

An outcome analysis and long-term viability of cryopreserved cultured epidermal allografts. Assessment of the conservation of transplantable human skin allografts¹

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ABSTRACT

PURPOSE: To assess the viability of cultured epithelium and preserved by freezing for periods varying from one month to one year.

METHODS: Samples of cultured epithelium were incubated in cryoprotectant medium (Group A), packed in aluminum envelopes and packed in polystyrene boxes. The boxes were subjected to a temperature of -70°C. After freezing for a period of time ranging from one to 12 months, cultured epithelial samples were assessed for their viability by vital staining (Trypan blue) and metabolic analysis based on glucose consumption and lactate production. Samples of not frozen cultured epithelium (Group B) were also tested for viability and the results obtained were used as comparison parameter for the variation of viability.

RESULTS: Statistical analysis between the group A and B indicate that the mean age of the donors ($p=0.51$) and the culture time ($p=1.18$) showed no statistical difference. In 30 days we obtained 37% of the original viability of cultured epithelium, 25% at six months and one year, less than 15%. This trend was confirmed statistically with a reduction of approximately 1.8% of the original viability epithelium cultured every 30 days of storage. In the analysis by lactate production, similar results were observed. In the analysis by the glucose consumption results were not significant. The viability indices show statistically significant difference between the group A and B ($p<0.0001$).

CONCLUSIONS: Although cryopreserved cultured epithelium showed significant reduction of viability, all samples remained viable. It was also found that the viability of cryopreserved cultured epithelial decreased as a function of storage time.

Key words: Tissue Culture Techniques. Tissue Banks. Cryopreservation. Burns.

Introduction

Cultured epithelial grafts has become a reliable technique in modern plastic surgery and has allowed reconstruction of different types of wounds defects and acute burns. In fact, the possibility to transfer skin has greatly improved the restoration of tissue defects following traumas, clinical disease or major traumas¹⁻⁹.

In burn care field, skin allografts play an important part in the surgical treatment. In fact, to limit metabolic and septic complications of major burns, wound excision must be performed as early as possible. However, due to insufficient availability of donor sites, frequently immediate autografting is complex and not available¹⁰. Thus, human skin allografts can be an alternative and the wound bed can be temporary covered. Allografts are also indicated to prepare the wound before the use of definitive cultured epidermal autografts, in order to provide a permanent dermal replacement underneath the epidermal sheets^{10,11}.

Introduced by Rheinwald and Green¹, the use of cultured epidermal autografts in burns has been based on a technique for effective keratinocyte cultures¹. In fact, cultured epidermal are taken as a permanent coverage of wounds, and they are known as an epoch-making treatment for large burns for which an adequate skin replacement is complex to achieve^{4,13}. However, usually acute burn patients have to wait for the growth of the epidermis once the epidermal culture requires almost one month. Contrary, cultured epidermal allografts are applicable anytime when necessary, and they were expected to be an important method in burn treatment.

For cultured epidermal allografts become an affordable alternative, there must be available in sufficient quantity, which is undermined when the graft is obtained directly from the culture flask. Moreover, the process is more efficient when the epithelium is produced and stored in a tissue bank to be used promptly.

In the literature, several authors have described the cultured epidermal allografts for freezing, expanding its availability and making your employment as a viable clinical alternative^{3,5,6,13}. Thus, the cryopreservation of cultured epithelium has been a constant search topic in many centers worldwide^{3,5,13-15}. However, in these studies the efficacy of the method was verified by short periods of time of seven and thirty days. Moreover, in these studies there is no mention of what would be the effect of preserving for longer periods on the viability of cells. Thus, we believe to be relevant new studies to verify the effectiveness of cryopreservation of cultured epithelium for longer periods. Thus, the aim of this study is to verify the effectiveness of the method of cryopreservation to -70°C in maintaining the viability of cultured

epithelium, preserved for the period 1-12 months.

