Isolation and Culture of Primary Human Oral Buccal Fibroblasts

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Abstract-

Introduction: Oral fibroblasts are the important cells for maintaining structural integrity of the oral mucosa. They can be activated and altered in various diseases like oral submucous fibrosis and other benign and malignant fibrous lesions. Fibroblast cell culture is an important in vitro tool allowing researchers to study the fibrotic diseases in detail and devise various therapeutic strategies. Different techniques of isolation of fibroblasts have been used so far and each has its own merits and demerits.

Objective: The objective of our study was to optimize the protocol of isolation of fibroblasts from oral buccal mucosa.

Methods: Primary cells were isolated from biopsy specimens of patients undergoing 3rd molar surgery. Tissue fragments were digested with trypsin for 10, 30 and 60 min., while some fragments were left as undigested explants. Primary cells were subcultured and maintained according to the standard protocols.

Results: By using combination of explant and enzymatic digestion for 10 min. we were able to isolate fibroblasts earlier by attachment of fibroblasts on 3rd day of culture and reaching 90% confluence on 14th day.

Conclusions: Our study revealed that combination of explant and enzymatic digestion for 10 min. is a faster method for isolation of oral fibroblasts as compared to longer time durations and undigested explants.

Keywords: Isolation of fibroblasts; Primary cell line; Explant-enzymatic technique

I. INTRODUCTION

Oral fibroblasts play a significant role in maintaining the structural integrity of the oral cavity. These cells are responsible for producing and organizing extracellular matrix components such as collagen and glycoproteins, which provide strength and support to the tissues in the mouth. Additionally, oral fibroblasts participate in the wound healing process by producing growth factors and cytokines that promote tissue repair. They also can differentiate into different cell types, contributing to the tissue regeneration process[1].

Oral fibroblasts also participate in the pathogenesis of several oral pathologies such as oral submucous fibrosis (OSF), fibromatoses, and fibrosarcoma. Behaviour of these cells can be altered in response to various pathological stimuli, leading to changes in the extracellular matrix and altered tissue organization[2].

Fibroblast cell culture is an important tool in cell biology and medical research. It allows scientists to study these cells in a controlled laboratory setting, which provides insights into the fundamental biology of fibroblasts, including their behaviour, growth, differentiation, and responses to different stimuli[3,4].

In the medical field, fibroblast cell culture is used in regenerative medicine to produce skin and other tissues for transplantation. It also helps in understanding the cellular and molecular mechanisms underlying various disorders, such as systemic sclerosis, fibrotic disorders, and wound healing[5].

Thus, fibroblast cell culture has significant implications in both basic scientific research and medical applications, making it an important tool in the advancement of medical knowledge and therapies.

Isolation of human primary fibroblasts involves various protocols. In explant technique, the tissue is cut into small pieces and placed in a culture dish, where the fibroblasts will grow out from the explant over time. In digestion technique, the tissue is treated with a mixture of enzymes, such as collagenase and trypsin, to dissociate the cells and isolate the fibroblasts. Both explant culture and enzymatic dissociation have their own advantages and disadvantages[6]. Hence, we decided to establish an efficient protocol for isolation of fibroblasts by comparing both techniques and study their morphological characteristics.

II. MATERIALS AND METHODS

Written informed consent was obtained from all study participants after explaining the objectives and rationale of the study. All information gathered during this study was kept confidential. The study was conducted after approval from the Ethics Review Committee of Ziauddin University, Karachi, Pakistan (3830521AIOM).

Patients were selected from the Department of Oral and Maxillofacial Surgery, Ziauddin Hospital, Karachi. Healthy patients above 18 years of age undergoing impacted 3rd molar surgery were selected for the study. Patients having habits such as

tobacco chewing, alcohol consumption and smoking; patients taking drugs that could affect fibroblasts, e.g., calcium channel blockers, cyclosporine phenytoin sodium, and steroids and those having any other debilitating diseases were excluded from the study.

