

# A novel primary culture method for rat choroidal epithelial cells

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## ABSTRACT

**الأهداف:** تأسيس طريقة لمزرعة الخلايا الظهارية المشيمية الأساسية.

**الطريقة:** أجريت دراسة وصفية مخبرية في جامعة شيان جياو تونغ، شيان، الصين خلال الفترة من سبتمبر 2009م حتى أغسطس 2010م. تم عزل الخلايا الظهارية المشيمية من أنسجة الفئران حديثي الولادة عددهم 36 فأر. تم فصل الأنسجة إلى مجموعات خلايا صغيرة بواسطة طريقة آلية وعمل مزرعة في طبق المزرعة البلاستيكي الذي يحتوي على مستنبت النسر المحدث مع 10% مصلى بقرى جيني، 10 نانوغرام/مل عامل النمو البشري في درجة حرارة 37 درجة مئوية في حاضنة مع 5% ثنائي أكسيد الكربون. تم فحص المزرعة بواسطة مجهر متباين الصفحات، والفحص المجهرى، والكيمياء المناعية.

**النتائج:** أظهرت الخلايا خصائص متكافئة للنمط الظهاري مع مظهر مرصوف في طبقات عديدة لمدة 7-9 يوم بعد الزراعة. اكتشف المجهر الإلكتروني خلايا ظهارية مشيمية عادية مع زغيبية في الغشاء الخلوي، وعضيات السيتوبلازم، ولحام موصل محكم للخلايا المجاورة. كما كانت إيجابية ضد مضاد النبروكسين المناعي.

**خاتمة:** أن طريقة المزرعة لا تحتاج أي أجهزة معقدة ومهارات عملية وتعتبر طريقة بسيطة وفعالة للحصول على الخلايا الظهارية المشيمية بعدد كافي ونقي من مزرعة أولية مختلطة لأنسجة الفئران.

**Objective:** To establish a method for the culture of primary choroidal epithelial cells.

**Methods:** This descriptive experimental study was carried out in Xi'an Jiaotong University, Xi'an, China from September 2009 to August 2012. Choroidal epithelial cells were isolated from the choroid plexus tissues of the lateral ventricles from neonatal rats

(n=36). The tissues were dissociated into small cell aggregates by a mechanical method, and cultured on plastic culture dishes containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 10 ng/ml epidermal growth factor at 37°C in an incubator with 5% humidified carbon dioxide. The cultured cells were examined by phase contrast microscope, electron microscopy, and immunocytochemistry.

**Results:** The cells showed typical morphologic characteristics of epithelial phenotypes with a cobblestone appearance in monolayer 7-9 days post-seeding. The electron microscopy spotted typical choroidal epithelial cells with microvilli on the cytomembrane, organelles in the cytoplasm, and tight junctions welding 2 adjacent cells. They were positive against anti-transferrin immunostaining.

**Conclusion:** This culture technique, which does not require complex equipment and operation skills, might be a simple and efficient method for obtaining choroidal epithelial cells in sufficient number and purity from mixed primary cultures of rat tissue.

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The choroid plexuses are heavily vascularized secretory organs within the ventricular system of the vertebrate brain, which are lobulated with a single continuous layer of cells derived from the ependymal lining of the ventricles. Despite being derived from the ependymal lining, these cells possess epithelial cell characteristics and are often referred to as choroidal epithelial cells. The choroidal epithelial cells are known to produce and secrete numerous growth factors and neurotrophins.<sup>1</sup> There are several reports describing the positive effects of treatments with neurotrophins and growth factors in models of brain diseases.<sup>1-3</sup> Although neurotrophic factors providing a scaffold for regenerating neurons have considerable therapeutic potential, there are practical difficulties in providing a continuous supply of these agents with syringe or pump procedures in the clinical situation. So, transplanting of cells that produce neuro-protective agents is a simple and pragmatic method.