Methods

The clinical and experimental protocol was approved by the Ethics Committee of the Plastic and Hand Surgery, Burn Center of Klinikum Bogenhausen – Munich and University of Sao Paulo School of Medicine. All the procedures strictly followed the existing regulations about clinical and experimental research.

Allografts were obtained from 19 patients submitted to conventional plastic surgeries in Plastic and Hand Surgery, Burn Center of Klinikum Bogenhausen – Munich. This collection of discarded surgical skin fragments was performed according to a protocol approved by the University Institutional Review Board. In all cases, patients gave their consent to this protocol after they received detailed information about its objective and risks.

After the skin was obtained by the surgeon, the skin was kept moist between layers of saline-saturated gauze until required for banking. These cells were prepared into cultured epidermal allografts and cryopreserved. All patients were examined for HIV, HBV, HCV, HTLV-1 and other bacterial infections, and the patients were confirmed to be negative to all of them.

From the culture fragment from each donor skin, a sample of cultured epithelium was obtained (Figure 1A). The cultured epithelial samples were randomly divided into two groups as follows:

Group A: 7 cultured epithelium samples not cryopreserved;

Group B: 12 cultured epithelium samples cryopreserved.

Samples of cultured epithelium in group A were evaluated at the time of adequate maturity. Samples of cultured epithelium in group B were evaluated for their viability following defrosting, after remaining frozen for varying periods 1-12 months.

In the present study, we used the technique described and standardized internationally by Rheinwald and Green¹ to obtain epithelium cultured keratinocytes.

Cryopreservation

In the cryopreserved group (B), after being washed in Eagle's Medium (DMEM-Gibco), the samples were incubated for 30 minutes in cryoprotective solution. This solution consisted of Eagle's essential medium (DMEM-Gibco), with Earle saline solution, 4 mmol L-glutamine, 4.5 mmol of L-selen methionine, tobramycin (100 microgram / ml) solution, 5% human albumin and 15% glycerol. Glycerol was chosen as the cryoprotective agent

because of its low cytotoxicity and was added gradually every five minutes until reaching its final concentration. After incubation in the cryoprotectant solution, samples of cultured epithelium were placed in envelopes formed from aluminum sheets, polypropylene coated internally and externally with polyester (Figures 1B, 1C).

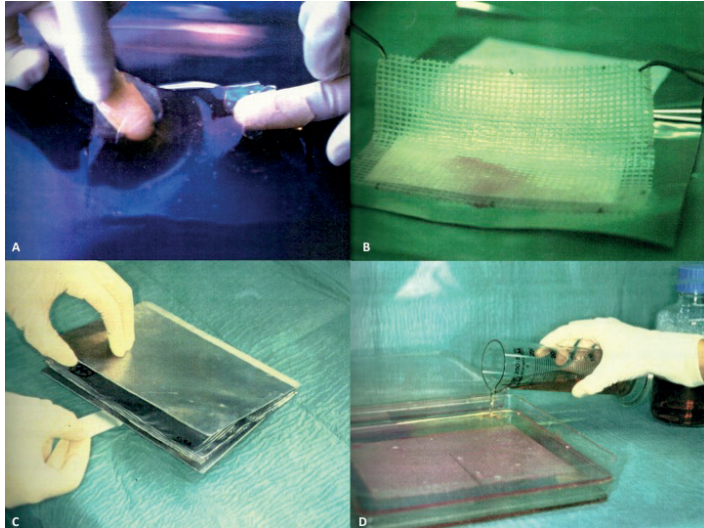


FIGURE 1 – **A.** Sample cultivated epithelium after their release from culture flask by means of enzyme treatment. **B.** Sample of cultured epithelium attached before packaging the bandage aluminum. **C.** Prior to freezing, the samples are placed between the aluminum sheets with a thickness of 1.0 mm. **D.** Irrigation of cultured epithelial samples with Eagle's medium after thawing.