Specimens were taken from three patients undergoing surgery for impaction. Non-inflamed tissue was excised from the buccal mucosa during surgical extraction of 3rd molar. Approximately 4 mm of tissue was collected from each patient.

After obtaining specimen, the tissue was washed with phosphate-buffered saline (PBS) (pH 7.4) + 100µl Penicillin/Streptomycin and transported in a falcon tube to the cell culture facility of Ziauddin University. The tissue processing was carried out in a biosafety cabinet and all the sterilization protocols were maintained before the experiment. The tissue was washed twice with PBS and minced. Tissue pieces were transferred to three 15 ml falcon tubes and digested with 2 ml Trypsin-EDTA (Gibco, UK) for 10, 30 and 60 min., represented as D1, D2 and D3 respectively. Following digestion, minced tissue was centrifuged. Supernatant was discarded and the pellet was suspended in working medium Dulbecco's Modified Eagle medium (DMEM) (pH 7) (Gibco, UK) complemented with 10% fetal bovine serum (FBS) (Gibco, UK), 100 µg/ml of penicillin, 100 µg/ml of streptomycin (Gibco, UK) in T25 flasks. For explant technique of isolation, the tissue pieces were directly cultured in DMEM and labelled as (E). All flasks were incubated at 37°C in humidified atmosphere of 5% CO^2 and 95% air for further proliferation.

Observation of cultures was done every day under live cell imaging inverted microscope (Floid cell imaging station) for migration of primary cells. Medium was replaced every 3rd day. Tissue fragments were left in the culture and removed on 7th day from D1 and on 10th day from rest of the flasks.

The cultured cells were identified as fibroblasts by the determination of morphological phenotypes[4]. When the cells reached 90% confluence, they were subcultured on 14th day. Medium was aspirated and cells were washed twice with PBS and trypsinized. After centrifuging the cells were seeded in sterile tissue culture treated flasks and maintained in CO^2 incubator.

Cells were cryopreserved using the standard protocol[7]. After reaching confluence the cells were trypsinized and centrifuged. The cell pellet was resuspended in 1 ml cryomedium containing 95% FBS and 5% DMSO. Cell suspensions were aliquoted in cryogenic vials and frozen gradually; at −20°C for 2 hr. then at −80°C in a deep freezer overnight. Then vials were transferred to a liquid nitrogen tank to store them at temperatures below -130°C.

III. RESULTS

Day 0

Cells were observed immediately after digestion. Tissue in D1 showed dissociation of spherical cells (Figure 1a). D2 showed migration of a greater number of cells as compared to D1 (Figure 1b) but it resulted in a mixed population of cells and other types of cells dominated. D3 showed almost complete tissue digestion (Figure 1c) and resulted in the release of a large number of mixed population of cells. While on the other hand, no migration of the cells from explant was observed in E (Figure 1d).

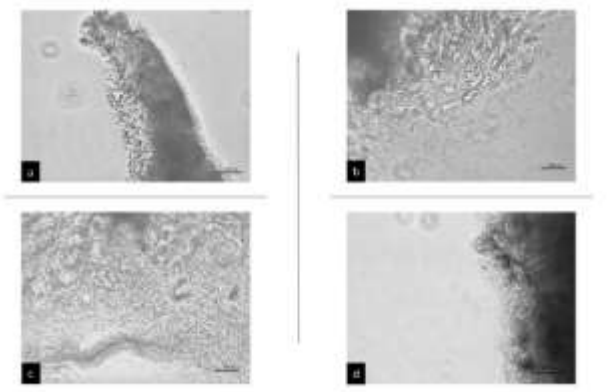

Figure 1: Photomicrograph showing migration of cells on day 0. Dissociation of small no. of cells in D1 (a). Greater no. of cells migrating from the tissue in D2 (b). Complete tissue digestion in D3 (d). No migration of cells from explant in (E) (c).