There is a growing body of literature reporting the transplantation of choroid plexus in several animal models.<sup>4-9</sup> These studies are similar in that they used large clusters or small fragments of choroidal epithelial cells. In preparing for transplantation, it is critical to obtain sufficient cells used for seeding. However, transplantation using single isolated choroidal epithelial cells has to our knowledge not yet been reported. Because of the intrinsic deficits of immortalized cell lines, choroidal epithelial cells derived from primary culture, are optimal. To overcome this, attempts have been made to establish and refine the methods for isolation of choroidal epithelial cells. Our goal was to establish a simple and fast method without aggressive manipulations for the culture of choroidal epithelial cells.

**Methods.** This study was carried out in Xi'an Jiaotong University, Xi'an, China from September 2009 to August 2012. An *in vitro* laboratory study was conducted gaining primary culture of choroidal epithelial cells from neonatal rat tissue using a mechanical method instead of an enzymatic method. The experiments were performed on at least 3 animals for each assay. The Xi'an Jiaotong University Animal Experimentation Committee approved protocols for animal care and experimental management. Ethical approval for the study was obtained from the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University. Principles of laboratory animal care were followed, and all procedures were conducted according to guidelines established by the National Institutes of Health. All efforts were made

to minimize the number of animals used and their suffering.

**Animals.** Neonatal Sprague-Dawley one-day-old rats (n=36) weighing 5-6 grams, male and female (1:1), were supplied by the Center of Experimental Animals, Xi'an Jiaotong University School of Medicine.

**Reagents.** Dulbecco's modified Eagle's medium (DMEM, low glucose) and fetal bovine serum (FBS) were purchased from Thermo (Waltham, MA, USA). Culture dishes of 35 mm were provided by Corning Costar (New York, NY, USA). Rabbit anti-transferrin (TTR) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and 4',6-diamino-2-phenylindole (DAPI) was purchased from Roche (Basel, Switzerland). Poly-L-lysine, fluorescein-conjugated goat anti-rabbit IgG, penicillin, and streptomycin were purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant rat epidermal growth factor (EGF) was obtained from PeproTech (Rocky Hill, NJ, USA). Stock solutions were prepared considering the manufacturers' recommendations on solubility and were stored at -20°C. For germ control, a cocktail of penicillin/streptomycin was added once initially. Basal culture medium was prepared from DMEM, and the growth medium was DMEM supplemented with 10% FBS and 10 ng/ml EGF. All other reagents used were of analytical grade.

**Dissociation of choroidal epithelial cells and primary culture.** The following procedures were performed under strict aseptic conditions. The materials and reagents were prepared before the cell isolation. All the procedures for dissociation of choroidal epithelial cells were carried out on ice to maintain cell viability as much as possible.

The animals were anesthetized with 10% chloral hydrate intraperitoneally, with blood drained as much as possible from the hearts using a syringe to minimize the amount of blood present in choroid plexus tissues, and immediately decapitated. The brains were removed from the skull by performing a longitudinal incision and subsequent removal of the brain from the skull using tweezers and kept in chilled phosphate-buffered saline (PBS). The lateral ventricles were subsequently dissected using a sterile scalpel, and the choroid plexus tissues were carefully extracted from both the lateral ventricles and collected. Then, choroid plexus tissues were washed in PBS to remove blood, minced with a sterile scalpel blade into small pieces less than or equal to one mm<sup>3</sup> in size, and immediately transferred into a beaker containing cold basal culture medium supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. The tissue pieces were mechanically

triturerated by repeated passages through a 20-gauge needle or a one ml pipette in order to dissociate tissues in clusters of around 10 cells. After centrifugation at 800 rpm for 5 minutes, the supernatant was discarded, and the growth medium was added. Viability of cells obtained during the extraction process was assessed by trypan blue exclusion test. After that, cells suspended in the growth medium, at appropriate concentrations (one neonate per dish), were seeded in 35-mm-diameter Petri dishes or 24-well plates, with or without glass coverslips previously coated by poly-L-lysine. The dissociated cells were then incubated in a 5% humidified carbon dioxide incubator at 37°C. After 48 hours, the medium was removed, unattached cells were removed by washing, and fresh medium was added to the culture. Thereafter, the culture medium was replaced every 3 days.

**Light microscopy.** All cultured cells were examined daily for growth, adherence, and presence of contamination under phase contrast microscope (Olympus, Tokyo, Japan).

