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Comparison of two cellular harvesting methods for primary human oral culture of keratinocytes

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Abstract The possibility of obtaining transplantable oral epithelia opens new perspectives for oral treatments. Most of them are surgical, resulting in mucosal failures. As reconstructive material this in vitro epithelia would be also useful for other parts of the human body. Many researchers still use controversial methods; therefore it was evaluated and compared the efficiency of the enzymatic and direct explant methods to obtain oral keratinocytes. To this project oral epithelia fragments were used. This work compared: time needed for cell obtainment, best cell amount, life-span and epithelia forming cell capacity. The results showed the possibility to obtain keratinocytes from a small oral fragment and we could verify the advantages and peculiar restrictions. We concluded that under our conditions the enzymatic method showed the best results: in the cells obtaining time needed, cell amount and life-span. Both methods showed the same capacity to form in vitro epithelia.

Keywords Primary cell culture · Keratinocytes · Oral keratinocytes · Cell culture techniques · Primary culture techniques

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Introduction

Several hereditary and genetic pathologies are found in the mouth (Laskaris and Scully 2005) which need to be corrected or repaired through surgical treatment (Lauer and Schimming 2001; Nadjmi and Jackson 1999). Many of these treatments are unable to obtain desired results, resulting in discontinuity of the oral mucosa. Several different techniques have been developed to solve these problems. One of these techniques is in vitro cell culture, in which the cells from human oral mucosa are cultivated. This option has had positive and promising results (Bodner and Grossman 2003; Sumi et al. 2000). Transplant of epithelium cultivated in vitro has been used successfully since 1980 to treat patients who have sustained third degree burns on large areas of their bodies. Long term studies of the evolution of the transplanted cultivated tissue have been done. They showed

normal histological features 5 years after transplantation. At this point, a higher degree of organization has been detected in the connective tissue under the transplanted areas when compared with areas treated with more traditional techniques (De Luca et al. 1990). Transplantation of cultivated oral epithelium, obtained from cells removed from a small section of intra-oral tissue is not restricted only to iso-type transplant. Its unique features allow it to be used as a transplant material for other types of reconstruction, such as the urethra and cornea, and for the ocular surface (Bhargava et al. 2004; Nakamura et al. 2004). Conditions of in vitro cultivation are ideal for these cells to proliferate as in the original organism, which makes the autogenous transplant of these kerotinocytes possible. In 1975, Rheinwald and Green published a revolutionary method for cultivation of keratinocytes in which they employed modified previously irradiated murine fibroblasts (3T3-Swiss albino) to form a Feeder-Layer (Rheinwald and Green 1975). This method made it possible to cultivate these keratinocytes with superior cell yield and for extended durations than what had been previously achieved. With the advantages offered by the availability of these cultured cells, it became apparent that an efficient cultivation method for oral keratinocytes could be established. Among the concerns of cell cultivation, the need for a high quantity of clonogenic cells with good performance stands out. Most of the time available fragments for cellular extraction inside the mouth are small, considering that the mouth itself is a small area (Bodner and Grossman 2003).

In 1910, Carrel and Burrows described a method for the extraction of epithelia cells called direct explant, which has been used since that time (Lauer and Schimming 2001). The cells from the initial tissue extraction affixed to a culture dish migrate out of the tissue (centrifugal direction) starting from the edges of the fragment, adhering to and multiplying on the culture dish. In 1952, Billingham and Reynolds pioneered a technique for the separation of epithelial cells using an enzyme (trypsin), thus called the enzymatic method, in order to obtain keratinocytes and at the same time prevent these cells from loosing their viability and culture potential (Billingham and Reynolds 1952).

Despite several published studies of both methods (enzymatic and direct explant) employed in keratinocyte cultivation, doubts are still present about which one would be the best choice in obtaining the greatest number of clonogenic cells, cell performance, and the best culture life-span.

