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RESEARCH ARTICLE

A Novel Epithelial and Fibroblastic Cell Isolation and Purification Method Using Primary Culture of Bovine Tongue Tissue

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Abstract

Cell lines provide a useful in vitro model to study in different biotechnological fields. The purity of the cell lines plays a pivotal role in research and production activities. This study aims to present a new method to establish pure cell lines by using bovine tongue tissue. For this purpose, the bovine tongue was obtained from the local slaughterhouse. After establishing primary cell culture, the cells were treated with EDTA (0.02%) for 3-5 min at 37°C. Primarily detached fibroblasts were collected into 5 mL EMEM (with 20% FBS), centrifuged and transferred to a new culture flask with EMEM (10% FCS) medium. The remainder cells in the primary flask were incubated for 8 hours with DMEM (10% FCS). Thereafter, the same process was applied with Na_2EDDA (0.01%), and the cells were washed twice with DMEM (with 20% FBS). Reincubation was carried out with decreased FBS concentration (10%) and EGF (10 ng/mL) at 37°C, 5% CO_2 in humidified air conditions. The same processes were repeated after 48 h. Conformational studies of pure cultures were done with immunostaining technique with anti-cytokeratin and anti-vimentin monoclonal antibodies where, after purification, fibroblasts displayed vimentin-positive and epithelial cells cytokeratin-positive. In conclusion, this study successfully demonstrated an easy, effective, and efficient method to generate pure novel cell lines from primary tissue culture or mixed cell cultures.

Keywords: *Primary cell culture, Fibroblast cell, Epithelial cell, Pure culture*

Sığır Dil Dokusu Primer Kültürü Kullanılarak Yeni Bir Epitel ve Fibroblastik Hücre İzolasyonu ve Saflaştırma Yöntemi

Öz

Hücre hatları, farklı biyoteknolojik alanlarda çalışmak için elverişli in vitro modeller olup, saflıkları araştırma ve üretimlerde çok önemli bir rol oynar. Bu çalışmanın amacı, sığır dil dokusunu kullanarak saf hücre hatlarının oluşturulması için yeni bir yöntem oluşturmaktır. Mezbahadan alınan sığır dil dokusundan primer hücre kültürü hazırlandıktan sonra, 37°C'de 3-5 dak EDTA (%0.02) ile muamele edilen hücreler, fibroblastlar yüzeyden ayrılmaya başladığında, %20 FBS içeren 5 mL EMEM ortamında toplandı, santrifüjlendi ve %10 serum içeren EMEM ortamında yeni bir kültür kabına aktarıldı. Eski kültür kabında kalan hücreler 8 saat 5 mL DMEM (%10 FBS'li) ile inkübasyonu takiben, aynı işlem Na_2EDDA (%0.01) ile uygulandıktan sonra hücreler, %20 FBS'li DMEM ile iki kez yıkandı ve bu defa %10 FBS ve 10 ng/ml EGF içeren DMEM ile inkübe edildi. 48 saat sonra aynı işlemler tekrarlandı. Saf kültürlerde sitokeratin ve vimentin için spesifik monoklonal antikorlar kullanılarak doku orijini doğrulaması amacıyla immün boyama protokolü uygulandı. Sonuçlarımız, immünositokimyasal analize göre, saflaştırılmış fibroblast hücrelerinin vimentin pozitif olduğunu ve saflaştırılmış epitel hücrelerinin de sitokeratin pozitif gösterdiğini gösterdi. Sonuç olarak, bu çalışma, primer kültürler ya da karışık hücre kültürlerinden saf yeni hücre hatları oluşturmak için kolay, etkili ve verimli bir yöntem ortaya koymuştur.

Anahtar sözcükler: *Primer hücre kültürü, Fibroblast hücre, Epitelyal hücre, Saf kültür*

INTRODUCTION

Cell cultures are major tools for biotechnological advances from diagnosis to vaccine production. By definition, cell culture is the environment in which cells separated from the tissue by spontaneous migration, mechanical or enzymatic dissociation, and is kept alive, reproduced *in vitro,* and studied away from physicochemical and physiological variables. The first step of culture is called primary culture or primary cell culture [1,2]. However, due to the preparation and cost constraints of primary cultures, cell lines are often used to study biological processes [3,4].

