การปรับวิธีการเลี้ยงเซลล์ปฐมภูมิของจระเข้น้ำจืดพันธุ์ไทย (*Crocodylus siamensis*) จากเซลล์หนังตาและหลอดเลือด

The Modified Cultured Method for Siamese Crocodile (*Crocodylus siamensis*) Primary Cell Culture derived from Eyelid and Blood Vessel

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บทคัดย่อ

จระเข้น้ำจืดพันธุ์ไทย (*Crocodylus siamensis*) เป็นจระเข้น้ำจืดที่พบได้ในภูมิภาคเอเชียตะวันออกเฉียงใต้ ประเทศไทยในอดีตอาจพบได้ตามธรรมชาติ และได้ถูกจัดอยู่ในกลุ่มเสี่ยงใกล้สูญพันธุ์ขั้นวิกฤตโดยสหภาพนานาชาติเพื่อการอนุรักษ์ธรรมชาติและทรัพยากรธรรมชาติ (IUCN) ซึ่งในประเทศไทยนั้นมีการอนุญาตให้มีการทำฟาร์มเพาะเลี้ยงเพื่อที่จะอนุรักษ์พันธุกรรมของพันธุ์แท้ และเพื่อเป้าหมายอื่นที่มีเหตุผล อย่างไรก็ตามความบริสุทธิ์ของจระเข้น้ำจืดพันธุ์ไทย ปัจจุบันลดลงมากเนื่องจากมีการผสมข้ามกับจระเข้พันธุ์น้ำเค็มซึ่งพบในหลายๆฟาร์ม วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาการเพาะเลี้ยงเซลล์ของจระเข้น้ำจืดพันธุ์ไทยโดยการปรับวิธีการเพาะเลี้ยงเซลล์ปฐมภูมิเพื่อใช้ประโยชน์ในการศึกษาต่อไปในด้านโครโมโซม การเป็นเซลล์เจ้าบ้านที่มีความจำเพาะต่อโรค และเป็นแหล่งทรัพยากรสำหรับการเป็นเครื่องมือในการวินิจฉัยอื่นๆ ตัวอย่างหนังตาและหลอดเลือดได้มาจากจระเข้ที่ได้รับบาดเจ็บและเสียชีวิตจากการต่อสู้กันเอง โดยตัวอย่างที่ถูกตัดย่อยเป็นชิ้นเล็กๆถูกนำไปเลี้ยงในอาหารเลี้ยงเซลล์ 2 สูตร คือสูตร 1) ไอเอ็มดีเอ็ม (Iscove's Dulbecco's Modified Eagle Medium) และเติมซีรั่มลูกวัว 10% และสูตร 2) ไอเอ็มดีเอ็ม ซึ่งเติมพลาสมาของจระเข้ 10% โดยทั้งสองสูตรมียาปฏิชีวนะและสารป้องกันเชื้อราเพื่อป้องกันการปนเปื้อนชื้อจุลินทรีย์จากนั้นนำไปเลี้ยงที่อุณหภูมิที่แตกต่างกันคือที่ 28 °C และ 37 °C ในตู้เลี้ยงเซลล์ซึ่งมีคาร์บอนไดออกไซด์ 5% พบว่าเมื่อตรวจสอบด้วยกล้องจุลทรรศน์ชนิดหัวกลับ ใน 2สัปดาห์หลังการเพาะแยกเซลล์พบเซลล์ลักษณะไฟโบรบลาสต์ (fibroblast-like) เซลล์ถูกเลี้ยงต่อไป จนกระทั่ง 10 passage และถูกเก็บไว้ทุกๆครั้ง ที่อุณหภูมิ -196 °C ในไนโตรเจนเหลว ได้ผลการศึกษาว่าอาหารเลี้ยงเซลล์ทั้งสองสูตร และที่อุณหภูมิทั้งสองระดับคือ 28 °C และ 37 °C สามารถใช้เลี้ยงเซลล์ปฐมภูมิจระเข้น้ำจืดพันธุ์ไทยได้สำเร็จ แต่การใช้ซีรั่มลูกวัว ในการเลี้ยงเซลล์จระเข้นั้นสามารถนำมาปรับใช้ได้ง่ายกว่าการใช้พลาสมาของจระเข้เองเนื่องจาก ซีรั่มลูกวัวนั้นหาได้ในทางการค้าทั่วไป