The purpose of this study was to compare the efficiency of the enzymatic and direct explant methods of obtaining human oral keratinocytes, as well as to evaluate the in vitro vitality of the cells when obtained using either method.

Materials and methods

This project was approved by the Research Ethics Committee of the Nuclear and Energetic Institute Research, under License Number 087/CEP-IPEN/SP. The experiments were carried out using human oral epithelial tissue from volunteers undergoing dental surgeries such as: third molar extraction, gum surgery, frenectomy, and dental implants (Klingbeil 2006). All the patients were healthy and did not smoke, drink alcohol or use other drugs which could interfere with the results. The samples were washed and disinfected in 70% alcoholic solution and physiological solution with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (40 μg/ml), and amphotericin B (2.5 μg/ml) (Klingbeil 2006), were measured and divided in two equal pieces before proceeding into the comparative tests.

Enzymatic method

The initial fragment was cut into small pieces of approximately 0.5 × 0.5 mm and immersed in a trypsin 0.05%/EDTA 0.02% solution under low agitation at 37°C. Supernatant solution with the cells was taken in intervals of 30 min and the trypsin action was neutralized with culture medium containing 10% fetal bovine serum (Klingbeil 2006). This mixture was centrifuged for 5 min at 1,500 rpm. After that, the supernatant was aspired and the cells resuspended in appropriate keratinocyte culture medium constituted from Dulbecco's Modified Eagle Medium (DMEM; Gibco, New York, NY, USA), F-12 Nutrient Mixture (HAM, Gibco, New York, NY, USA) (2:1), with 10% Bovine Serum Product, Fetal Clone III (Hyclone, Logan, Utah, USA), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (2.5 μg/ml) glutamine (4 mM), adenine (0.18 mM) (Sigma–Aldrich,

St Louis, MO, USA), insulin (5 μg/ml) (Sigma–Aldrich, St Louis, MO, USA), hydrocortisone (0.4 μg/ml) (Sigma–Aldrich, St Louis, MO, USA), cholera toxin (0.1 nM) (Sigma–Aldrich, St Louis, MO, USA), triiodotyronine (20 pM) (Sigma–Aldrich, St Louis, MO, USA) (Benassi et al. 1993). This proceeding was repeated until it was impossible to visualize any cell pellet. The cells obtained during the extraction process were counted. The cells (6×10^3 cells/cm^2) were seeded on a feeder-layer 3T3-Swiss albino, a murine fibroblasts lineage (ATCC/catalogue number CCL-92). The integrated keratinocytes/feeder-layer was mixed with the keratinocyte culture medium described above and this medium was substituted every 2 days. After that the first change Epidermal Growth Factor (EGF) (R&D Systems, Minneapolis, MN, USA) concentration 10 ng/ml was added (Benassi et al. 1993). The cells were kept in a humid incubator at 37°C containing 5% CO_2 and were removed by trypsinization from the culture flask. When the sub-confluence stage was reached these cells were counted and propagation in culture was done on a new feeder-layer.

Direct explant method

After washing and disinfection a tissue sample was cut into small pieces of approximately 1 mm^2 in size and placed with epithelial surface directly over the culture dish area (Carrel and Burrows 1910). They were placed in a ratio of approximately one fragment per cm^2 on the dish. These small pieces were slightly humidified with physiological solution before being placed in the culture dish.

The culture medium was prepared as DMEM with 10% fetal bovine serum (Bovine Fetal Serum, Cultilab, Campinas, SP, Brazil), glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (2.5 μg/ml), was dropped between those fragments, which were then incubated for approximately 24 h in a humidified atmosphere at 37°C with 5% of CO_2.

After 24 h this culture plate was flooded with the same culture medium described above (Kedjarune et al. 2001; Klingbeil 2006). The culture plates were examined daily by inverted microscope. When the migrated cells reached a 2–5 mm diameter, the medium was changed to the culture medium described on the enzymatic method enriched with EGF.