Day 3

On 3rd day of culture, attached cells with small extensions resembling spindle shaped fibroblasts (f1 type) were seen in D1 (Figure 2a). Expansion of same spherical cells was observed in D2 (Figure 2b) and D3 (Figure 2c). Culture mixture still showed heterogenous population of cells. Migration of spherical cells was also observed from explant tissue (E) (Figure 2d). No contamination was observed in cultures.

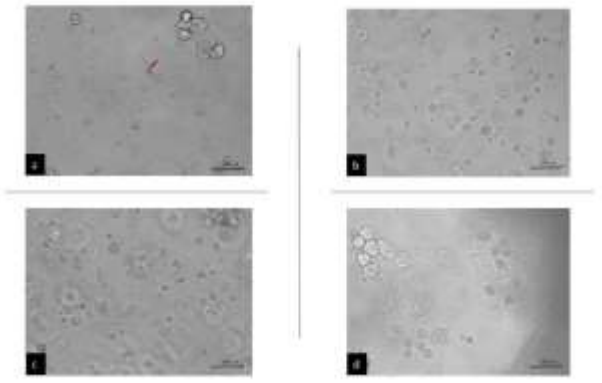

Figure 2: Attached spindle shaped fibroblast seen on 3rd day in D1 (arrow) (a). No attachment seen in D2 (b). Mixed population of cells observed in D3 (c). Spherical cells migrating in E (d).

Day 7

As the culture progressed proliferation of spindle shaped (f1 type) fibroblasts was seen in D1 (Figure 3a). Fibroblast attachment was also observed in D2, D3 and E on 7th day (Figure 3b-d).

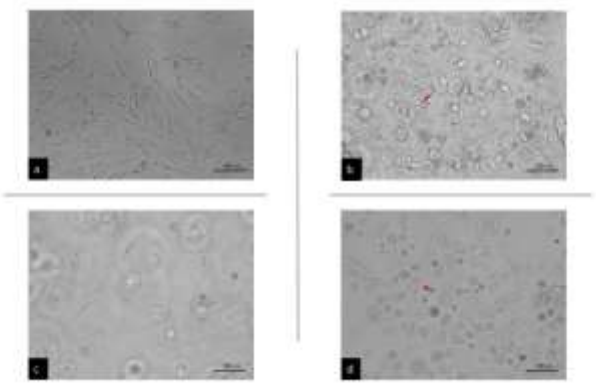

Figure 3: Proliferation of fibroblasts in D1 (a). Attachment of fibroblasts in D2 (b), D3 (c) and E (red arrows) (d).

Day 10

D1 was 70% confluent on 10th day of culture (Figure 4a). Not much fibroblast proliferation was observed and mixed population of cells other than fibroblasts dominated the cultures in D3, D4 and E (Figure 4b-d).

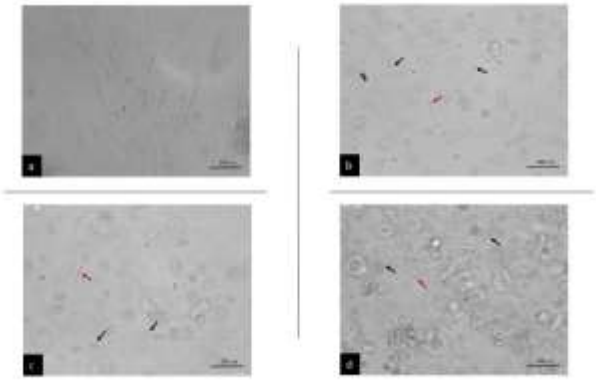

Figure 4: Monolayer of fibroblasts in D1 (a). A very few no. of fibroblasts (red arrows) and mixed population of other cells (black arrows) in D2 (b), D3 (c) and E (d).