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Cell lines originating from primary cultures have either a limited lifespan or immortal, capable of continuous proliferation [5]. Although cell lines are a powerful tool, attention should be paid when using them instead of primary cells. They must display and maintain functional characteristics as close as possible to the primary cells. However, animal cell lines are one of the main tools in vaccine production and biotechnological studies, such as generating artificial tissues, gene editing, synthesis of biological compounds and therapeutic proteins. It is a vital experimental process that is easier to control than *in vivo* [6,7]. In this process, maintaining high standards is essential for all scientific studies. Although it is also necessary for the reproducibility, reliability, acceptance, and implementation of the results obtained, most researchers can ignore this issue [4,7,8]. About 32.755 articles of studies with misidentified cell lines are in circulation [9]. Establishing a pure cell line from primary culture provides delicate and proper data to assess scientific research. Many methods have been developed for cell purification from primary culture. Each has advantages and disadvantages that are considered in the goals of the research [10]. Fibroblasts are widely predominant in the mixed culture of epithelial and fibroblastic cells. Intercellular junctions of fibroblasts are weaker than epithelial cells. For this reason, it is easier to remove fibroblastic cells first in primary cultures. Isolation and purification of cells from tissues by maintaining their native characteristics are substantial. Otherwise, they may hamper our understanding of their physiological, biological, growth, and differentiation characteristics [11]. Therefore, the advantages of working with well purified, identified, and contamination-free cell lines are indisputable. For this purpose, the purification step of the novel cell lines to be created from the primary cultures and confirmation of the histological origin are of great importance.

Epithelial and mesenchymal (connective) tissues constitute the two main classes of vertebrate tissues. The epithelial tissues of ectodermal or endodermal origin containing small amounts of intercellular substances. The mesenchymal tissues are mesodermal origin and have significant amounts of extracellular proteins [12]. Cell metabolism or cell membrane protein expression is highly critical during the purification process [13]. Immunocytochemistry (ICC), which is used to verify the cell cultures' tissue origins, is one of the methods performed using monoclonal antibodies specific to the relevant tissue intermediate filament. Intermediate filaments, cytoskeletal elements, differ from tissue to tissue. While the intermediate filaments of epithelial cells are generally composed of specific cytokeratin combinations, fibroblast cells consist of several intermediate filaments, mainly vimentin and collagen type 1 [14].

This study aimed to generate an easy, effective, and efficient method to develop pure novel cell lines from primary culture or mixed cell cultures using bovine tongue tissue.

Material and Methods

Establishment of Primary Cell Culture

Bovine tongue tissue was obtained from the local slaughterhouse. After washing with cold phosphate-buffered saline (PBS) and sterilization with 70% ethanol, the tongue was transported to the laboratory in ice-cold PBS supplemented with antibiotics. The upper layer of tissue was chopped with scissors into small fragments after removing the *stratum corneum* layer under sterile conditions [15,16]. Then, tissue fragments were digested in an enzyme solution containing collagenase I (0.4 mg/mL, Sigma, USA) and dispase (1.2 mg/mL, Sigma USA) for 45 min at 37°C. After 2 times centrifugation, the resulting pellet was collected and dispersed in DMEM/F-12 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, France), 5 µg/mL insulin (Sigma, USA), 10 ng/mL epidermal growth factor (Sigma, USA), 100 nM/mL transferrin (Sigma, USA), 100 U/mL penicillin (Sigma, USA), 100 µg/mL streptomycin (Sigma, USA), and 2.5 µg/mL amphotericin-B (Sigma, USA) [17-20]. The cell density was determined using the trypan blue dye exclusion (0.4%, Sigma, USA) method [21]. The cells were transferred into T75 cell culture flasks at $1x10^6$ cells/mL and allowed to incubate (37°C, 5% CO_2). The culture was examined every 48 h with an inverted phase-contrast microscope, and the growth medium was changed. On the first, third and fifth days of the culture, proliferation was checked with an inverted phase-contrast microscope and monitored by micrography (Olympus IX71, Japan).

