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# **Transplantation of cultured human keratinocytes in single cell suspension: a comparative in vitro study of different application techniques**

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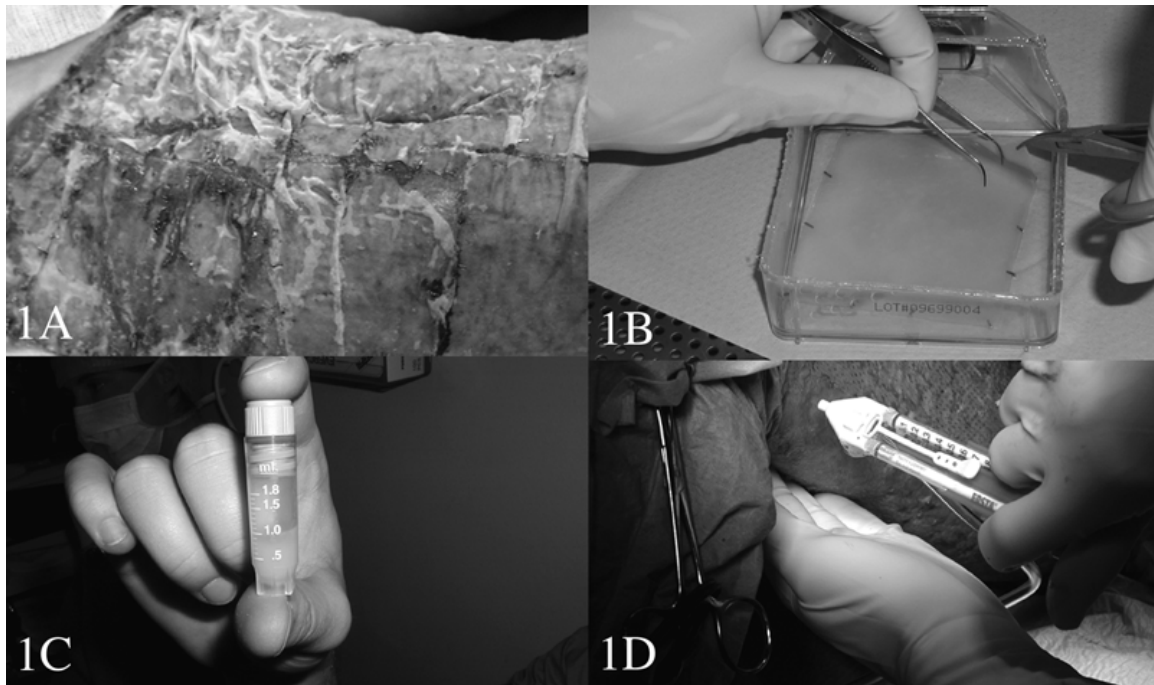
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## ***Abstract***

Transplantation of autologous cultured keratinocytes in single cell suspension is useful in the treatment of burns. The reduced time needed for culture, and the fact that keratinocytes in suspension can be transported from the laboratory to the patient in small vials, thus reducing the costs involved and be stored (frozen) in the clinic for transplantation when the wound surfaces are ready, makes it appealing. We found few published data in the literature about actual cell survival after transplantation of keratinocytes in single cell suspension and so did a comparative in vitro study, considering commonly used application techniques. Human primary keratinocytes were transplanted in vitro in a standard manner using different techniques. Keratinocytes were counted before and after transplantation, were subsequently allowed to proliferate, and counted again on days 4, 8, and 14 by vital staining. Cell survival varied, ranging from 47% to >90%, depending on the technique. However, the proliferation assays showed that the differences in numbers diminished after 8 days of culture. Our findings indicate that a great number of cells die during transplantation but that this effect is diminished if cells are allowed to proliferate in an optimal milieu. A burned patient's wounds cannot be regarded as the optimal milieu, and using less harsh methods of transplantation may increase the take rate and wound closing properties of autologous keratinocytes transplanted in a single cell suspension.