The envelopes were placed between two sheets of aluminum with 1.0 mm thickness. The slides were wrapped with 40 layers of cellulose tissue and placed in a polystyrene boxes. The boxes were placed in refrigerators at a temperature of - 70°C for 4 hours. After this period, the boxes were removed from the refrigerator, the envelopes were removed from the boxes and returned to the refrigerator without aluminum sheet. Thus, the epithelium remained stored until the moment of use (Figure 1).

For defrozing process, the envelope was removed from the refrigerator and placed in 37°C saline solution for a period of three minutes. The epithelium was removed from envelope then washed with Eagle medium for removal of cryoprotectant (Figure 1D). The cryopreserved epithelium was then prepared according to the methodology for analysis of cell viability.

Assessment of cell viability

The evaluation of the viability of cultured epithelial samples was analyzed by means of vital dye (Trypan blue) and metabolic parameters based on glucose consumption and lactate production. The cultured epithelial samples of group B were

evaluated after remaining cryopreserved for a period of one to 12 months. After defrozing, the segment of epithelium was transferred to a solution of 0.025% trypsin and incubated at 37°C for five minutes. After digestion with trypsin, keratinocytes were centrifuged at 100 rpm for five minutes. After this step, a 100 µL of Trypan Blue was added to the 100 µL cell suspension.

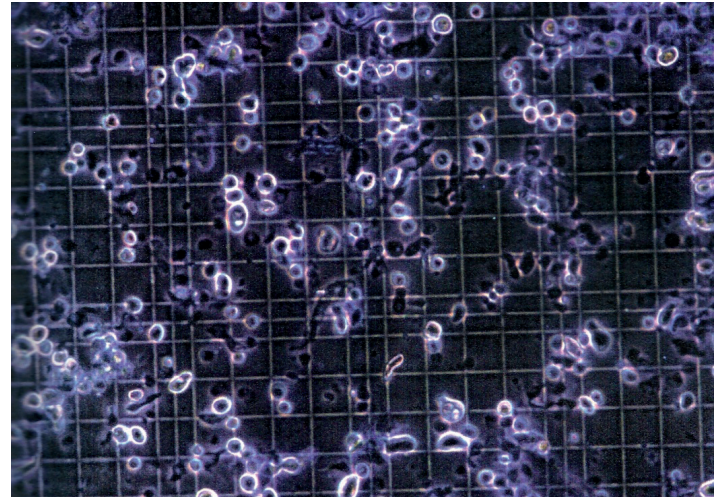


FIGURE 2 - Microscopy showing keratinocytes on a Neubauer chamber to count cells stained and unstained by Trypan method blue (x500).

The solution was placed on the square of Neubauer and under microscopic observation proceeded to count cells stained and unstained by blue Thypan. Boundary lines of the Neubauer ruling are the center lines of the groups of three (Figure 2). The central square millimeter is ruled into 25 groups of 16 small squares, each group separated by triple lines, the middle one of which is the boundary. The ruled surface is 0.1 mm below the cover glass, so that the volume over each of the 16 small squares is approximately 0.00025 cubic mm. By counting viable and unviable cells, it was possible to obtain the percentage of viable cells in each sample. The mean number of viable cells determined in the pattern group A (N) for normal proportion of viable cells in the epithelium not cryopreserved. This pattern served to verify the change in the proportion of viable cells in the epithelium cultured cryopreserved.

To determine the amount of cells remained viable after cryopreservation, we established the an index, called Preserved Viability Index (PVI). The PVI was determined by the amount of viable cells in the epithelium cryopreserved (P) in relation to the percentage of viable cells in the epithelium unfrozen (N).

$$\text{PVI (\%)} = \text{P} / \text{N} \times 100$$

P= % of viable cells in the cryopreserved epithelium

N= average of viable cells in the non-cryopreserved epithelium (group A).