ABSTRACT

Siamese crocodile (*Crocodylus siamensis*) is a freshwater crocodile. Its wildly distributed habitat was Southeast Asia. It was commonly found in wild nature of Thailand in the past and presently declared as critically endangered by International Union for Conservation of Nature (IUCN). In Thailand, crocodile farming is allowed in order to conserve pure bred and facilitate rational purpose. However, the purity of Siamese crocodiles' genetic has been decreased due to cross-breeding with saltwater crocodile in many farms. The objectives of this research were to study cell culture method by modifying the cell culturing methods and maintain Siamese crocodile genetic material as primary cell culture for further study on crocodiles' chromosome, host specific disease and providing resource for other diagnosis tools. Eyelid and blood vessel were obtained from the newly dead Siamese crocodile suffering from fighting. The specimens were then excising into small pieces and cultured with two supplement formulas including i) Iscove's Dulbecco's Modified Eagle Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS) and ii) IMDM supplemented with 10% crocodile plasma. The antibiotic and antifungal were added in both types of media for preventing contamination. Thereafter the cultured flasks were incubated at 28 °C and 37 °C with 5% CO_2 supplied. The fibroblast-like cells were observed under the inverted microscope at 2 weeks later. The cells were subcultured until 10 passages and preserved at every single passage under -196 °C in liquid nitrogen. The result indicated that both supplement formulas were successfully applied for primary cell culture at the temperature of 28°C and 37°C. The supplementation by FBS is more applicable than crocodile plasma due to commercially available.

คำสำคัญ: จระเข้น้ำจืดพันธุ์ไทย เซลล์ปฐมภูมิ หนังตา หลอดเลือด การเลี้ยงเซลล์

Keywords: Siamese crocodile, Primary cells, Eyelid, Blood vessel, Cell culture

INTRODUCTION

Siamese crocodile (*Crocodylus siamensis*) is small size fresh water crocodilian. The International Union for Conservation of Nature and Natural Resources (IUCN) had released the red list status of *C. siamensis* confirmed as critically endangered species (CR). It is listed under the appendix I of CITES represented in captivity (IUCN. 2016). Geographically report of its natural habitat is restricted in Southeast Asia. The existing condition of Siamese crocodile is critical, the vulnerable factors in the past decade were skin trade, commercial hunting, losing habitat that made the number diminished. Few numbers of *C. siamensis* in Cambodia, Lao, Indonesia and Thailand were detected in nature. Several conservation orientated programs were surveyed and approached, nevertheless the number were increasing, but continue to be hunted for commercial trading. Pure genetic line of *C. siamensis* has found in Cambodia as captive farm holdings, (Bezuijen et al. 2008, IUCN. 2016, Jintana et al. 2014). In Thailand, the Siamese crocodile are also held, captive on farm. Regarding the accorded of genetic purity conservation is concerned as many crocodilian species can cross-breed and can also back cross. Species Identification of *C. siamensis* were reported using the Karyotyping analysis of metaphase chromosomes from cells culture derived from eyelid and aorta. (Wilairat et al. 2017). To date, several of endangered animal species were cultured and reported as the genetic resource of fibroblast cells. Primary cell cultures are advantageous for cell genetic study and keep the purity of species. Those were kept

in cell bank for retrospective and progressive studies. (Wilairat et al. 2016). Thus, in this study we aimed to collect cell culture of *C. siamensis* by applying modified culture method. In addition cell culture is necessary for disease diagnosis obtained the host species specific, Virus Isolation and Virus Neutralization test. (Shilton et al. 2012).

RESEARCH METHODOLOGY

Specimen Collection

The specimens taken from eyelid and blood vessel were collected from newly dead Siamese crocodile suffering from fighting. The tissue were then washed with phosphate buffer saline (PBS) containing Penicillin-Streptomycin (10,000 U/mL) and Amphotericin B 250 µg/ml for 3 times.