## ***Introduction***

Cultured keratinocytes have been used for about 20 years in the treatment of burns and other cutaneous wounds. In 1975, Rheinwald and Green [1] described a reliable method of culturing human epidermal cells in stratified and coherent layers, and so cultured epidermal sheet autografts became available to complement autologous split thickness skin grafts in treating major burns or other large wounds (Figure 1A) [2]. However, producing confluent grafts of keratinocytes puts heavy demands on laboratory skills, comprises manual labour, and is expensive, which limits the use of the autografts in many ways. The sheets are only 8-10 cells thick, which make them fragile and difficult to handle, and means that they have to be placed on a supportive backing material to be possible to transfer from laboratory to patient (Figure 1B). When the autografts have been detached from the culture vessel, they must be transplanted the same day, which requires the wound surfaces to be ready for grafting at the same time as the autografts are ready to be transplanted. Because the transplanted epidermal sheets are quite unstable and prone to blistering, care must also be taken, not only during production and transplantation, but also during dressing of the applied grafts, mobilisation of the patient, and changing of dressings [3]. The production of stratified grafts inevitably involves some degree of maturation and differentiation, of the keratinocytes, which reduces their proliferative capacity, and this in turn may affect the take-rate and wound-healing capacity [4]. Extrinsic factors including preparation of the wound, nutritional state, and dressings used influence their success.



*Figure 1A*  
 Cultured epithelial autografts transplanted to wound surface with a polyamide-mesh backing material. Picture taken five days after transplantation.

*Figure 1B*  
 Cultured epithelial autograft has been enzymatically released from culture vessel's bottom. Polyamide-mesh backing material (Surfasoft®) is being attached by folding the autograft's edges over the backing material and securing them with surgical micro-clips.

*Figure 1C*  
 Transport and storage vial for cultured cells, e.g. cultured autologous keratinocytes. Depicted vial contains approximately  $20 \times 10^6$  cultured cells ready for transplantation.

*Figure 1D*  
 Autologous cultured keratinocytes from the vial in picture 1C has been aspirated into the thrombin fraction of the Tisseel Duo Quick™ tissue glue syringe and are being spray-painted on the wound surface using the Duploject™ Spray set.

The attention to, and understanding of, these shortcomings have led to a progressive development of techniques of skin culture and an increased use of suspensions of single cells of keratinocytes being transplanted instead of sheet grafts. Fraulin et al [5], in 1998, described a novel technique in which they used an aerosol device to spray epithelial cells

on wounds in pigs. They noted that re-epithelialisation was quicker than in unsprayed controls. Navarro et al [6] developed this technique further by combining it with meshed split thickness skin grafts. They reported faster healing and a better quality of cells when they were sprayed. Further advantages of suspension transplantation are the reduced time needed for culture, and avoidance of the manual labour of releasing cell-sheets from culture flasks and attaching the cell-sheets to backing materials. By culturing and transplanting the cells in a suspension rather than as a sheet, the use of enzymes like Dispase<sup>®</sup> can be avoided. Keratinocytes in suspension can then be transported from laboratory to patient in a handful of small vials (Figure 1C) and be stored (frozen) at the clinic to be transplanted when the wound surfaces are ready [7]. The single cell suspension of keratinocytes can then be transplanted to the patient with whatever method is available such as being spray-painted on the wound surfaces with or without fibrin-glue (Figure 1D) [8, 9]. Today, transplantation of keratinocytes in a single cell suspension overgrafted with meshed allogeneic donor skin is a common approach in the treatment of burns [9, 10]. Techniques used in clinics today include spraying cells, with or without the additional use of tissue or fibrin glue, painting the cell suspension with a brush, or dripping the cell suspension on to the wound bed using a syringe. At our burn unit we have used the Tissomat applicator together with Tisseel Duo Quick<sup>™</sup> tissue glue and the Duploject<sup>™</sup> Spray set (all from Baxter Medical AB, Kista, Sweden) to transplant cultured keratinocytes. To distribute the cells satisfactorily, pressures as high as 200 kPa must be used. This has long been thought to damage the cells, both by the passing of the spray nozzle and by the high velocity impact on to the wound bed. Harkin et al [11] recently examined the viability of keratinocytes delivered by aerosol, using the Tissomat

applicator and found that the viability after transplantation (93.7% at 70 kPa and 90% at 138 kPa) was similar to the viability of the cells just recently detached from the culture dish (94%). When they adjusted the Tissomat applicator to deliver 207 kPa, they showed that fewer cells survived (73.3%), but not significantly so.

If a brush is used to distribute the cells to the wound (the method of choice in some clinics) it probably causes shear forces that damage the cells, and may leave fibres from the brush in the wound. Drips from the cell suspension, when using a syringe, may well cause an uneven distribution of the cells, if the suspension runs off the surface, pools in cavities, and leaves some areas uncovered. The fact that surprisingly few research workers have studied the viability and survival of transplanted cells, encouraged us to design a comparative in vitro study that took into consideration the methods used in clinics today.