The metabolic parameters were measured by glucose consumption and lactate production. After collecting the sample for staining, the epithelium was incubated in 50 ml of culture solution containing essential Eagle medium and using Ham F-12 solution in a ratio of 3 to 1, fetal bovine serum 10%, hydrocortisone (0.4 micrograms / ml) and 0.01 glucose monohydrate. The cultured epithelial samples were incubated for 16 hours at glasshouse 37°C with 5% CO₂.

After incubation for 16 hours, samples were collected for measurement of glucose and lactate. Because the initial concentrations of glucose and lactate in solution were known, the value obtained from the absorbed glucose and lactate produced by the difference of the initial and final concentrations. The glucose concentration was determined by the enzymatic method (glucose dehydrogenase) and analyzed photometrically. The lactate level was also made by enzymatic method and analyzed by special instruments (Technicon RA-1000).

Statistical analysis

The values of the age of donor skin, epithelial cultivation time, period of preservation of samples, lactate production, glucose consumption rate and viability of cultured epithelium samples were subjected to statistical analysis with a significance level of 5% (p<0.05). We used the following statistical models: Test of equality of means (Student-t test), non-parametric test of Mann-Whitney, chi-square test, Pearson correlation test and nonparametric Spearman correlation. Results were expressed as mean ± SD.

Results

Skin allografts obtained from 19 donor patients were analyzed. Samples of cultured epithelium in group A (n=7) were evaluated at the time of adequate maturity. Samples of cultured epithelium in group B (n=12) were evaluated for their viability following defrosting, after remaining frozen for varying periods 1-12 months. Statistical analysis between the mean values of group A and B indicate that the mean age of the donors (p=0.51) and the culture time (p=1.18) showed similar values and no statistical

difference.

Group A (non-cryopreserved cultured epithelium samples)

The mean values obtained with group A are demonstrated in Table 1. The mean patient donor age was 35.4 years (range 21-44 ys; SD = 7.21) and average cell culture 26.86 days (range 22-30 days; SD = 2.61). This group observed a lactate production and glucose consumption of 78.12 μmol (range 58.3-94.4 μmol) and 175.1 μmol (range 147-215 μmol) respectively. By analyzing the correlations between the data of group A, it was possible to establish a viability index for non-cryopreserved cultured cells. The cell count in a Neubauer chamber with Trypan blue ranged 95-189 with an average of 136.28. By calculating the percentage, we observed that 27.56% of the cells were viable and 72.44% were non-viable.

TABLE 1 - Group A. Cultured epithelium samples not cryopreserved.

| | Age (ys) | Culture period (days) | Lactate (μmol) | Glucose (μmol) | No. of observed cells | No. of Non-stained cells | No. of Non-stained cells (%) |
|---------|----------|-----------------------|----------------|----------------|-----------------------|--------------------------|------------------------------|
| Average | 35.43 | 26.86 | 175.1 | 78.12 | 136.28 | 37.57 | 27.56 |
| SD | 7.21 | 2.61 | 33.6 | 14.28 | 33.88 | 9.86 | 2.41 |

SD: standard deviation

Group B (cryopreserved cultured epithelium samples)

The data obtained from group B are demonstrated in Table 2. The mean patient donor age was 38.83 years (range 1-86 ys; SD = 21.0) and average cell culture period was 29.42 days (range 16-40 days; SD = 6.69). This group observed a lactate production and glucose consumption of 40.0 μmol (range 0-275 μmol) and 25.69 (range 8.33-52.77 μmol) μmol respectively. The cell count in a Neubauer chamber with Trypan blue ranged 120-219 cells with an average of 146. The average number of unstained cells was 10.67 ranging from four to 18 cells. By calculating the percentage, we observed that 7.18% of the cells were viable and 92.82% were non-viable. The average IVP was 26.5% for the cryopreservation period of 186 days (average), ranging from 32 to 375 days. The average lactate production of cryopreserved samples was 23% in the control group. The average consumption of glucose was equal to 33% of control group. The viability indices show statistically significant difference between the group A and B. The relations between the values of Groups A and B are demonstrated in Table

3 and Figure 3.