Primary Cell Culture

Both eyelid and blood vessel tissues were excising into small pieces and then transferred into 25 cm^2 tissue culture flasks which containing 5-7 ml of i) Iscove's Dulbecco's Modified Eagle Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin-Streptomycin (10,000 U/mL) and Amphotericin B 250 µg/ml and ii) IMDM supplemented with 10% crocodile plasma, Penicillin-Streptomycin (10,000 U/mL) and Amphotericin B 250 µg/ml. Then place cultured flasks in 5% CO_2 supplied incubator at 28°C and 37°C. Every week, cell culture medium was replaced. Observe the cell proliferation, when cells perform the growth and confluence on the surface of tissue culture flask using inverted microscope and counting cells with hemocytometer. Cells viability was examined with 0.2% tryphan blue solution staining. Total cells number of $1x10^6$ cells/ml were subculture for the next passage.

Cryopreservation and Recovery

The grown cells were tripsinized with 0.1 % trypsin in PBS. Numbers of cells were counted by hemocytometer. The 10^6 cells/ml were preserved in cryopreservative reagent with two formula of tissue culture medium i) IMDM containing 10% DMSO, 20% FBS, Penicillin-Streptomycin (10,000 U/mL) and Amphotericin B 250 µg/ml and ii) IMDM containing 10% DMSO, 20% crocodile plasma, Penicillin-Streptomycin (10,000 U/mL) and Amphotericin B 250 µg/ml. Cells in cryopreserved vials were kept in cryopreserved box and preserved at -196°C in liquid nitrogen. Cells viability assessment was performed.

Cells viability assessment

Cells recovery was performed after 1 month of cryopreservation and 3 months apart from the first cryopreservation. In brief we thawed the cells at 37°Cand washed with cells culture media without serum and centrifuged at 1,500 rpm/min. The cells pellet were kept and resuspended with the culture media in each of tested formulae. Then place cultured flasks in 5%CO_2 supplied incubator at 28°C, and 37°C, respectively. Cells growth and morphology were observed since the initial stage of culture and after subculture or recovered cells under inverted microscope. Number of cells viability or survival cells and time of confluent cells were recorded. Cells Image were kept and recorded.

RESULTS

Eyelid tissue of Siamese crocodile cultured with adapted IMDM with 10% FBS containing antifungal and antibiotics that were incubated at two temperature conditions of 28°C and 37°C. The observation under microscopic view found the cell proliferation of confluence within 30 days, active growing showed in Fig 1A, 1B. Cells grew multi- directional from the originated tissue at the first culture. After subculture each week,

confluence cells were observed by a week. Cells performed adherent characters and contact of inhibition grown. The morphology of cells was fibroblast. Besides the eyelid tissue cultured with in IMDM 10% crocodile plasma including antifungal and antibiotics performed the active growing cells similarly in both incubation temperatures (Fig 2A, 2B). The cells morphology was fibroblast-like as well. The next passages were still found the same shape of cells. Proliferation time of confluence since initial culture, subculture obtain similarity of cells characters.

Likewise the blood vessel at the initial phase of culture in IMDM with 10% FBS including antifungal and antibiotics by 30 days, confluence were found, cells grew actively as shown in Fig 3A, 3B. Cells in the same microscopic view had smaller size and similar size as of cells from eyelid tissue under the different incubation temperatures. Blood vessel cultured with IMDM 10% crocodile plasma including antifungal and antibiotics. Fig 4A, 4B achieved the same cells morphology of epithelial-like in shape.

Cells from all kind of tissues culture with the different formula media and temperature conditions had the viability assessment of more than 80% after subculture. An overview of confluent cells morphology characters of adhesion on cell culture flasks were firmly, none of granulated vesicle or gigantic cells were found. These cells grew rapidly, well arranged, densely packed in the flask surface before subculture, demonstrated the active and healthy cells. Nevertheless, the size of originated tissue were different by manual excisions, morphology of cells derived from different tissue types were still found well adhered which indicated the characters of active living cells.