The aim of our study was to compare and evaluate different commonly used techniques of transplantation of single cell suspension, from the point of view of cell survival, attachment, and proliferation of cells.

## ***Material and methods***

### *Cell culture*

Normal human keratinocytes were isolated and expanded in vitro. Briefly, biopsy specimens of skin from surgical waste were transferred to the laboratory in gauze soaked in physiological saline. Keratinocytes were isolated within 24 hours and the tissue was kept in +8°C until use. The skin was rinsed twice in phosphate buffered saline (PBS) with antibiotics and mycotics (50 IU/ml penicillin, 50 µg/ml streptomycin). Subcutaneous fat

was removed with scissors and the remaining tissue (dermis and epidermis) was cut into roughly 1 cm<sup>2</sup> pieces and incubated in Dispase<sup>®</sup> 15 ml (16.7 mg/ml, 1.04 U/ml) for 18 hours at +8°C. After incubation the epidermis was lifted off the dermis with pliers and transferred to 0.02% EDTA 2 ml and 0.25% trypsin 2 ml. The tissue was incubated in +37°C at 95% humidity and 5% carbon dioxide for roughly 10-15 minutes, during which time it was repeatedly removed from the incubator and triturated with a Pasteur pipette to dissociate the cells. After incubation and trituration, the action of trypsin was inhibited by transferring the cell suspension to washing medium (10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin and centrifuged at 400 g for 5 minutes. The supernatant was removed and the cell pellet re-suspended in culture medium 15 ml and seeded into a 75 cm<sup>2</sup> culture flask (BD Falcon, Stockholm, Sweden). Culture medium used was keratinocyte-serum-free media (Ker-SFM) supplemented with 200 µl/100 ml bovine pituitary extract, 3.3 µl/100 ml endothelial growth factor and penicillin-streptomycin. Medium was changed every second day throughout the study. When sub confluence had been achieved (for illustrations of different states of confluence, see Figure 3A-B) the primary culture was split 1:3 by rinsing the culture twice with 2 ml EDTA 0.02%, and subsequently adding 0.02%/EDTA 2 ml and 0.25% trypsin 2 ml and incubating for 10-15 minutes. The effect of the trypsin was then inhibited. The supernatant was removed and the cell pellet re-suspended in culture medium 12 ml and divided into three culture flasks 75 cm<sup>2</sup>. An additional 13 ml of culture medium was then added. Cells of the second passage were used in the study and the cultures were incubated in +37°C, 95% humidity, and 5%



carbon dioxide. All media and supplements were bought from Invitrogen AB, (Lidingö, Sweden) and EDTA and trypsin from Gibco<sup>®</sup>/Invitrogen AB, (Lidingö, Sweden).

#### *Preparation of single cell suspensions*

The cells were detached from the culture flasks and the effect of trypsin was inhibited as described. The supernatant was removed and the cell pellet re-suspended in culture medium 10 ml. Cells were counted in a haemocytometer by triplicate samples, cell suspension 10 µl mixed with trypan blue 10 µl (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and incubated in room temperature for 5 minutes.

#### *Application techniques*

Well-dispersed cell suspension was aspirated into 1 ml syringes (BD Plastipak<sup>™</sup> Becton Dickinson, Madrid, Spain) and applied by dripping or spraying the cells into individual 100 mm Ø Petri dishes from a distance of 10 cm (Table 1, Figure 2A-F).