TABLE 2 - Group B. Cultured epithelium samples cryopreserved.

| | Age (ys) | Culture period (days) | Conservation period (days) | Lactate (μmol) | Glucose (μmol) | No. of observed cells | No. of Non-stained cells | No. of Non-stained cells (%) |
|---------|----------|-----------------------|----------------------------|----------------|----------------|-----------------------|--------------------------|------------------------------|
| Average | 38.83 | 29.42 | 186.17 | 40.0 | 25.69 | 146.0 | 10.67 | 7.18 |
| SD | 21.0 | 6.69 | 104.50 | 80.48 | 13.97 | 27.99 | 4.01 | 2.01 |

SD: standard deviation

TABLE 3 - Relationship and significance of the mean percentage of viable cells in groups A and B.

| Cels | Group A | Group B | Chi = | Significance |
|------------|-----------|-----------|--------|--------------|
| Viable | 27.57 (%) | 7.31 (%) | 203.51 | P < 0.00001 |
| Non-Viable | 72.43 (%) | 92.69 (%) | - | - |

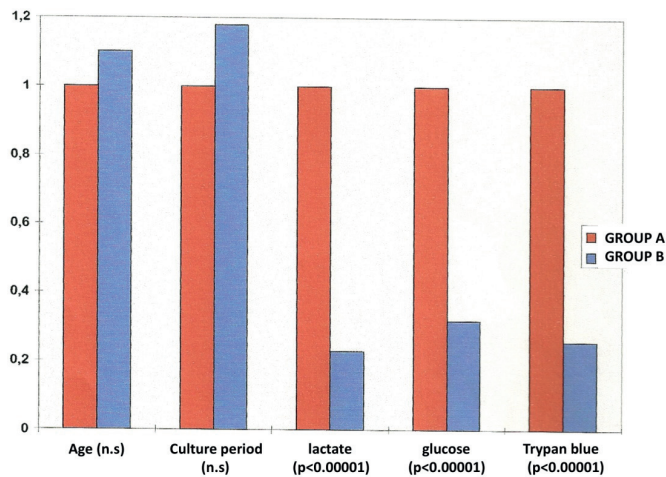


FIGURE 3 - Graphic representation of the values relations between Groups A and B.

The viability of the sample when assessed by vital staining showed significant correlation with respect to time cryopreservation (p=0.0001). In analyzing the data, it was possible to obtain a function to determine the number of viable cells versus time for cryopreservation. These data are shown in Figure 4.

When analyzing the time of cryopreservation and lactate production, the similar trend was observed. From this equation, it was possible to plot the variation of lactate production as a function of time cryopreservation (Figure 5). Moreover, the present analysis revealed statistical correlation between the index of viability detected in vital staining and lactate production. Applying the simple linear regression with their correlation coefficients it is possible to demonstrate the production of lactate in proportion of viable cells by vital staining test (Figure 6).

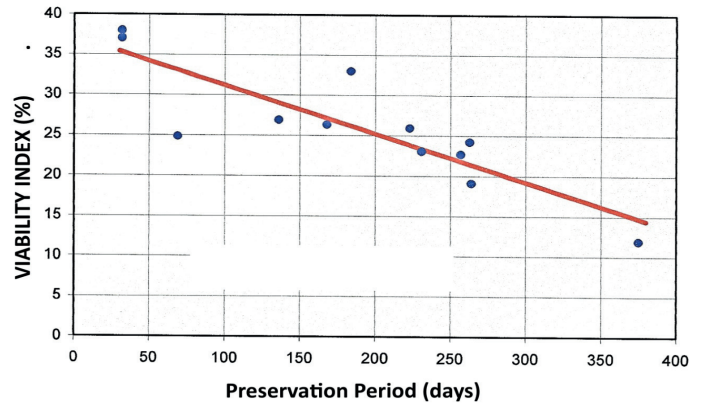


FIGURE 4 - Linear regression representing the index variation preserved viability versus time of preservation. Equation of the line, correlation coefficient and its significance.