Purification of Epithelial and Fibroblastic Cells

In primary culture, fibroblastic cells usually predominant and mixed with epithelial or surrounded the epithelial islets. Herein, a combined protocol was created by utilizing some parts of the techniques used in different studies to obtain the purity of two different cell groups. When the culture was 70% confluent cell layer, the conventional detaching procedure with trypsin (0.25%) was applied after washing with Ca^{+2}-Mg^{+2} free PBS [13,21]. The next step was EDTA (ethylenediaminetetraacetic acid, 0.02%) addition and incubation (3-5 min at 37°C) [22]. Fibroblasts were collected in 5 mL EMEM (Eagle's minimum essential medium, Multicell-Wisent, Canada) with 20% FBS, centrifuged (800 rpm, 5 min) and transferred to a new culture flask in EMEM with 10% serum. The residual cells in the primary culture flask were washed twice with DMEM (with 20% FBS) and cultivated with 5 mL DMEM (with 10% FBS) in 5% CO_2 atmosphere at 37°C for 8 h. Following incubation, the same process was applied with Na_2EDDA (disodiumethylenediamine-N,N'-diacetic acid, 0.01%) to complete the removal of the fibroblasts. Then, the cells were washed twice with DMEM containing 20% FBS, reincubated with decreased FBS concentration (10%) and epithelial growth factor (EGF) (10 ng/mL) at 37°C humidified conditions [19,23-25]. The same processes were

repeated after 48 h, and the separation and purification of fibroblast and epithelial cells from primary cell culture were completed.

Immunophenotyping

Immunostaining was conducted based on the general immunocytochemistry procedures. For this purpose, ab M3515 and ab M0821 (Dako, USA) were used after 1/50 dilution and ab M0725 (Dako, USA) were used after 1/100 dilution as primary antibodies. As secondary antibodies for cytokeratin, goat anti-mouse IgG FITC (fluorescent isocyanate) (Dako, USA) and for vimentin, goat anti-mouse IgG TRITC (tetramethylrhodamine-isothiocyanate) (Southern Biotech, USA) were used after 1/100 dilution. Hep-2 (human larynx epidermoid cells) cell line was originally derived from an epidermoid carcinoma of the larynx, but it contains HeLa marker chromosomes and were derived via HeLa contamination [26]. Hep-2 cells positive for cytokeratin were used as the positive control group for purified epithelial cells. It was obtained from HÜKÜK (Cell Culture Collection, HÜKÜK, Ankara). MRC-5 cell line established from normal human fetal lung tissue positive for vimentin was used as a positive control group for purified fibroblast cells [27]. It was obtained from ATCC (American Type Culture Collection, Rockville, MD). MRC-5 cells and Hep-2 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS.

Purified epithelial cells, fibroblasts, and the control group cells were separately inoculated to an 8-well chamber slide at 200 cells/well (Lab-Tek II, Thermo Fisher Scientific) left incubation for 24 h in 37°C, 5% CO_2. After the incubation period, the cells were fixed with 400 µL 4% paraformaldehyde for 15 min at 37°C, left in 400 µL permeabilization solution [(0.1 M Tris-Cl (pH 7.4) + 50 mM EDTA (pH 8) + 0.5% TritonX-100)] for 15 min at room temperature to allow antibodies to penetrate cellular membranes. Following PBS washing, the cells were treated with a blocking buffer for 2 h to prevent nonspecific interaction of antibodies in different cellular compartments. Primary antibodies (M3515, M0821, M0725, Dako, USA) were diluted according to the manufacturer's recommendation, applied to the cells and incubated at room temperature for 2 h. After PBS washing, secondary antibodies were applied and incubated at room temperature for 90 min [28]. Following incubation, the cells were stained with DAPI [2-(4-amidinophenyl)-1H-indole-6-carboxamidine] and were evaluated under fluorescence microscope (Olympus IX71, Japan).

Results

Primary Cell Culture

Primary cell culture was established by disaggregation of the tongue tissue using a combined enzymatic and mechanical method. In order to dominate the culture in general and to show cell morphologies in the inverted phase-contrast microscope images of the primary culture, the magnification of images was taken as 100x and 200x. Epithelial-like cell foci formation was observed on the 1st and 3rd days following the attachment of the cells to the culture surface *(Fig. 1-A,B)*. On day 5, proliferation of dispersed fibroblast-like cell populations and epithelial-like cell islets was observed *(Fig. 1-C)*.