##### *1 Drop (Figure 2A)*

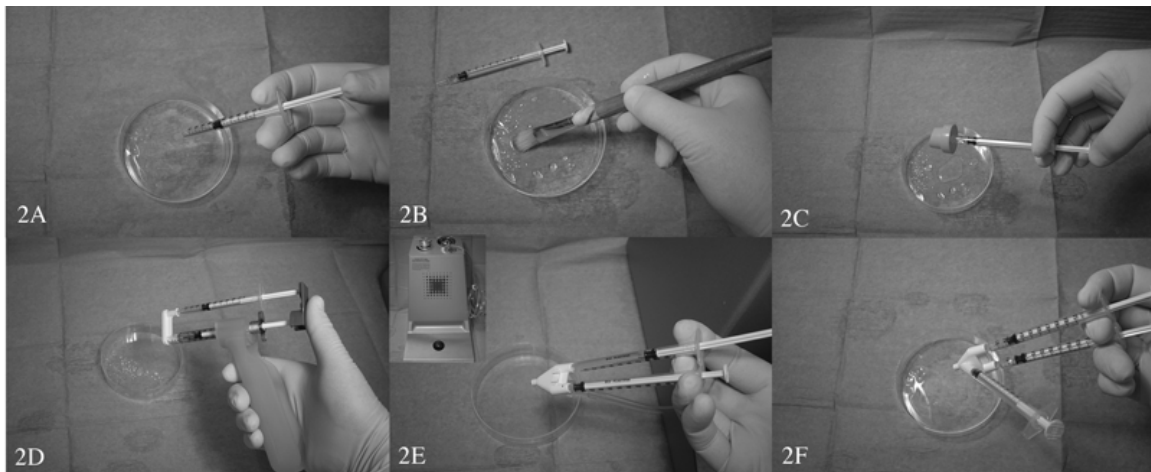
The cell suspension was dripped on to the surface while the syringe was moved over the area of the Petri dish, aiming for an even distribution of the cells and using as much pressure as needed to obtain a steady flow or drip of suspension.

##### *2 Paint brush (Figure 2B)*

An artist's paintbrush (Dekorima Symphony size 16, Dekorima AS Sandefjord, Norway) was used. The cell suspension was dripped on to the Petri dish (as for the first group). The cells were then smeared with the brush over the surface with 10 strokes in different directions to paint the surface with the cell suspension.

*Table 1: Application techniques used in this study to transplant cultured keratinocytes.*

1) Drop	Dripped with a standard 1 ml syringe.
2) Paint brush	Dripped with a standard 1 ml syringe and dispersed on the culture surface with a paintbrush.
3) Spray nozzle	Sprayed through a spray nozzle attached to a 1 ml syringe.
4) Harvest <sup>®</sup>	Sprayed with the Harvest SK/S Spray Applicator Kit <sup>®</sup> .
5) High pressure	Sprayed with the Tissomat and Duploject <sup>™</sup> spray kit with 200 kPa air pressure.
6) Low pressure	Sprayed with the Tissomat and Duploject <sup>™</sup> spray kit with 50 kPa air pressure.
7) Duploject <sup>™</sup> nozzle	Sprayed with the Duploject <sup>™</sup> spray nozzle attached to a 1 ml syringe.



*Figure 2A-F: The different transplantation techniques used in this study for transplanting cultured keratinocytes in suspension: (A) – Drop, (B) - Paint brush, (C) – Spray Nozzle attached to a syringe, (D) – Harvest SK/S Spray Applicator Kit<sup>®</sup>, (E) – High pressure and Low pressure spraying using the Duploject<sup>™</sup> spray kit. (Insert: Tissomat application device adjusted to deliver 200 kPa or 50 kPa respectively), (F) – Duploject<sup>™</sup> nozzle.*

### *3 Spray nozzle (Figure 2C)*

The spray nozzle (Male valve, ST300 T3K016K20, LINDAL Group, Bad Oldesloe, Hamburg), an ordinary spray nozzle for distributing substances such as hairspray, was attached to the syringe and the suspension sprayed over the surface for an even distribution of cells. The force applied was just enough to create a fine mist.

### *4 Harvest<sup>®</sup> (Figure 2D)*

The Harvest SK/S Spray Applicator Kit<sup>®</sup>, referred to as Harvest<sup>®</sup> (Harvest Technologies GmbH, München, Germany), is designed to deliver two liquid components simultaneously, such as cell suspension and tissue glue. The Harvest<sup>®</sup> nozzle was attached to the syringe and to prevent back-flush from the second canal this was blocked. The suspension was then sprayed evenly over the surface.

### *5 High pressure (Figure 2E)*

The Tissomat applicator is designed to deliver and regulate pressures up to 1000 kPa and is often used with a Duploject™ spray set to transplant cells together with Tisseel Duo Quick™ tissue glue. The Duploject™ spray set consists of a special Duploject™ spray nozzle with a two-channel spray system that allows two fluids to be delivered simultaneously under the same pressure and flow. It comes with a tube to fit onto the Tissomat applicator.