**Electron microscopy.** For cells of primary culture, scanning electron microscopy was performed. Briefly, the cell-seeded coverslips were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C, postfixed in 1% osmium tetroxide for 2 hours at 4°C, dehydrated through a graded series of ethanol, and dried to the critical point. The samples were then mounted in a metal base, sputtered with gold, analyzed and photographed under the scanning electron microscope (SEM) (Jeol, Tokyo, Japan).

Cultured choroidal epithelial cells were processed for transmission electron microscopy. The cells were fixed overnight with 2.5% glutaraldehyde at 4°C. Post-fixation was carried out with 1% osmium tetroxide for 2 hours, and dehydration was carried out in graded ethanol. Then, the cells were embedded in Epon-812. Ultrathin sections with the thickness of 100 nm were cut with a microtome and stained with uranyl acetate followed by lead citrate and examined under a transmission electron microscope (Hitachi, Tokyo, Japan).

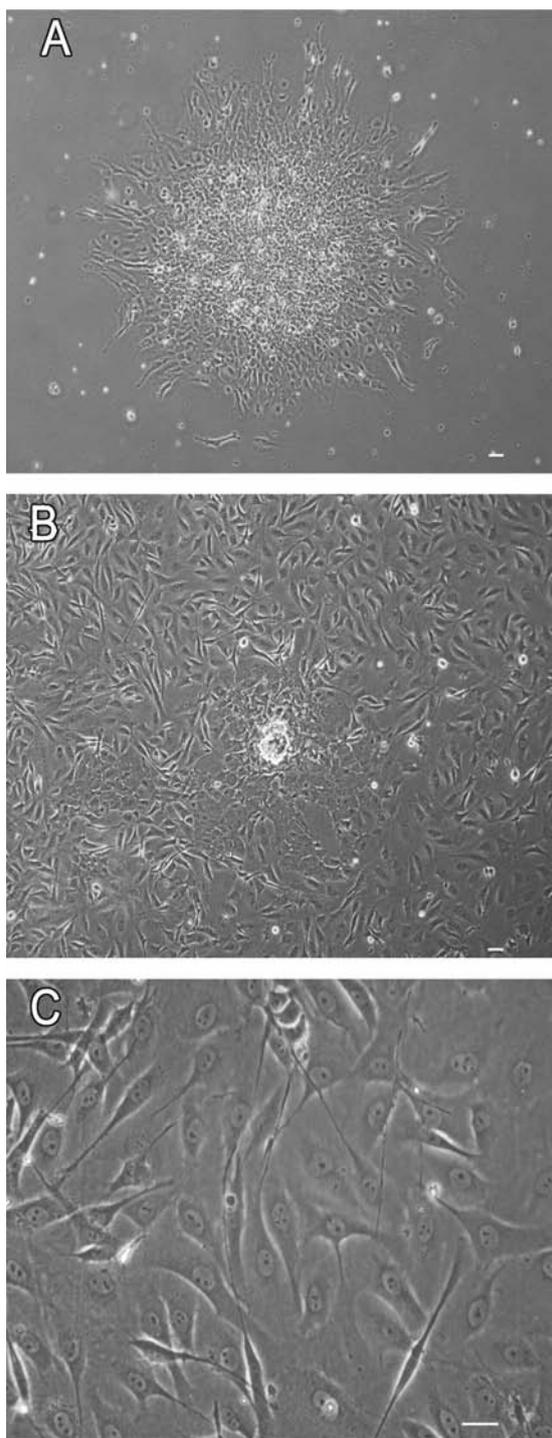
**Immunocytochemistry.** Rabbit anti-transferrin, a marker of choroidal epithelial cells, was assayed by immunostaining. When the cells reached 80% confluence, glass coverslips adhered by cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cell slips were washed with PBS 3 times for 5 minutes each time. Nonspecific reactions were blocked with 10% normal goat serum for 30 minutes. The cells were subsequently incubated with 200-fold diluted rabbit anti-TTR at 4°C overnight. After 3 washes in PBS, a secondary antibody, fluorescein-conjugated

goat anti-rabbit IgG diluted 1:100, was added, and the cell-seeded coverslips were incubated for 2 hours at room temperature. Cell nuclei were counterstained using DAPI. Then, the sections were washed in PBS again, air-dried, and cover slipped. Fluorescence microscopy was carried out by using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a mercury lamp power supply.<sup>10</sup> Negative controls were performed, with primary antibodies omitted.

