mitogenic activity [30].

REFERENCES

[1] Kunzi-Rapp, K., Genze, F., Kufer, R., Reich, E., Hautmann, R.E. and Gschwend, J.E.: J. Urol., 166(4): 1502-1507 (2001).

[2] Defuow, D.O., Rizzo, V.J., Steinfeld, R., Feinberg, R.N.: Microvasc. Res., 38: 136-147 (1989).

[3] Romanoff, A.L.: The avian embryo: Structural and functional development. New York: MacMillan, pp 1039-1041 (1960).

[4] Billet, F.S., Collini, R. and Hamilton, J.: Embryol. Exp. Morph., 13: 341-356 (1965).

[5] Tuan, R.S.: J. Exp. Zool.,1: 1-13 (1987).

[6] Richardson, M. and Singh, G.: Curr. Drug Targets Cardiovasc. Haematol. Disord., 3: 155-185 (2003).

[7] Ausprunk, D.H., Knighton, D.R. and Folkman, J.: Dev. Biol., 38: 237-249 (1974).

[8] Spanel-Borowski, K.: Res. Exp. Med. (Berl), 189: 69-75 (1989).

[9] Burton, G.J. and Palmer, M.E.: Scanning Microsc., 3: 549-557 (1989).

[10] Melkonian, G., Cheung, L., Marr, R., Tong, C. and

Talbot, P.: Toxicol. Sci., 68: 237-248 (2002).
[11] Melkonian, G., Munoz, N., Chung, J., Tong C., Marr, R. and Talbot P.: J. Exp. Zool., 292: 241-254 (2002).
[12] Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z.: Faseb. J., 13: 9-22 (1999).
[13] Jadhav, J., Gonjari, G., and Kanase, A.: Drug Invention today; 3(6): 62- 68 (2011).
[14] Jadhav, J., Mane, A. and Kanase, A.: Int. J. Drug Dev. Res., 3(4): 307-317 (2011).
[15] Schlatter, P., Konig, M.F., Karlsson, L.M. and Burri, P.H.: Mictrovasc. Res., 54: 65-73 (1997).
[16] Patan, S., Haenni, B., and Burri, P.H.: Anat. Embryol. (Berl), 187: 121-130 (1993).
[17] Patan, S., Haenni, B., and Burri, P.H.: Mictrovasc. Res., 53: 65-73 (1997).
[18] Djonov, V., Schmid M., Tschanz, S.A. and Burri, P.H.: Circ Res., 86: 286-292 (2000).
[19] Tiziana, T., Francesca, R. and Pier, P.C.: 22, 6549-6556 (2003).
[20] Gilbert, S.F.: Developmental Biology, 7th ed. Sunderland: Sinauer Associates, pp 517 (2003).
[21] Ausprunk, D.H., Knighton, D.R. and Folkman, J.: American J. Pathol., 79: 597-618 (1977).
[22] Lesson, T.S. and Lesson, C.R.: J. Anat. Londen, 97: 585-595 (1963).
[23] Narbaitz, R.: J. Anat., 124: 347-354 (1977).
[24] Packard, M.J. and Packard, G.C.: Respiration and Metabolism of Embryonic Vertebrates (Semour, R.S. ed); Dr. W. Junk Publishers, place of publication, pp 155-177 (1984).
[25] Weiss, J.W., Launois, S.H., Anand, A. and Garpestad, E.: Prog. Cardiovasc. Dis.; 41: 367-376 (1999).
[26] Jadhav, J., Mane, A. and Kanase, A.: J. Pharm. Res., 5(1): 208-211 (2012).
[27] Jadhav, J. and Kanase A.: J. Pharm. Edu. Res.; 3(2): 101-106 (2012).
[28] Jadhav, J., Thorat, S., Jamale, J. and Gonjari, G.: J. Pharm. Res., 1(4): 339-344 (2013).
[29] Ribatti, D., Urbinati, C., Nico, B., Rusnati, M., Roncali, L. and Presta, M.: Devlop. Biol., 170: 39-49 (1995).
[30] Flamme, I., Schulze-Osthoff, K. and Jacob, H.J.: Development, 111: 683-690 (1991).