The syringe was attached to the Duploject™ spray set according to manufacturer's directions and subsequently connected to the Tissomat applicator, which was adjusted to

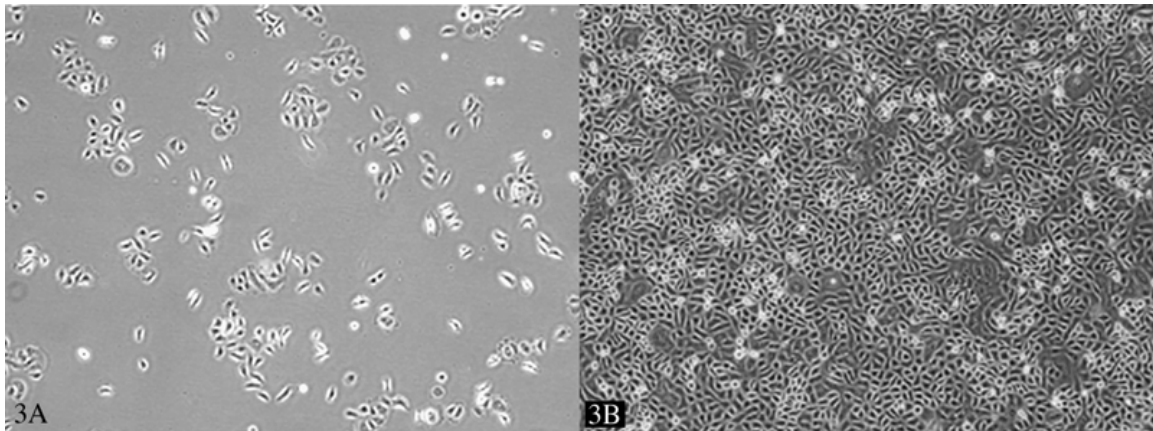
deliver a pressure of 200 kPa. To prevent back flush from the second canal, this was blocked and the suspension was then sprayed over the surface.

#### *6 Low pressure (Figure 2E)*

The Tissomat application device was adjusted to deliver a pressure of 50 kPa and the suspension was then sprayed over the surface as above.

#### *7 Duploject™ nozzle (Figure 2F)*

The syringe was connected to the Duploject™ spray nozzle. To prevent back flush from the second canal it was blocked, and the suspension was sprayed evenly over the surface without any additional air pressure from the Tissomat application device.



*Figure 3A-B: Phase contrast microphotographs of a non-confluent keratinocyte culture (A) and a confluent keratinocyte culture (B).*

#### *Assessment of keratinocyte viability*

After application of the cell suspension, culture medium 7 ml was added instantly to each individual Petri dish, giving a total of 8 ml in each dish. The number of living and dead

cells was counted in a haemocytometer, by triplicate samples of cell suspension 10  $\mu$ l, each mixed with trypan blue 10  $\mu$ l.

#### *Attachment of cells and proliferation assay*

Three samples of 2 ml from the 8 ml cell suspension from each event were seeded into 3 wells of a 6-well cell culture plate (Falcon, BD Plastipak™ Becton Dickinson, Stockholm, Sweden), and then incubated at +37°C, 95% humidity, and 5% carbon dioxide until it was time to sample them.

