Pluronic F127 as a drug vehicle used in chick embryo chorioallantoic membrane shell-less model

Tonglin Shi[1], Zongwei Li[1], Quanbin Zhang[2], Dawen Zheng[3] and Zhuoyu Li[1]*

[1]Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan, Shanxi, PR China
[2]Central Laboratory of Taiyuan Central Hospital, No. 1, East Sandao Alley, Xinghualing District, Taiyuan, Shanxi, PR China
[3]Cornea Department of Shanxi Eye Hospital, No. 100, Fudong Street, Taiyuan, Shanxi, PR China

Abstract: The developing vascular network is grown on the surface of chick embryo chorioallantoic membrane (CAM), so CAM is widely used as an *in vivo* model to study the angiogenesis. Because the CAM is hindered or wrinkled by the vehicle, the drug effect is difficult to be observed. In the present study, we firstly introduced the pluronic F127 aquogel to deliver drugs for the CAM model. The biocompatibility and advantage of this vehicle was shown by applied ranibizumab-pluronic F127 mix on the CAM. The results were showed that, the growth of blood vessels was not impaired by pluronic F127 gel, and the gel was almost imperceptible on the CAM, at the same time, the degradation of blood capillaries caused by ranibizumab was clearly visible. In conclusion, pluronic F127 was a good vehicle for angiogenesis research.

Keywords: Chick embryo chorioallantoic membrane (CAM); Shell-less culture; Pluronic F127; Angiogenesis

INTRODUCTION

The angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. It is a common feature of many pathological tissues, such as rheumatoid arthritis, wet form of age-related macular degeneration (AMD), retinopathy of prematurity (ROP), inflammatory diseases, etc (Carmeliet and Jain, 2000; Cheung *et al*., 2010). Judah Folkman described that angiogenesis is of crucial importance for tumor progresses. The nutrient and oxygen required by developing cancer was mainly obtained from newly formed blood vessels (Folkman, 1971). This theory led to the idea that inhibiting the angiogenesis can suppress the progression of malignancies.

The CAM model is probably the most widely used *in vivo* assay for studying angiogenesis, because of physiologically relevant, reliable and technically straightforward (Nguyen *et al*., 1994; Ribatti *et al*., 1996). The lack of a mature immune system in 7-8-day old chick embryo allows almost any drugs application (Folkman, 1975). The drugs always topically applied by kinds of delivery materials onto the CAM. The drug effects can be estimated by measuring density of the vessels based on the images captured by stereomicroscope. But the clear images were always difficult to obtain, since the CAM was hindered or wrinkled by drug vehicles. For example, the filter paper is opaque, which leads to the impossibility of direct investigation. Furthermore, polyethylene ring, another vehicle always used in the CAM experiments would wrinkle the CAM because of the pressure.

**Corresponding author:* e-mail: Lzy@sxu.edu.cn

In the present study, we firstly introduced a novel drug delivery substance, pluronic F127 (PF127), a well-known colorless hydrogel, to achieve topically administration, and used a recognized anti-VEGF agent, ranibizumab to show the advantages of this vehicle.

MATERIALS AND METHODS

Materials and chemicals

Ranibizumab was a product of Novartis (Stein, Switzerland). The normal saline (NS, 0.9% NaCl solution) was used to be the solvent for PF127, or ranibizumab-PF127 mix. The chick embryos (hy-line brown breed) on EDD (embryo development day) 3 were purchased from Shanxi Shiyu Animal Science and Technology Development Co., Ltd (Taiyuan, China). PF127 was purchased from Sigma-Aldrich.

The CAM model

The culture of shell-less embryos was modified from Dohle *et al.* (2009). Briefly, fertilized eggs were carefully wiped by semi-dry sterilized gauze using warm water to remove dirt, feathers and excrement. Sprayed with 75% ethanol, and air-dried in a laminar flow hood. Before crack, the eggs were stood horizontally for at least 5 min to assure embryos on the top-side of the eggs. Keep embryos upright, the eggs were cracked, and only embryos with intact yolk sac were explanted into 1.5 cm×9 cm glass Petri dishes. Embryos were incubated at 38% and 90% of relative humidity (RH) was used with mandatory air supply.

Determination of working concentration of PF127

20%, 25% and 30% (w/w) of PF127 were statically dissolved in NS separately at 4^{o}C overnight to ensure

homogeneous solution formed (Matthew *et al*., 2002). The solutions were filter sterilized by pre-cooled 0.45µm membranes. The embryos at EDD 8 were taken from incubator to room temperature (approx. 25^{o}C), then 20 µL of the PF127 solutions at various concentrations were pipetted onto the CAMs using pre-cooled pipette tips. Of which concentration formed a piece of uniform, flatten gel was used for the following studies.