Purification and Subcultivation

Purification of the fibroblastic and epithelial cell populations was based on the 0.02% EDTA and 0.01% Na_2EDDA treatment of the primary culture. The culture was monitored at the 5th passage level by microphotography for morphological

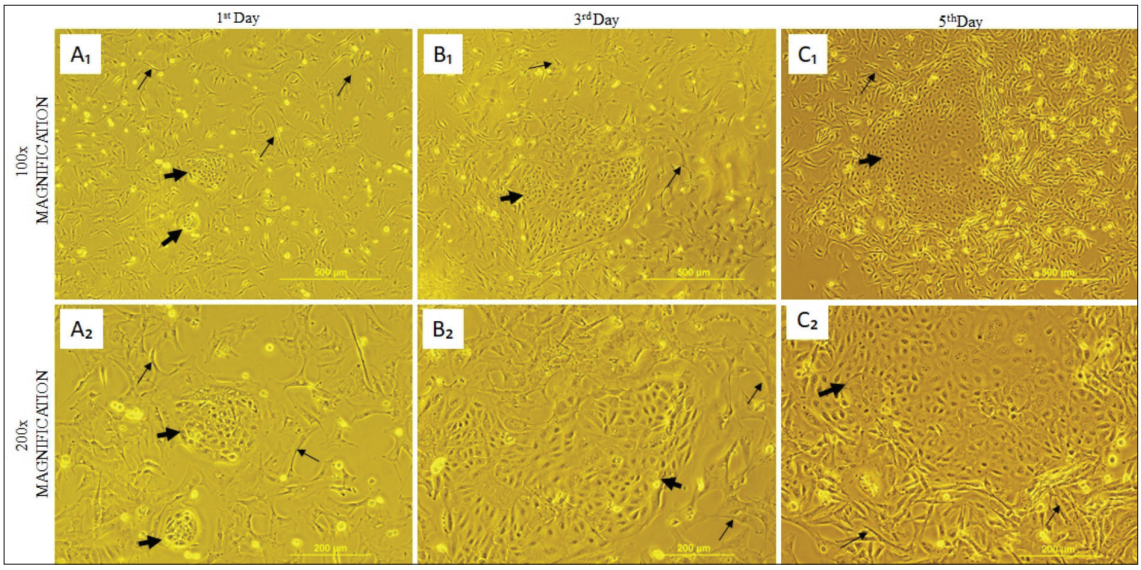

Fig 1. Phase contrast microscope images of the primary culture on day 1, day 3 and day 5. *Thin arrows* indicate fibroblast cells, and *bold arrows* indicate epithelial cell foci (magnification 100X for A_1, B_1, C_1 and magnification 200X for A_2, B_2,C_2)

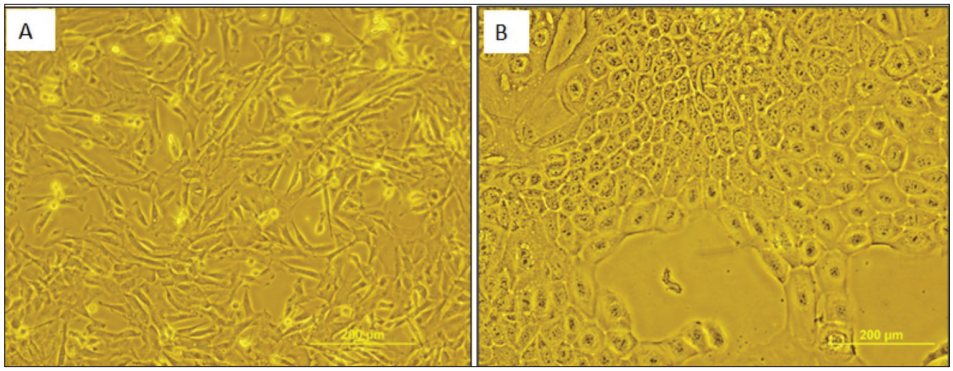

Fig 2. Phase contrast microscope images from passage 5 of purified cells. (A) Fibroblast cell line presents fibroblast-specific elongation and spindle morphology, (B) epithelial cell line presents regular polygonal and islet-shaped proliferative morphology specific to epithelial cells (magnification 200X)