**Results.** The viability of the harvested cells before seeding was more than 95% as assessed by the trypan blue exclusion method. Interestingly, under the phase contrast microscope, single cells did not attach to the substratum and died in less than 48 hours. So, single cells seemed to be unable to proliferate or proliferated slowly. Only small clusters could attach to culture dishes and grow. The primary cultured cells proliferated in an island-like monolayer after incubation for 2 days (Figure 1A), and they became confluent within 7-9 days (Figure 1B). The cells migrated out around the fragments after being seeded from adhesion to the dish. Morphologically the choroidal epithelial cells appeared to be of polygonal shape with irregular cytoplasmic processes and a cobblestone appearance (Figure 1B). The cells had a high nucleus-to-cytoplasm ratio, and each cell contained a pale nucleus with one or several nucleoli (Figure 1C).

Intrastructurally, the scanning electron micrograph of the choroidal epithelial cells showed that most epithelial cells had thin microvilli and a minority of cells lacked microvilli (Figure 2A). Junctional complexes such as tight junctions, desmosomes, and interdigitations were displayed formed between the intercellular boundaries with the transmission electron micrograph (Figure 2B). Regarding the morphological features of the choroidal epithelial cells as assessed by electron microscopy analysis, our results were similar to that reported in the Opossum by Ek et al.<sup>11</sup> The cytoplasm contained cell organelles, including Golgi complex, mitochondria, and rough endoplasmic reticulum (Figure 2C). The nucleus was indented, located either centrally or basally. In addition, there was marked cell division, but interestingly, the neogenetic cells had a smooth surface devoid of microvilli (Figure 2D).

The choroidal epithelial cells were ascertained by staining with anti-TTR antibodies. As can be seen in Figure 3, almost 100% of choroidal epithelial cells were positive for TTR. The contaminated cells detected in the cell culture were few.