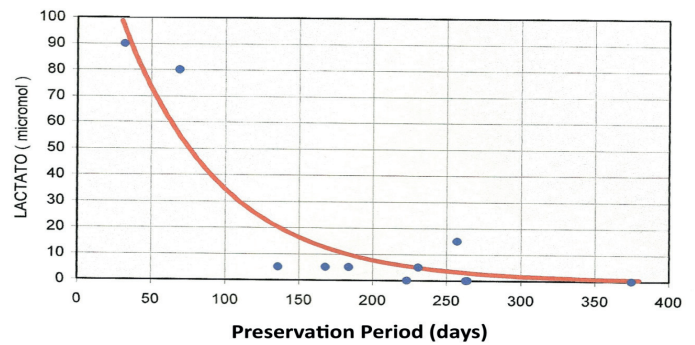


FIGURE 5 - Exponential regression representing the level of lactate produced by the tissue (μmol) according to preservation time (days). Equation of the exponential curve.

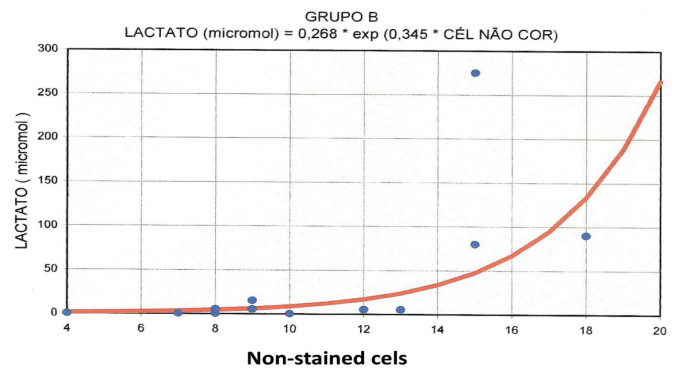


FIGURE 6 - Exponential regression representing the level of lactate produced by the tissue (μmol) according to viable cells (non-stained) in days. Equation of the exponential curve.

Discussion

The development of an efficient and economically viable method for conserving tissue is an important step for the clinical use of cultured tissue. The proper preservation of tissues and cells make possible to create tissue banks that may be useful in the treatment of various diseases and injuries.

Concerning the allografts properties, it is well accepted that the take and characteristics of skin allografts are related mainly to tissue viability^{23,24,33}. Although Webster firstly introduced the concept of the use of skin conservation at 48°C, the decline in viability of skin allografts with storage time has only been studied more recently^{23,24,26,27,32}. Thus, the present study sought to develop and evaluate the applicability and efficiency of the method for the preservation of cultured epithelium. The methodology utilized in the present study is derived from the epithelium of cultured keratinocytes developed by Rheinwald and Green¹. The cryopreservation method used was the adaption of the method developed by May and Roberts utilized for skin cryopreservation¹⁷.

Another important issue is the cryopreservation technique and the method of freezing. In fact, to achieve viable skin grafts following cryopreservation, it has been shown that cells should be cooled gradually and thawed rapidly²². Udoh *et al.*²² demonstrated that freezing is more suitable than rapid freezing for cryopreservation of cultured epithelial grafts. Nonetheless, it is known that when cells are frozen too slowly, survival rate decreases. May *et al.*²⁸ cryopreserved human cultured epithelial cells to assess their viability after thawing, and observed that viability was highest when cells were frozen at 1°C/min and thawed in a water bath maintained at 37.8°C. Similarly, the same author analyzed the viability of cells by either freezing porcine skin to -70°C at 18°C/min in a programmable-temperature freezer and storing the box in a constant-temperature freezer maintained at the same temperature²⁹. The authors observed that viability, measured by glucose metabolism, was about 80% in both groups after 24hs of cryopreservation, and that there were no significant intergroup differences.