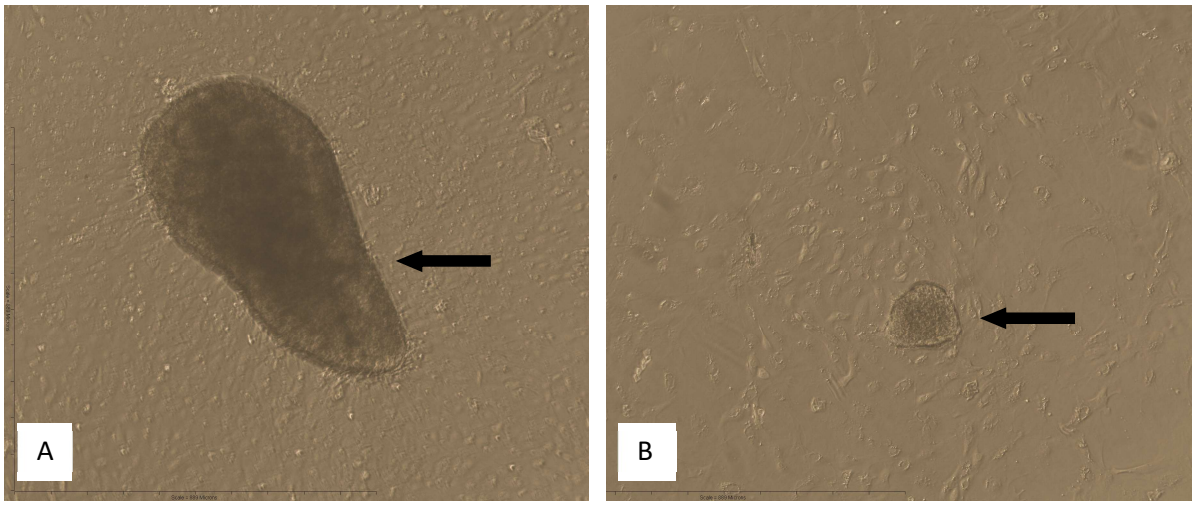

Fig 1. Eyelid of Siamese crocodile at the initial phase of culture in IMDM 10% FBS including antifungal and antibiotics after 15 days of culture.100X magnification under inverted microscope. Black arrows represented a piece of eyelid tissue (A- incubated at 28°C, B- incubated at 37°C)

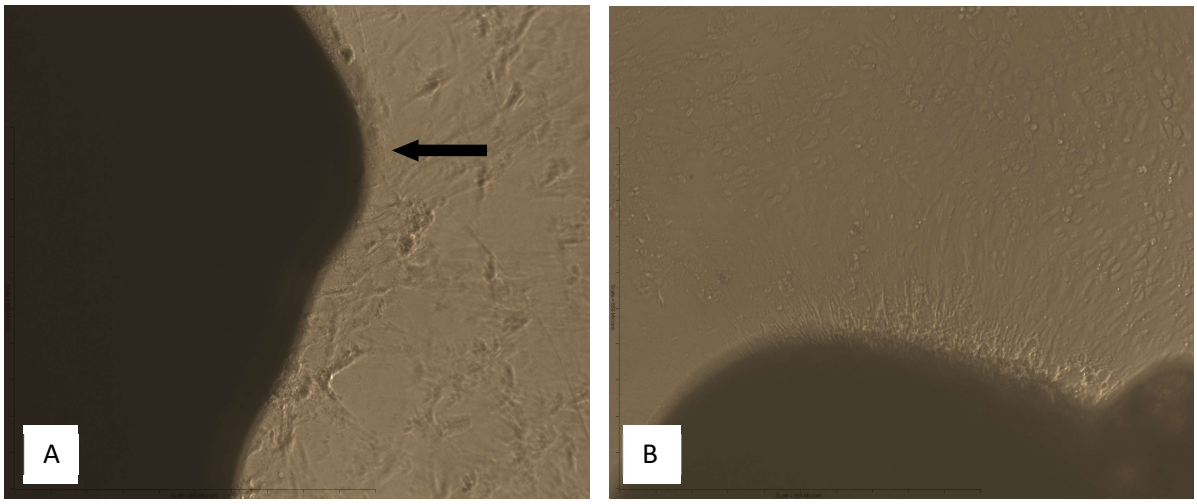

Fig 2. Eyelid of Siamese crocodile at the initial phase of culture in IMDM 10% crocodile plasma including antifungal and antibiotics after 15 days.100X magnification under inverted microscope. Black arrows represented a piece of eyelid tissue (A- incubated at 28°C, B- incubated at 37°C)