When the cells around the sample fragment started to change their morphological aspect raising their volume, that meant that these cells reached the confluence in their own micro-system; they were then removed by trypsinization, counted, and started their further propagation on a feeder-layer as described by the enzymatic method.

Keratinocytes propagation on feeder-layer

After determination of keratinocyte primary culture in both methods, the propagation of this cell in subsequent cultures was done. The passages were fulfilled every time the culture reached the sub-confluence stage. At that moment, cell trypsinization and centrifugation were done. The cells were counted and seeded (6×10^3 cells/cm^2) on a new feeder-layer. The medium was changed every 2 days (Benassi et al. 1993).

Colony formation efficiency

The efficiency was obtained from seeding 100 cells on a feeder-layer (cell dish: 28.3 cm^2). These cells were sustained for 14 days and the medium was changed every 2 days. After this growing period the colonies were macroscopically visible. To make the colonies visible, the plates were incubated with 2% Rodamin B diluted in 4% formaldehyde solution. The macroscopically colonies were counted with a special marker pencil and the very small colonies were counted under a microscopy to confirm the abortive colonies, when necessary. The CFE was calculated considering the percentage of the colonies obtained in relation with the number of seeded cells (Klingbeil 2006).

Life-span

The cultured cell life-span is reached during the progressive passages. Colony formation efficiency was concomitantly observed in each passage. Cell generation number was calculated by using the following equation:

$$x = 3.322 \log \frac{N}{N_o}$$

x = Generation number; N = Cell number after each trypsinization; N_o = Seeded cell number multiplied by the correspondent CFE (Colony forming efficiency).

In the case of epithelial cells (keratinocytes), only some of them when cultivated will adhere to the dish and will start duplication, therefore CFE must be employed (Barrandon and Green 1985).

Epithelium production

The keratinocytes obtained in both methods were seeded in a 1.88 cm^2 deep dish on a feeder-layer. Culture medium was initially changed every 2 days and then every day after the cells reached the confluence stage. After the first confluent day, a duplicate from the formed epithelium was removed through dispase enzyme action (0.25%, Neutral Protease-grade II, Boehringer–Manheim, Germany) during 1 h at 37°C.

The same proceeding was successively repeated during some days after the confluence (2, 4, 7, 12, 15, 18, 21 and 28 days). The removed epithelium was stored in a 10% formaldehyde solution.

Histology analysis

The conserved epithelium was embedded in paraffin, sectioned, stained with hematoxylin–eosin, and examined with light microscopy for cellular architecture.

Statistical methods

The results obtained were statistically analyzed through two methods. A Wilcoxon test for non-parametric analysis and a *t*-student test for parametric analysis.

Results

Extent of obtained fragments

The fragments were previously measured and their respective area was calculated. The average, standard deviation, and range are represented on Table 1

Table 1 Fragment size: average, standard deviation, and variations

Fragment area (cm^2) $n = 35$	Mean	SD	Minimum variation	Maximum variation
	0.65	0.57	0.12	2.00

($n = 35$). A summary for calculated results is presented on Table 2.

Substantial time for the first cell harvest

Only 20–31 fragments have released cells and could be compared. Average time and standard deviation to obtain the first keratinocytes are presented on Table 3 ($n = 20$). These results showed that not all the samples used on Table 1 were able to release cells. The first harvest in the enzymatic method was done at the moment when the cells reached the sub-confluence stage and when the cells around the fragment sample became bigger in the direct explant method, showing the first sign of the differentiation process. This difference area is presented in Fig. 1 as a gray

Table 2 Fragment result summary (in cm^2)

Minimum	IQ	Mean	Middle	IIIQ	Maximum
0.12	0.24	0.45	0.65	0.88	2.3

IQ (first quantile) = area value below which is 25%

IIIQ (third quantile) = area value below which is 75%

Table 3 Time needed in both methods to obtain the first keratinocyte harvest, standard deviation and *P*-value