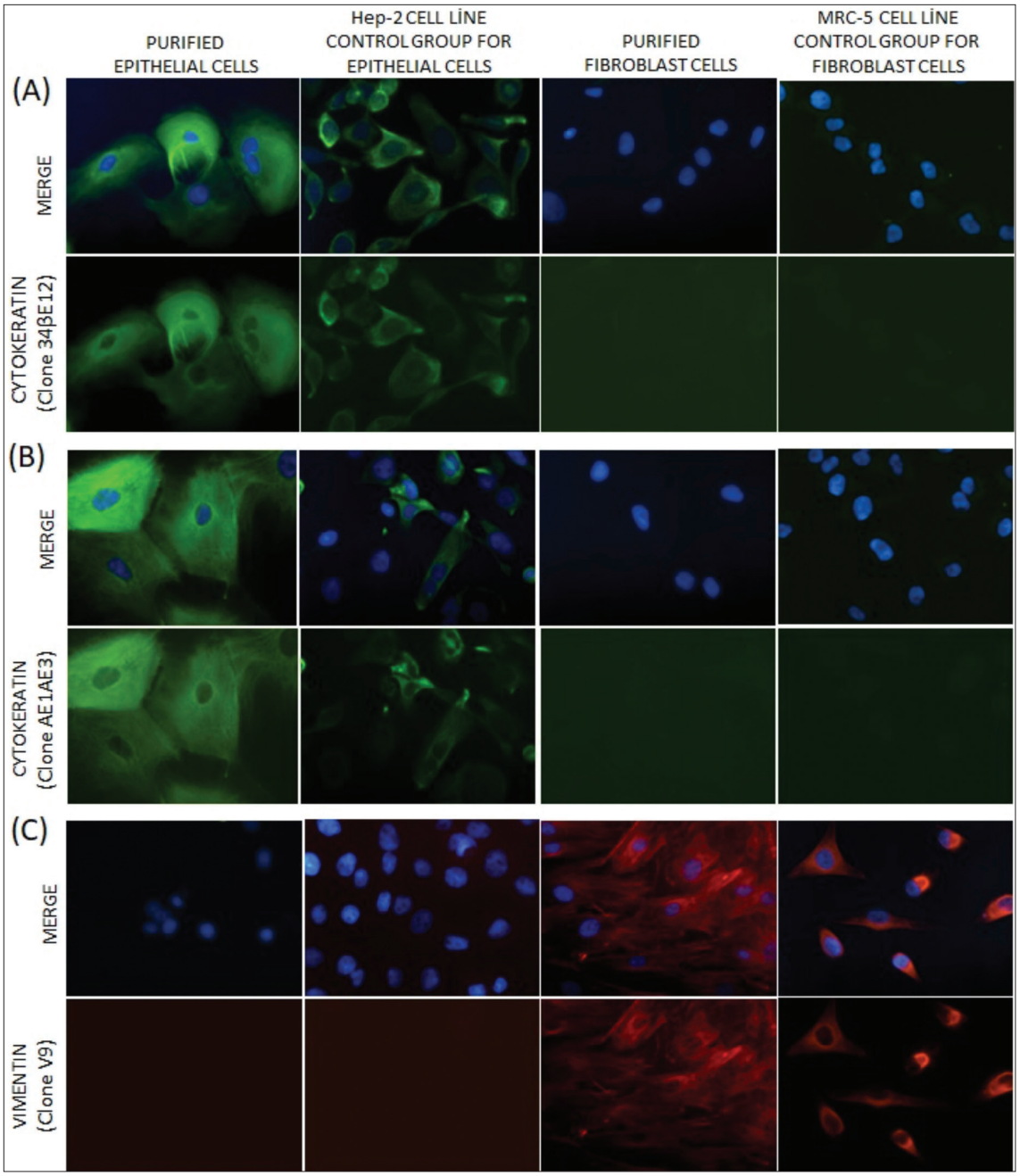

Fig 3. Intermediate filament protein expression of purified epithelial and fibroblast cells. **A, B:** Purified epithelial cells and positive control cells were positive for different cytokeratin expressions, while negative for vimentin expression. **C-** Purified fibroblastic cells and positive control cells were positive for vimentin expression, while negative for cytokeratin expression (magnification 400X)

inspection. While fibroblasts performed scattered, elongated, spindle-shaped and uniform appearance *(Fig. 2-A),* epithelial cells were standing out with their polygonal shape and islets in different sizes *(Fig. 2-B)*.

Immunophenotyping

After purification, all of the cells were stained with specific markers to examine their epithelial and fibroblastic characters; for cytokeratin and vimentin expression. The purified epithelial cells expressed strong cytokeratin *(Fig. 3-A,B)* and negative for vimentin *(Fig. 3-C);* on the contrary, purified fibroblasts expressed strong vimentin *(Fig. 3-C)* and negative for cytokeratin *(Fig. 3-A,B)* as expected.