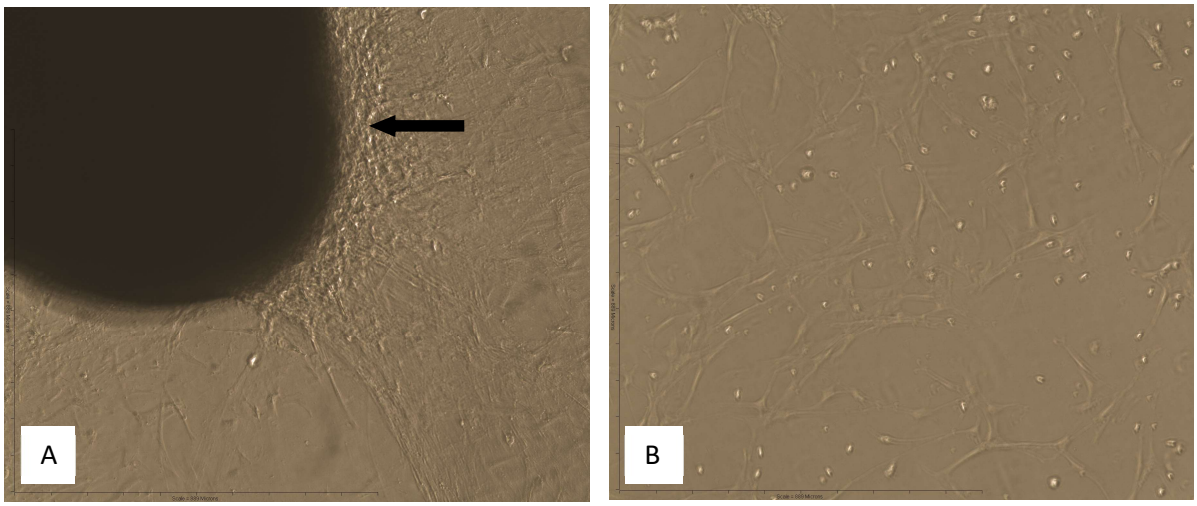

Fig 3. Blood vessel at the initial phase of culture in IMDM 10% FBS including antifungal and antibiotics after 15 days.100X magnification under inverted microscope. Black arrows represented a piece of blood vessel tissue. (A- incubated at 28°C, B- incubated at 37°C)