Time needed to obtain the first keratinocyte yield (days) $n = 20$	Enzymatic method		Direct explant method		*P*-value
	Mean	SD	Mean	SD	
	11.90	2.36	14.2	2.76	0.0088

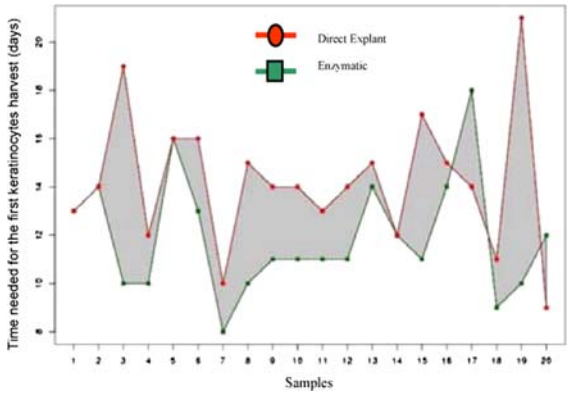

Fig. 1 Difference between the time needed (gray area) for the first keratinocyte harvest in both methods. *x*-axis = number of samples; *y*-axis = number of days

shadow. Samples are located in the x axis and the y axis represents the time (days) ($P = 0.0088$).

Initial cellular yield in the first cell harvest

The cellular yield data obtained by both methods in the first cell harvest is presented on Table 4 ($n = 31$). The number $n = 31$ since not every fragment have released cells and not all of the 35 ($n = 35$) have adhered on the dish ($P = 0.0000331$).

Cell morphology features by the first cell yield

Figure 2 shows the enzymatic method for keratinocytes at the sub-confluence and cells by the direct explant method in the moment that they were removed from the culture dish.

Colony forming efficiency (CFE)

Colony forming efficiency was used in both methods to compare cell types obtained in the first passage and to visualize cell evolution in subsequent passages.

Table 4 Initial cell yield for the first keratinocyte harvest, mean, standard deviation, and P-value

Cell yield on the first keratinocyte harvest (per cm^2) $n = 31$	Enzymatic Method		Direct explant method		P-value
	Mean ($\times 10^5$)	SD	Media ($\times 10^5$)	SD	
	43.50	43.90	6.52	9.41	0.0000331

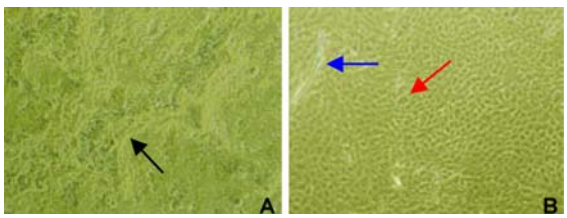

Fig. 2 Photographic images of Keratinocytes culture. **a** Sub-confluence cell stage (enzymatic method). *Remaining feeder-Layer* (↖). **b** cells at the moment they were removed from the plate (direct explant method). *Beginning of differentiation, cells expanded their size* (←), *cells were accumulated and started the desquamation* (↙). Image enlarged 100 times with blue filter

Macroscopic images from colored colonies in the plaques are shown in Fig. 3.

Keratinocytes life-span on feeder-layer

The cell duplicated data are presented in Fig. 4. All cells were provided by the same volunteer. About 127 duplications were achieved when cells were cultivated on feeder-layer.

Epithelium production

Epithelia were produced with cells obtained from both methods: enzymatic and direct explant. Figure 5 shows epithelium images from several days after cellular confluence. These epithelia were made up by keratinocytes cultivation on feeder-layer.