Drug application

20µM of ranibizumab was mixed with 25% (w/w) of PF127 solution and dissolved thoroughly overnight at 4$^{o.}$ On EDD 8, 20µL ranibizumab-PF127 mix or their control was topically added on the CAMs by pre-cooled pipette tips. In order to show the advantages of PF127 compared to traditional vehicles, the sterilized filter paper and polyethylene ring (cut from the pipette tip, diameter 5 mm, wall thick 0.5mm, height 2 mm) as the vehicles were also placed on the same CAM, topically applied with 20 µL of the corresponding concentrations of ranibizumab solutions (in NS) on/in them. Then the embryos were returned to the incubator. NS was added on/in the filter papers/polyethylene rings every 24h to avoid drugs not to dry. The culture condition was as same as mentioned above. After 48h incubation, images were obtained by an Olympus SZX16 stereomicroscope (2×/0.3NA objective).

RESULTS

25% (w/w) of PF127 was suitable for the drug delivery

Because the gelating temperature of PF127 was various upon the concentrations, while a piece of flat and uniform thickness gel should be use, so the working concentration of PF127 was firstly determined. 20%, 25% and 30% (w/w) of PF127 solutions were applied on the CAMs and incubated at 38^{o}C for 48 h. The results showed that (fig. 1) the 20% of PF127 was too watery to use, on the wettish surface of the CAM, after 48h incubation, it rarely been the gel (image not shown); on the other hand, 30% of which was difficult to form the thin gel layer, it gelated too fast at apprx. 25^{o}C of room temperature, because its lower gelating temperature (fig 1a). The 25% of PF127 solution was suitable for the drug application. An almost invisible vehicle membrane for investigation (fig. 1b) could be formed by this concentration of PF127. On the other hand, PF127 was bio-friendly, no anti-angiogenic activity was observed through compared to the area where there any solutions deposited (fig 1b, where out of the circle).

High quality images could obtain through transparent PF127 gel

We used ranibizumab, a commercial anti-VEGF monoclonal antibody for the treatment of wet type of age-related macular degeneration, as the agent to show the dependability of PF127 and advantages against traditional vehicles (fig. 2). Two days after administration, we topically captured images by a stereomicroscope. The CAM under the filter paper was not directly accessible, and difficult to get clear images (fig. 2a). The polyethylene ring was better than the filter paper, because there was no shield between the CAM and objective lens, but the perfect records were also hard to be obtained, due to the pressure from the wall of ring wrinkled the CAM (fig. 2b). On the other hand, the location where PF127 deposited was easy to investigate, the gel was almost imperceptible, vascular degradation is clearly visible (fig. 2c).

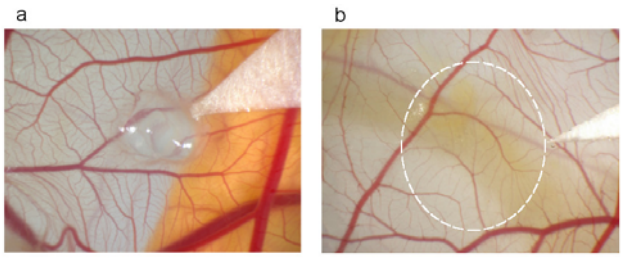

Fig. 1: The PF-127 sols on CAMs. (a) The 30 % (v/v) of PF-127 could not form a flatten membrane. (b) 25 % of PF-127 was suitable to use, the vehicle membrane formed at this concentration was almost invisible. The area where deposited PF-127 sol (inner of the circle) compared with the other area of the CAM (out of the circle), suggested that the PF-127 sol under this concentration had no toxicity to the CAM. The white triangle next to circle was a sterile filter paper to indicate where the PF-127 deposited.

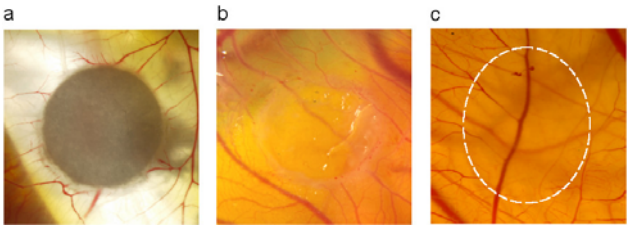

Fig. 2: The different vehicles used in CAM model with ranibizumab to show the advantages of PF-127 (c) compared with the filter paper (a) and the polyethylene ring (b, the ring was removed before capturing images).

DISCUSSION

Angiogenesis is linked to many diseases, including cancers. The CAM model is mostly used in angiogenic researches because of simple, easy to manipulate, and reliable (Nowak-Sliwinska *et al*., 2010). In CAM model, drugs were always topically applied on the membrane, the membrane absorbs drugs to show the biological effects, while ensure minimum damage to the embryos. There are kinds of substances to achieve topically administration. Here, we introduced PF127 to deliver drugs. The PF127 is a kind of nonionic triblock copolymer. It has been reported to be the least toxic of commercially available copolymers commonly used in cell culture for its cell

cushioning effects (Gilbert *et al*., 1986). Recently, PF127 was used as a pharmaceutical aid to deliver drugs. To the best of our knowledge, there were no previous studies reported its application in the CAM model.