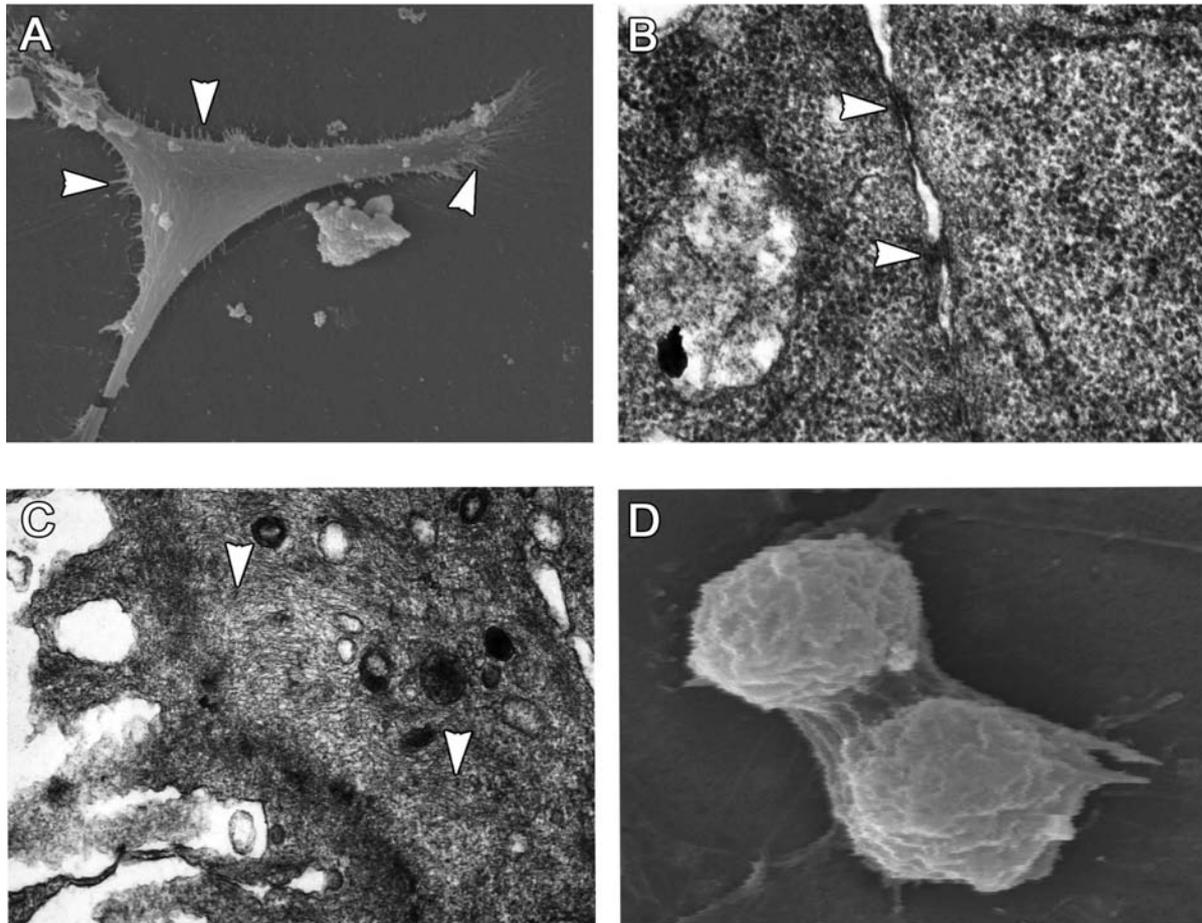
**Figure 1** - Phase contrast microscopy of primary-cultured choroidal epithelial cells showing: A) On day 2 of culture ( $\times 100$ , bar:  $50\mu\text{m}$ ) - the cells proliferate in an island-like monolayer. B) On day 9 of culture ( $\times 100$ , bar:  $50\mu\text{m}$ ) - the cells have a cobblestone appearance. C) On day 9 of culture ( $\times 400$ , bar:  $20\mu\text{m}$ ) - the cells have a high nucleus-to-cytoplasm ratio.

**Discussion.** We have developed a simple and efficient method to obtain choroidal epithelial cells in sufficient number and purity from mixed primary cultures of rat tissue. The technique was established in rats, because this species is commonly used for the study of central nervous system diseases. Our protocols have 3 important points. First, the processing time is considerably reduced, and no sophisticated skills are required. Second, the cells are plated at low density in primary cultures, and third, we have not encountered fibroblast contamination in this work.

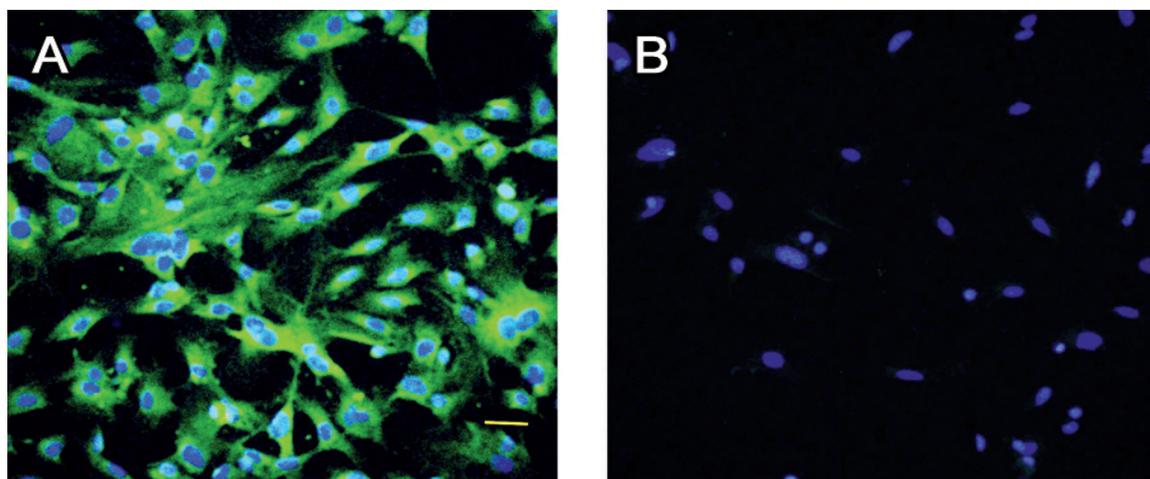
Nathanson<sup>12</sup> reported a method to culture choroidal cells, but it was a very short-term model. Most traditional/conventional methods for the isolation of choroidal epithelial cells utilize an enzymatic method for dissociation by treatment with collagenase or pronase, and so forth.<sup>13-16</sup> These methods, although provide certain numbers of choroidal epithelial cells with reasonable purity, require sophisticated operation skills. Further, the requirements for large numbers of animals and the tedious cell isolation procedures have been major drawbacks of these methods.