Thus, there is a consensus that the slow cooling of a tissue treated with cryoprotectants is the best way to avoid the deleterious effects of the change in physical state of the cell^{22,28,29}. In this sense, the slow freezing can be obtained by freezing with programmable cooling rate. However, some freezers and depend on the volume of liquid nitrogen used for cooling process makes the method expensive and unfeasible economically. Therefore, less expensive and simple methods of cryopreservation have practical and economic advantages enabling the implementation of a tissue

bank where financial resources are limited¹⁷. However, as freezing is achieved passively and the storage temperature is higher, the quality of the cryopreserved and maintaining tissue viability may be different.

In the search for simpler and less expensive methods, some authors have described the cryopreservation of skin, but with the preservation period of 30 days^{15,17}. In addition, other authors advocated the use of glycerol-preserved skin allografts. However, in recent study comparing the use of glycerol preserved and cryopreserved allogenic skin for the treatment of severe burns, the histological structural integrity observed that cryopreservation confirmed to be the more viable product³⁴. Thus, questions about the possibility of cryopreservation for longer periods (up to 1 year) encouraged this study in terms of long-term cryopreservation. In addition, in our study we utilized a cryoprotectant in order to improve the cell survival rate in cryopreservation. Similar as other authors^{22,34,35}, the glycerol was utilized as cryoprotectant once this molecule enter the cells, reduce the ice crystal formation, thereby protecting cells from freezing lesion.

To determine the effectiveness of a method of preserving organs and tissues is important to select an appropriate criteria to measure cell viability. In the literature, some authors have studied and evaluated different tissues in terms of properties of cell reproduction, the integrity of the cell membrane¹⁸ and activities of intracellular metabolism^{19,20,24,25}. In addition, more recently the skin viability can be evaluated by Tetrazolium reduction assays utilizing the Methyl Thiazolil Tetrazolium test (MTT)^{23,35} and the delivery of growth factors to the wound as described by Rennekampff *et al.*¹⁰. To be able to synthesize these factors, cells of the allograft must be viable and functional. In spite of the presence of different techniques available, some studies have evaluated the property of cell reproduction as evidence of the presence of viable cells. However, this method proved ineffective for keratinocytes analysis since the presence of large numbers of differentiated cells in this culture have made this evaluation inaccurate.

Some authors pointed out that the physical evaluation of the integrity of the cells is a determinant factor for the viability of tissue culture¹⁸. In fact, the disruption of the cell membrane is an early and irreversible phenomenon of the cell death. The vital dyes have been widely used for testing viability in cell biology. Moreover, the exclusion test using Trypan Blue staining is recognized as accurate method to evaluate the integrity of the cell membrane and thus cell death. Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane,

in a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method.

Although the exclusion test by vital staining is an appropriate parameter of viability, it does not necessarily indicate the metabolic state of the cell. Therefore the assessment of viability was complemented by the evaluation of metabolism of the tissue. In fact, several studies on viability by metabolic parameters were based on glucose oxidation, or conversion of C-glucose to CO₂. However, the main reactions of respiration of the epidermis in vitro is related to the β-oxidation of lipids¹⁹. Moreover, most of glucose consumed by the epidermis or epithelium in culture is converted to lactate and only a small part of the glucose metabolites is oxidized. Therefore, it was more convenient to assess the metabolism via glucose consumption and lactate production as we perform in the present analysis. In fact, according to some authors lactate dehydrogenase activity and oxygen consumption can be performed with predictable results^{24,25}.

In the present study, the results obtained with the cultured epithelial samples indicated a consumption of 4.88 μmol of glucose/hour/100g of tissue, and this fact revealed an intense uptake of glucose by the epidermis as previously described in the literature²⁰. With respect to lactate production, it was observed a 10.94 μmol/hour/100g of tissue, thus indicating that most of the glucose uptaked was converted into lactate by the glycolysis and in agreement with previous studies²⁰.