#### *Sampling*

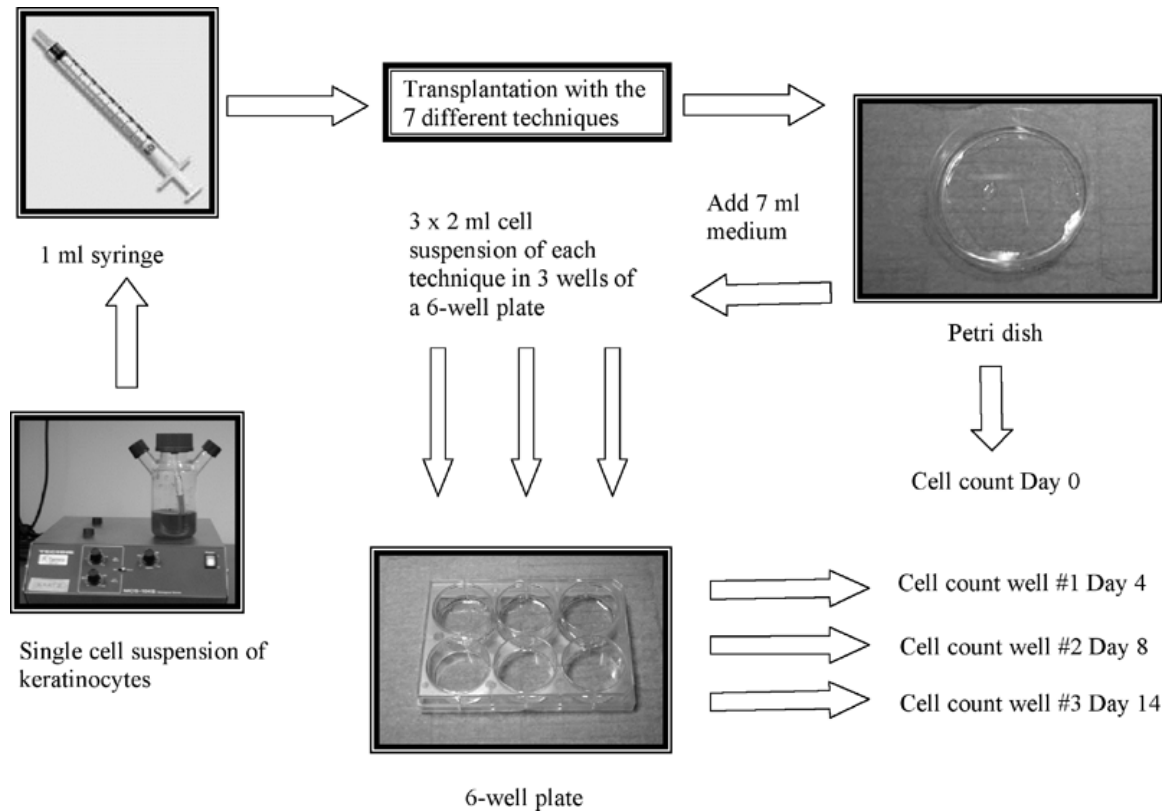
At 4, 8, and 14 days after transplantation, cells from each group were counted in triplicates by haemocytometer to assess cell proliferation and the living:dead ratio. The cells were detached from the wells with EDTA and Trypsin, and incubated with trypan blue before they were counted.

Figure 4 summarises the study set up.

#### *Statistics*

Each experiment was performed three times using cultures established from separate tissue donors on each occasion. We analysed the data from day 0, day 4, day 8, and day 14 by using two-way variance analysis with the cultures from the different tissue donors as explaining variables. We used a statistical model without interaction, since the data did not confirm significant interactions between the different methods. The triplicate samples from each donor-culture and time point were regarded as random repetitions without

mutual order within the combination tissue donor-method. The significance of difference was assessed using Tukey's simultaneous pair wise analysis of variance test, with a 95% confidence interval. Results are presented as mean if not otherwise stated.



*Figure 4: Figure illustrates the workflow of the transplantation process. Well-dispersed cell suspension was aspirated into 1 ml syringes and applied by dripping or spraying the cells into individual 100 mm Ø Petri dishes from a distance of 10 cm. After transplantation the cells from each group was counted, added 7 ml of culture media and subsequently seeded into 3 wells of a 6-well plate for further culturing. At day 4, 8 and 14 one well from each group was harvested and counted to determine proliferation.*

## **Results**

### **Viability and proliferative capacity of transplanted keratinocytes**

After harvest of the cultures and before transplantation, the trypan blue exclusion method was used to assess the viability of the cells in the stock suspension. We found that 94% of