In this paper, we describe a simple and rapid method for the isolation of choroidal epithelial cells from neonatal rats. The choroidal epithelial cells were obtained from the lateral ventricles of one-day-old rats by using the method of mechanical dissociation. The subsequent transfer and incubation in plastic dishes resulted in quick and selective adhesion of choroidal epithelial cells, while other contaminating cells remained suspended in the medium. Isolation and purification of choroidal epithelial cells is achieved on the basis of the anatomical and histological characteristics of the choroid plexus in lateral ventricles from neonatal rats. Growing cells in a medium that contains simple ingredients may minimize the possibility of potential contamination. The purification of choroidal epithelial cells in adult rats is difficult due to the unwanted growth of non-choroidal epithelial cells in primary cultures, especially fibroblasts. The choroidal epithelial cell isolation procedure developed in the present study has proved efficient in avoiding a noticeable contamination from fibroblasts by using neonatal brain cells. The erythrocytes will not adhere to plastic during the culture procedure, and therefore all the erythrocytes can be eliminated by medium change. In our experiment, a highly pure population of choroidal epithelial cells was determined by morphologic appearance, and cell surface marker expression.

For culturing the neonatal choroidal epithelial cells in vitro, mechanical disintegration is a critical step. Using enzymes for the choroid plexus tissue dissociation



**Figure 2** - Electron microscopy of primary-cultured choroidal epithelial cells showing: A) Scanning electron microscopy ( $\times 1500$ ). The cell has thin microvilli (arrowheads). B) Transmission electron microscopy ( $\times 40000$ ). The junctional complexes are displayed between the intercellular boundaries (arrowheads). C) Transmission electron microscopy ( $\times 20000$ ). There is a lot of rough endoplasmic reticulum (arrowheads). D) Scanning electron microscopy ( $\times 3000$ ). There are 2 neogenetic cells.



**Figure 3** - Immunocytochemistry of primary-cultured choroidal epithelial cells showing: A) The cells were immunostained with an antibody against transthyretin (blue). B) Control ( $\times 200$ , bar:  $50\mu\text{m}$ ). Blue is cellular nuclei.

procedure strongly reduces cell survival. In our method, enzymes are not needed for cell dissociation, as choroid plexus can readily become small cell aggregates through mechanical disintegration. This isolation method is relatively effective, and the cells isolated in the present investigation show a relatively high proliferation potential. Also, those previously established techniques for obtaining choroidal epithelial cells, like enzymatic dissection of the choroid plexus, are time-consuming, and expose the cells to oxidative stress, which influences cell proliferation. In contrast, the method in this paper does not require complex equipment and skills. In addition, reducing the enzyme digestion period can effectively shorten the whole culture process, and the ingredients of the growth medium are cheaper. The choroidal epithelial cell monolayers are easily detached from the plastic surface and can be subjected to transplantation.

In addition to its utility for transplantation, this technique can be applied to basic investigations, such as the physiology of the choroid plexus epithelium cells. Although we have only used our method with rat choroid plexus tissue, we expect that our method will be suitable for generating choroidal epithelial cells from a variety of other mammalian species. Then the characteristics of the cells cultured, such as barrier properties (electrical resistance), permeability (transport function), and secretory function (growth factors), needs to be fully investigated.

In conclusion, we report a novel method for the *in vitro* culture of choroidal epithelial cells that yields, on a weekly basis, large numbers of primary cells.

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