The analysis of the group A showed a significant correlation between the number of cells not stained and total number of cells subjected to the test by vital dye exclusion. In fact, it was found that the ratio between viable and non-viable cells is kept within certain limits, being possible to establish an index for viability of cultured cells. Furthermore, the rate of viability exhibited by cryopreserved cultured epithelial samples without considering the time at which the tissue was maintained frozen show values remained relatively close by different ratings. The index of viability of cryopreserved and cultured tissue showed similar values in the three assessment methods employed. In this analysis, it was observed a rate of cell viability by 23%, 33% and 29% of the tests employing lactate production, glucose consumption and Tryphan Blue respectively. Furthermore, there was a significant correlation between the results obtained with Tryphan blue and lactate production and freezing period. However, in this analysis there was no correlation with the test glucose production. This fact may be partly explained by the process of metabolism of glucose.

Thus, it can be considered that even when the number of viable cells was reduced, the glucose metabolism was maintained even with the cell membrane disrupted. The molecule of glucose enter the reactions of glycolysis cycle, but the sequence of reactions would be stopped before the glucose was converted to lactate.

In the present study, the evaluation by testing vital dye exclusion and lactate production provided more reliable data. Thus, within the main objectives of the study, the test with the method of Trypan Blue was more appropriate. The test demonstrated a simple method to perform and provided results more accurate and useful. For this reason, we adopted the indices obtained by vital staining as a parameter to evaluate the method of preservation.

Regarding the freezing period and cell viability, the present study showed interesting and expected results. After cryopreservation for an average period of 186 days, the average viability by vital staining was close to 26%. However, when the rate of viability was related to the time of cryopreservation, the results show a clear tendency to decrease viability in function of time. This result was similar as observed by other authors utilizing different methodologies²². In fact, Udoh *et al.*²² previously confirmed viability of the allograft by conducting a colony forming efficiency test and flow cytometry. In this analysis, the author concluded that the viability of the cells in flow cytometry was 89.3% after one month cryopreservation, 61.7% after six months, and 6.6% after one year. In the present study, the correlation and linear regression statistics indicate that the process of freezing and thawing lead to a decrease of 63% of viability of the tissue. Thus, around 30 days of storage the epithelium present 35% of their original viability, with 6 months it present 26% and after one year the epithelium have less than 15% of viability. Over a longer period, close to 620 days, there would be no viability (0%) of cryopreserved epithelium. The present data confirm previous studies which have shown that the storage of the tissue 4°C^{31,35} and -70°C³⁰ result in a reduction of viability after a few weeks. In the clinical situation of need storage for longer periods, the best indication would be the use of liquid nitrogen in order to maintain proper temperature and thus increase tissue viability. According to some authors, when viable cells are subjected to long-term cryopreservation, the samples should be stored at a temperature below -130°C, since at temperatures higher than that, ice crystals form and recrystallization occurs in the cells, causing cellular lesion^{21,22}. However, in spite of the benefits cryopreservation using liquid nitrogen has its disadvantages. Some authors mentioned that it is difficult to store a large number of specimens, and tissue samples must be tightly sealed. In addition the liquid nitrogen must be continually replenished²².

The results of this study demonstrated a variation on viability of cryopreserved epithelial as a function of storage time. These data provide important parameters for establishing the period in which the cultured epithelium can be safely preserved and utilized. The final answer to this question will be obtained by determining the degree necessary for the viability of cultured epithelium can be used successfully³³. The perspective is that, in the near future, the cultured epithelial grafts preserved in the tissue bank it will be safely used in the clinical practice.

Conclusions

The method employed maintained the viability of the epithelium for one year. The process of freezing, storage and thawing led to a significant reduction in the viability of cultured epithelium. In addition, the viability of the tissue dropped directly over the storage time. At 30 days of preservation 37% of the original viability of the tissue was maintained, at six months only 26% and at one year less than 15% viability of the tissue was observed.

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