The PF127 aqueous sols (10-35%, w/w) were thermo sensitive, which viscosity increased with an increase in temperature (Tirnaksiz and Robinson, 2005). Thus, the drugs could be added into PF127 solutions in lower temperature (e.g. 4^{o}C), in which, especially to protein drugs would be more stable compared to those drug delivery material, such as agarose (it melt at approx. 90^{o}C and gel at 35-40^{o}C) used in CAM research (Katrancioglu *et al*., 2012). On the other hand, as mentioned above, those commonly used drug delivery substances, such as filter paper or polyethylene ring, shielded the CAM or wrinkled CAM because of pressure and could not expand with CAM growth. In addition, the drug or solvent loss would happen under the condition of filter paper or polyethylene ring used because of leakage or evaporation of water. All of those defects were irreparable, especially in long-term investigation, and leading to false results. Nevertheless the PF127, as a soft aquogel, its area expanded kept pace with CAM growth; moreover, it was considered as a sustained release material which could lock the water, prolong peptide release and reduce peptide degradation (Wenzel *et al*., 2002). Besides all of above advantages, PF127 aquogel is colorless. In present study, 25% (w/w) of its solution was worked well for direct observation, photos for vascular or capillary structures of CAMs were easy to take. However, it should be noted that, this working concentration of PF127 was suitable at approx. 25%, it might vary slightly at different working conditions.

In conclusion, our study indicated that, PF127 could be an ideal drug vehicle applied in CAM model, because of colorless, non-toxic and sustain drug release. It could be widely used in angiogenesis research.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (No. 31271516, No. 31201072), The National Basic Research Program of China (973 Program, No. 2014CB543001), Shanxi Province Science Foundation for Youths (2012021028-4) and oversea scientists (20111009/20111010), Research Fund for the Doctoral Program of Higher Education of China (20111401110011). Conflicts of interest: The authors have declared that no conflict of interest exists.

REFERENCES

Carmeliet P and Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature,* **407**(6801): 249-257.

Cheung N, Mitchell P and Wong TY (2010). Diabetic retinopathy. *Lancet*, **376**(9735): 124-136.

Dohle DS, Pasa SD, Gustmann S, Laub M, Wissler JH, Jennissen HP and Dünker N (2009). Chick ex ovo culture and ex ovo CAM assay: How it really works. *J. Vis. Exp.* **33**: e1620, doi:10.3791/1620.

Folkman J (1971). Tumor angiogenesis: Therapeutic implications. *N. Engl. J. Med.,* **285**(21): 1182-1186.

Folkman J (1975) Tumor angiogenesis: A possible control point in tumor growth. *Ann. Intern. Med.,* **82**(1): 96-100.

Gilbert JC, Hadgraft J, Bye A and Brookes LG (1986). Drug release from Pluronic F-127 gels. *Int. J. Pharm.,* **32**(2-3): 223-228.

Katrancioglu N, Karahan O, Kilic AT, Altun A, Katrancioglu O and Polat ZA (2012). The antiangiogenic effects of levosimendan in a CAM assay. *Microvasc. Res.*, **83**(3): 263-266.

Matthew JE, Nazario YL, Roberts SC and Bhatia SR (2002). Effect of mammalian cell culture medium on the gelation properties of Pluronic F127. *Biomaterials,* **23**(23): 4615-4619.

Nguyen M, Shing Y and Folkman J (1994). Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. *Microvasc. Res.,* **47**(1): 31-40.

Nowak-Sliwinska P, Ballini J-P, Wagnières G and van den Bergh H (2010). Processing of fluorescence angiograms for the quantification of vascular effects induced by anti-angiogenic agents in the CAM model. *Microvasc. Res.,* **79**(1): 21-28.

Ribatti D, Vacca A, Roncali L and Dammacco F (1996). The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis. *Int. J. Dev. Biol.,* **40**(6): 1189-1197.

Tirnaksiz F and Robinson JR (2005). Rheological, mucoadhesive and release properties of pluronic F-127 gel and pluronic F-127/polycarbophil mixed gel systems. *Pharmazie,* **60**(7): 518-523.

Wenzel JGW, Balaji KSS, Koushik K, Navarre C, Duran SH, Rahe CH and Kompella UB (2002). Pluronic F127 gel formulations of deslorelin and GnRH reduce drug degradation and sustain drug release and effect in cattle. *J. Control. Release,* **85**(1-3): 51-59.