Discussion

Today, scientific studies with living systems pose several ethical problems. For this reason, studies on cell culture techniques have been used in the creation of tiny copies of the living environment. Development of the new methods with advancing technology in this field, day by day cell culture areas is becoming more prevalent. The cell lines purified from primary cultures play a pivotal role in producing biotechnological products, cell-based diagnosis and treatments. Fibroblasts can grow faster and differentiate at a higher rate *in vitro*. Because of these properties, fibroblast contamination or dominance is common in primary cultures [23,29]. In this study, two different purified cell populations, epithelial and fibroblast cells, were formed from primary or mixed cell cultures using a novel purification method. In the preparation of primary culture from the bovine tongue, collagenase and dispase enzymes were used together to isolate fibroblasts and epithelial cells from tissue and healthily separate them. Although dispase, a neutral protease, is an effective agent to separate the epidermis from the dermis and is a gentle agent that preserves cellular membrane integrity, sometimes it may be insufficient to separate epithelial cells from the tissue alone. Therefore, it has been used together with the collagenase I [30,31]. In the secondary culture, serum concentration, a mitotic stimulant for fibroblasts, decreased to 5% in the medium and partially reduced the dense fibroblast cell population. Thus, a preliminary preparation was made to remove fibroblasts from the culture in the next step [17]. More robust binding properties than fibroblasts characterize epithelial cells. Keratinocytes compose muscular cell-cell adhesions in a calcium-rich environment. They need extracellular calcium for the formation of adhesive bonds and desmosomes. Different methods, such as enzymatic and chelation, can separate or remove fibroblasts from cultures.

Na_2EDDA is a chelate that binds calcium ions and enables epithelial cells to stay in cell culture flask by strengthening their connections [22]. Singer et al.[32] used the 1B10 monoclonal antibody, which they produced against human thymic fibroblasts, to remove them in culture and recognize fibroblasts with different origins (skin, thymus, synovia) without discrimination. Although this method is complex and relatively expensive, they have shown that the fibroblasts in the culture are eliminated through the complement-mediated cytotoxic effect of the 1B10 monoclonal antibody following 0.02% EDTA treatment in mixed cultures. Drewa et al.[24]. removed fibroblasts from keratinocyte culture using long-term Na_2EDDA treatment. Fibroblasts in keratinocyte cultures produced in DMEM/F12 medium containing autologous serum and some additional additives for epidermal grafting were treated with 0.01% Na_2EDDA until removing in another successful but also time-consuming study, fetal bovine epithelial primary cell culture was treated with 0.01% Na_2EDDA for 2-3 min, then incubated in 2% serum media for 6, 12 and 24 h and again treated with 0.01% Na_2EDDA. These procedures were repeated until the complete removal of the fibroblasts [25].

In this study, primarily, Pal and Grover's method, treatment the culture with antibiotic in Hanks' balanced salt solution, which is a practical and economical method to remove fibroblasts, was used [23]. Still, the fibroblasts could not be eradicated completely. Then, Jeon and Hwang's purification method was applied, the cells were purified, but it was observed that epithelial cells lost their membrane integrity in the 0.05% Na_2EDDA solution used in the first step and granulations were appeared in the cells following subculture [19]. After these experiences, a protocol that combines the different stages of the methods mentioned earlier has been developed. According to this protocol, the serum concentration in secondary culture was reduced to 5%. The dominance of fibroblasts was limited, followed by short trypsinization (0.25%), which saves time in the long-term Na_2EDDA method of Drewa et al.[24]. At the last stage, fibroblasts and epithelial cells were purified by treatment with 0.02% EDTA and Na_2EDDA (0.01%), which binds calcium ions. In the microscopic examination, it was observed that their viability and morphology were healthy. Tissue origins of purified cells were confirmed by using immunostaining. It was observed that epithelial cells expressed cytokeratin and fibroblast cells expressed vimentin.

In conclusion, this study demonstrates an economical, effective, and easy method that can be applied to remove fibroblasts from the environment in primary or mixed cultures and create different cell lines by purifying epithelial and fibroblastic cells.

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Conflict of Interest

The author declare that have no conflict of interest.

Availability of Data and Materials

Datasets analyzed during the current study are available in the author on reasonable request.

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