Histology analysis

Figure 6 shows the cellular architecture from the sectioned stained epithelia examined with light

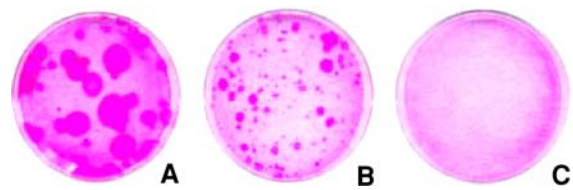

Fig. 3 Macroscopic images of CFE dishes with types of colonies found after the test. **a** Developing colonies; **b** Developing colonies and abortive colonies; **c** Absence of colonies

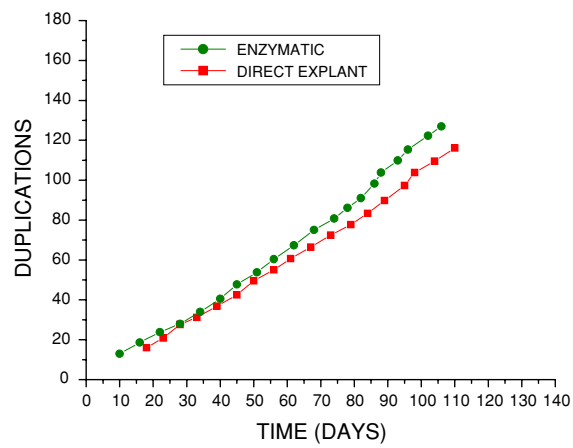

Fig. 4 Number of duplications in each method during the studied period

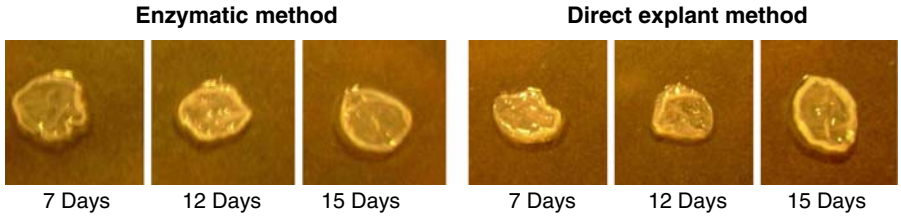

Fig. 5 Epithelia images after the keratinocytes have reached the confluence stage. Under each epithelia, the number of the days after confluence

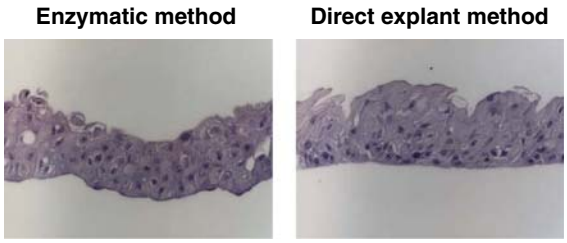

Fig. 6 Cellular architecture from the sectioned stained epithelium, after 15 days confluence

microscopy. Epithelia stratification was observed in both methods.

Discussion

The two methods to obtain cells were compared from the beginning of the respective processes. Then, cell propagation and culture technique, for both methods, were exactly the same. After cell extraction, both culture conditions were continued in the same manner, making it possible to apply statistical analysis.

Keratinocytes cultivation proceedings from several tissues has been used for different purposes (Bhargava et al. 2004; Bodner and Grossman 2003; Nakamura et al. 2004; Sumi et al. 2000); however, a substantial clonogenic cell amount with high efficiency will always be a point of concern, especially in the mouth, where available sample area will always be reduced thus supplying small fragments (Bodner and Grossman 2003).

In this work, each volunteer had his/her own reason for surgical therapeutic indication. This explains the variance size represented on Tables 1 and 2. This variation will always be present in such studies, since it is only possible to define the right fragment size to be removed in specified therapeutic surgical procedures.

The average time needed to obtain the first keratinocyte (Table 3) was 11.9 days for the enzymatic method and 14.2 days for the direct explant method. The *t*-student test was applied for statistical analysis. The result ($P = 0.0088$) suggests that the time (days) needed to get the first keratinocyte yield by the direct explant method is significantly higher when compared with the time needed by the enzymatic method. This variation is shown as the gray area in Fig. 1.