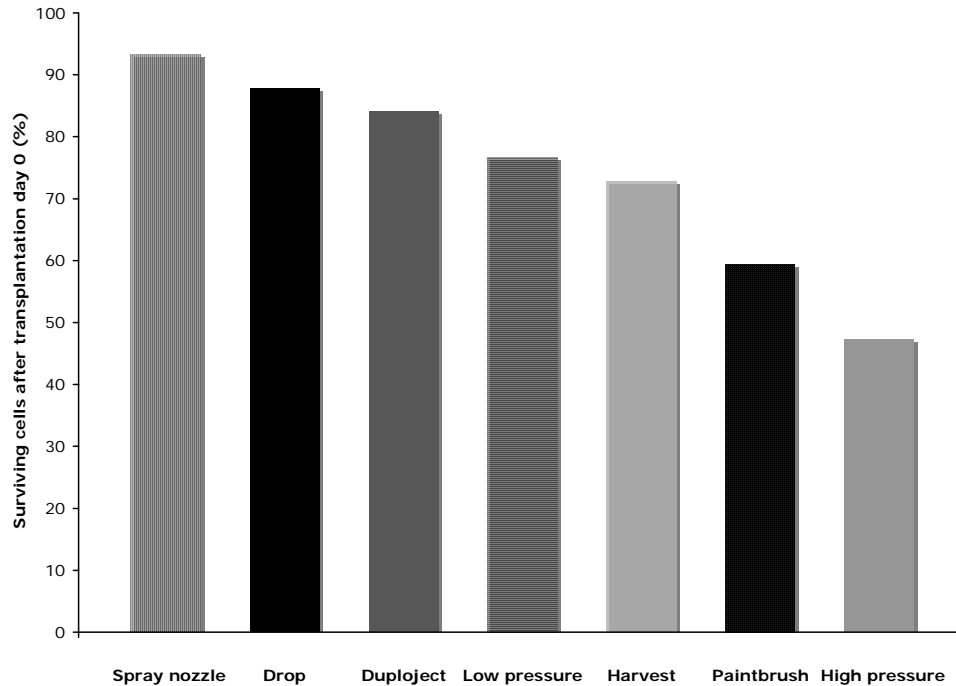
the cells remained viable after treatment with trypsin and EDTA and re-suspension in Ker-SFM. After the cells had been transplanted there was a variable cell death, see below. Apart from the High pressure group, all techniques showed significantly better survival at all time points, except for the Paintbrush group and the Harvest<sup>®</sup> group, which showed significantly fewer survivors at day 14 (Table 2).

*Table 2: Cell viability in percentage (% of original cell number) at the different time points after transplantation for each technique used.*

Technique	Day			
	0	4	8	14
Drop	87.8	44.0	46.3	55.4
Paint brush	59.4	34.1	35.4	39.7
Spray nozzle	93.2	45.7	44.8	48.4
Harvest <sup>®</sup>	72.8	35.6	40.2	46.0
High pressure (200 kPa)	47.3	22.5	26.1	39.8
Low pressure (50 kPa)	76.6	35.3	35.1	49.8
Duploject <sup>™</sup> nozzle	84.1	43.8	42.1	53.9

#### *Day 0*

The highest cell survival at day 0 was seen in the Spray nozzle group, which was significantly higher than the High pressure group ( $p < 0.001$ ), the Paintbrush group ( $p < 0.001$ ), the Harvest<sup>®</sup> group ( $p < 0.001$ ), and the Low pressure group ( $p < 0.001$ ). It also had larger numbers, but not significantly so, compared with the Duploject<sup>™</sup> and Drop groups (Figure 5).



*Figure 5: Percentage (%) viable cells after transplantation with seven different techniques. The highest cellular survival rate after transplantation was found after using technique Spray nozzle. This technique showed significantly more viable cells than the High pressure, Paintbrush, Harvest<sup>®</sup>, and Low pressure groups and showed tendencies towards higher numbers than Duploject<sup>™</sup> and Drop, but not significantly so. (\*\*\*,  $p < 0.001$  compared with Spray nozzle).*