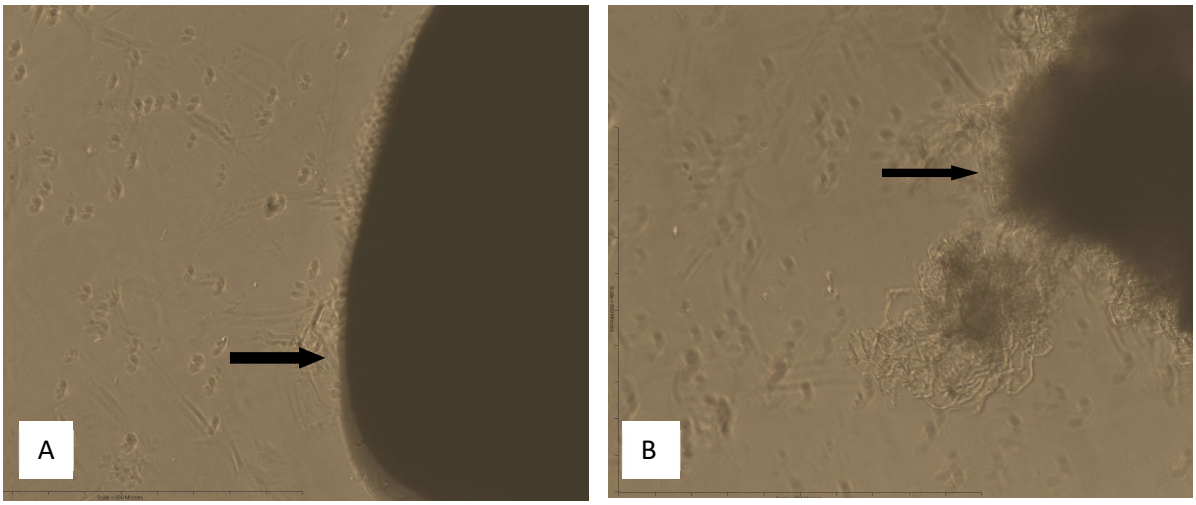

Fig 4. Blood vessel at the initial phase of culture in IMDM 10% crocodile plasma including antifungal and antibiotics.100X magnification under inverted microscope. Black arrow represented a piece of blood vessel tissue. (A- incubated at 28°C, B- incubated at 37°C)

DISCUSSION

To date diagnosis of crocodile disease is necessary to maintain cell culture**.** Crocodile primary cell culture had been developed from different tissues into cell lines, noted in Salted water crocodile (*C. porosus*) which were successful from kidney, heart, trachea, lung, gonad, subcutaneous and liver. These major mentioned sources of tissue gave the cell morphology of fibroblast-like except kidney which was epithelial-like (Alberts et al. 2002). Crocodile primary cell culture is necessary for disease diagnosis in conjunctivitis, pharyngitis and vasculitis encephalitis syndromes caused by herpes virus. They were tested *in vitro* using virus Isolation and viral neutralization test. (VNTs) assays *(*Shilton et al. 2014, Melville et al. 2012).

The major components in culture media are nutrient and fetal bovine serum (FBS). It is essential for cell growth and proliferation. FBS is widely used at 10% of concentration, though it may variable application. It obtained cocktails of the factors and most universal effective supplement. (Lodish et al. 2000). It may contain some adverse factor of endotoxin. That may cause allergy. Nevertheless, it is widely provided and commercially supplied (Gstraunthaler. 2003). Crocodile serum/plasma and its importance for replacement are concerned as its species specificity. In this study we succeeded in primary cell culture of *C. siamensis* derived from skin eyelid and blood vessel applying both type of supplements. Crocodile plasma might be more implied precisely specific growth factors than commercial FBS in crocodile primary cell culture; however the FBS is more commercially and simply found in any cell biology laboratory. In general, laboratories that maintain cell culture usually set the incubator for the standard condition related the temperature of 37 °C, besides if the insect cells are needed the 28 °C incubator is required. There was the report of salted water crocodile primary cell line monolayer in 28 °C (Shilton et al. 2016). *C. porosus* cell culture has proven for incubation in the extensive range of temperature of 20-30°C (Melville et al. 2012). Consequently, geography, climate zone and species itself might be involved. Geographical information of Southeast Asia which is in tropical zone and occupied the natural habitat of *C. Siamensis* has the broad range of thermal condition 22-37°C, drastically in summer

temperature could reach above 40°C. It is presumably that crocodile cells could be grown whether incubation temperature is quite different in wide range.

Crocodilian is the big family consisted of 23 species, Once is realized that it is free ranging in the wild and presented as the hydrology parameter of ecosystem (Mazzotti et al. 2009). On the other hand, crocodile farming is well raised industry in Australia *C. porosus*, definitely in captivity form. It is problematic concern to maintain the genetic purity. The distinct of example is hybridization between two species of *C. porosus* and *C. siamensis*, that is commercial objective preference for skin trade value and yield. (Charles. 2000). Consequently, pure bred crocodile existed to be complicated situation (Bezuijen et al. 2008). Thus far, primary cell culture is relevant. It would serve as one source to keep reliable inherent cells. Hence, in this study exhibited markedly in correct morphology of fibroblast-like from skin eyelid and epithelial-like from blood vessel.

CONCLUSIONS

Crocodile primary cells were successfully cultured by some modification on culture media and the optimal temperature for incubation and growth. The applied formula of culture media which supplementation of FBS and crocodile plasma both gave the favorable result of growth fibroblast-like cells from two types of tissue. However, FBS supplemented media would be used for laboratory routine application. We found that culture of crocodile cells derived from eyelid and blood vessel could be achieved at the incubation temperature of 28 °C and 37°C**.** These cells would be kept as *C. siamensis* genetic material source for further work.

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