The concern in obtaining a good cell amount and the best cell quality with high clonogenic capacity is emphasized when the direct explant method is employed. Moreover, this method needs more time according to the reports by Breidahl et al. (1989) and Kedjarune et al. (2001). When compared with the enzymatic method, the absence of a feeder-layer supports cell differentiation well.

The first cell yield (Table 4) in the direct explant method ($6.52 \times 10^5/cm^2$) was significantly lower when compared with the results obtained in the enzymatic method ($4.35 \times 10^6/cm^2$) disagreeing with the findings from Kedjarune et al. (2001). Other authors like Breidahl et al. (1989), differ on the method preference. Freshney (2000), highlights that the direct explant method should be chosen when the tissue sample is short. The results of this work showed another direction. The value obtained through Wilcoxon statistical analysis ($P = 0.0000331$) means that the first cell yield obtained by enzymatic method is significantly higher when compared with the results obtained by direct explant method.

A difference in the keratinocytes morphology can be observed in Fig. 2 in the first cell yield for both methods. Cells obtained through enzymatic method show the morphology appearance of basal keratinocytes. This means no differentiated cells (Fig. 2a). When these keratinocytes cells are obtained through direct explant method it was possible to observe cell

differentiation signs, such as cell volume growth (Fig. 2b) (Barrandon and Green 1985).

When the primary culture was examined by inverted microscope a great difference came up between the methods. In the enzymatic method, the cells must achieve the sub-confluence stage where the feeder-layer has to be almost entirely absent and no other kind of influence will take place when these cells have to be removed by trypsinization. On the other hand, there will always be a need to interfere in the direct explant method, since there is an optimal time to remove cells from the dish. Therefore there is a concern to obtain the largest cell quantity, while avoiding cell morphological changes.

The feeder-layer along with an adequate culture medium (Benassi et al. 1993) in the enzymatic method collaborates with the delay of the differentiation process providing a higher cell duplication capacity (Rheinwald and Green 1975). Colony formation efficiency provides the clonogenic difference from seeded cells and helps to predict the number of duplications that these cells were able to generate (Barrandon and Green 1985; Klingbeil 2006).

The images in Fig. 3 showed different colony types, which are possible to be obtained according to the keratinocytes culture evolution. The evolution colonies are those that present basal cells in their majority with a high clonogenic potential. They are macroscopic and microscopically visible (Barrandon and Green 1985). Abortive colonies are those composed of differentiated cells with a limited cell clonogenicity (Barrandon and Green 1985). Irreversible cell senescence process is translated by cell differentiation. At the end of their life-span, these keratinocytes are not able to form colonies anymore (Dish C).

Figure 4 presents the duplication quantity that these keratinocytes were able to achieve in both methods. The cells for this experiment were provided by the same volunteer. In the enzymatic method where the feeder-layer was used, cells were able to achieve 127 duplications within 120 days reaffirming the evolution of the cultivation technique developed by Rheinwald and Green (1975).

It was possible to obtain epithelia through both methods from the confluence of keratinocytes in culture. These epithelia showed the same aspects when compared with the epithelia obtained in the study from De Luca et al. (1990), and Green et al. (1979). It was also possible to observe a stratification process by histological analyzes, according to Staiano-Coico et al. (1986).

Conclusion

The enzymatic method under the conditions of this work provided the best results for human oral keratinocytes primary culture. When compared with the results obtained in the direct explant method, it was possible to obtain the best cell yels within the shortest time.

Technical handling involved in the direct explant method at the beginning of the process was less than the handling required for the enzymatic method. The next advantage was that the direct explant method did not initially require a feeder-layer in order to obtain cells. However, when cell propagation is desired, the feeder-layer employment is needed and it also prevents the appearance of other undesired cells in the keratinocytes culture, like fibroblasts. This work did not encounter such problems.

The use of both cultivation protocols was adequate for obtaining and propagating oral keratinocytes in culture, and also showed the possibility of formation of a stratified epithelium.

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