Day 14

D1 showed 90% (Figure 5a) and E showed 20% confluence on 14th day. Large epitheloid (f5 type) fibroblasts were observed in E (Figure 5d). D2 and D3 didn't show any further attachment or proliferation of fibroblasts, therefore, terminated (Figure 5b & 5c).

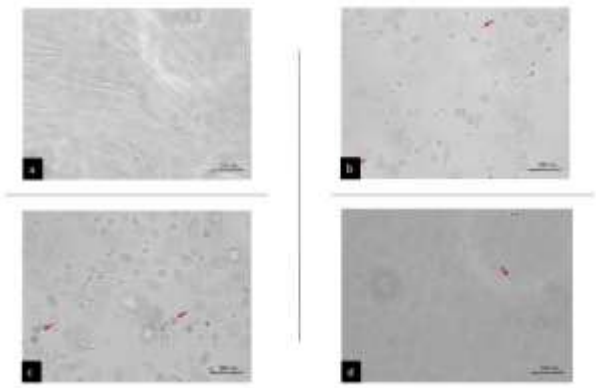

Figure 5: D1 showing 90% confluence (a). Cell death observed in D2 and D3 (b, c). E showed 20% confluence and f5 type fibroblasts (arrow) (d).

IV. DISCUSSION

Explant and enzymatic isolation are two common methods used to isolate human primary fibroblasts. Explant isolation involves taking a small piece of tissue (such as skin or fibrous tissue) and placing it in tissue culture flasks. The cells will naturally migrate out of the tissue and adhere to the flask, where they can be grown and studied. This method is simple and does not require the use of enzymes, but it is not always reliable and may result in a mixture of cell types[7].

Enzymatic isolation involves the use of proteases and other enzymes like trypsin, collagenase and dispase to digest the extracellular matrix and separate the fibroblasts from the surrounding tissue[8]. The enzymes are added to a tissue explant, and the cells are then dissociated and plated in culture flasks. This method is more reliable and provides pure population of fibroblasts, but it can be more time-consuming and complex than explant isolation[5,9].

In this study we established a primary cell line of oral mucosal fibroblasts and compared partial digestion and explant technique of isolation. In partial digestion the tissue fragments were left in the flask after digestion with trypsin. Tissue was digested for 10 min., 30 min., and 60 min. and digestion time was also compared in terms of attachment and proliferation of cells.

After digestion, heterogenous population of cells was noted till 3rd day, however, D1 showed attachment of fibroblasts at day 3. Consistent with our finding, Patil et al. also observed early fibroblast attachment (4th day) by using explant-enzymatic technique[7]. Trypsin causes hydrolysis of peptide bonds and acts to break down the extracellular matrix proteins such as collagen, fibronectin, and laminin. These proteins provide structural support to cells and tissues and hold cells together [10,11]. Salehinejad et al. observed cessation of growth of mesenchymal stem cells in the trypsin treated group after 30 days[12]. On the contrary in our study fibroblasts proliferated and became 90% confluent in D1 on 14th day. Leaving the tissue pieces in primary culture after digestion provides the benefit of release of growth factors which help in proliferation of fibroblasts and reduces the cost of added growth factors[6].

Attachment of fibroblasts was not observed until 7th day in D2 and D3. Moreover, not much proliferation was seen afterwards. This

might be due to the long duration of exposure of cells to the enzyme that could have damaged the membranes and interfered with the viability of the cells[12,13]. Cells in the explant flask took longer to proliferate and were only 20% confluent on 14th day. This agreed with the previous study done for the isolation of mesenchymal stem cells which showed that explant took 4 weeks to become confluent[12].

V. CONCLUSION

The purpose of this study was to develop a faster method of isolation of fibroblasts. Hence, we concluded that with the use of trypsin for 10 min., fibroblasts migrated and proliferated at a faster rate as compared to 30 min., 60 min. digestion and undigested explants. Leaving the pieces of tissue after digestion provided the added benefit of the release of growth factors necessary for the proliferation of fibroblasts.

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