#### *Day 4*

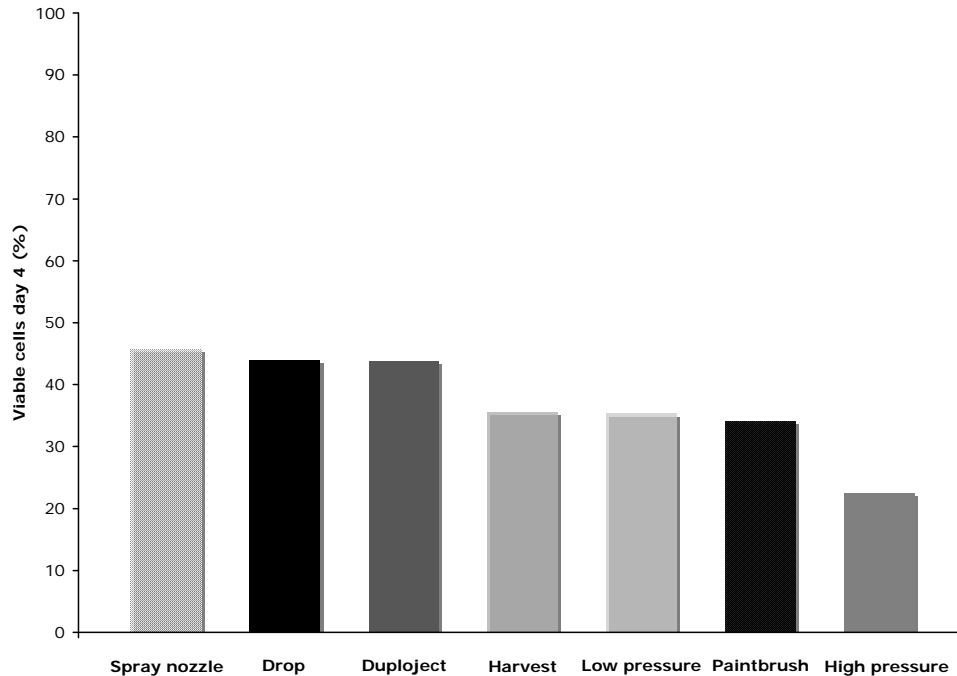
On day 4 the proliferation assays showed the best viability in the group Spray nozzle, which had significantly higher survival than the High pressure ( $p < 0.001$ ), Paintbrush ( $p < 0.01$ ), Low pressure ( $p < 0.01$ ), and Harvest<sup>®</sup> ( $p < 0.01$ ) groups and a slightly higher number (but not significantly so) than the Duploject<sup>™</sup> and Drop groups (Figure 6).

#### *Day 8*

The cell count for proliferation assays on day 8 showed a shift in the highest number of viable cells from the Spray nozzle group to the Drop group, which showed significantly higher surviving numbers than the High pressure ( $p < 0.001$ ), Low pressure ( $p < 0.001$ ), or



Paintbrush ( $p < 0.001$ ) groups, and higher survival numbers, but not significantly so, than the Harvest<sup>®</sup>, Duploject<sup>™</sup> and Spray nozzle groups.



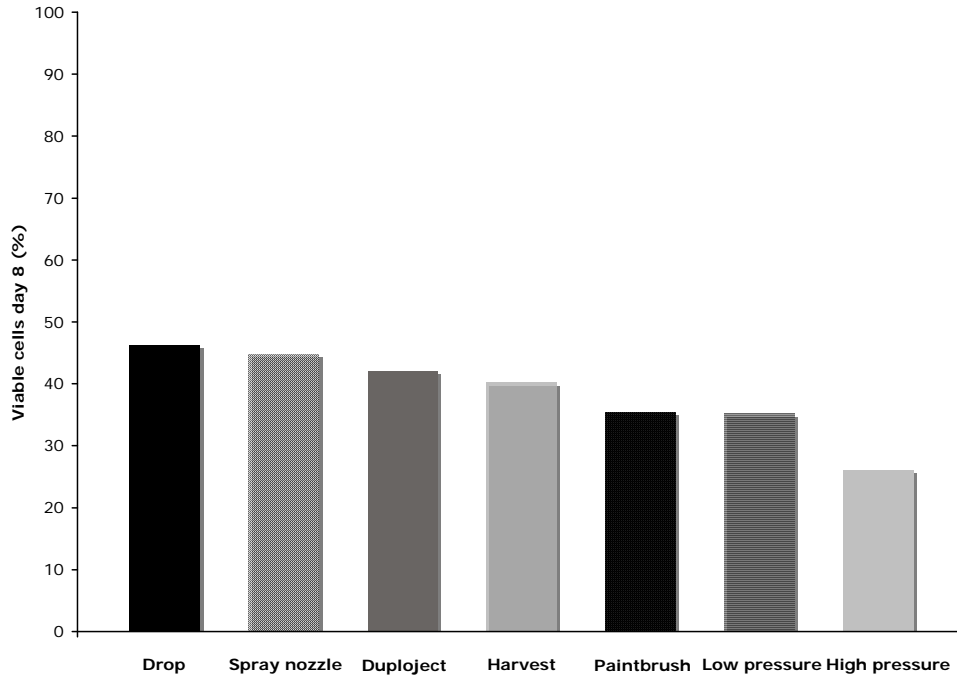
*Figure 6: Percentage (% of initial cell number) viable cells after 4 days of culturing. The proliferation assays showed highest viability with the Spray nozzle group, showing significantly higher numbers compared with the High pressure, Paint brush, Low pressure, and Harvest<sup>®</sup> groups, and a tendency towards higher numbers compared with the Duploject<sup>™</sup> and Drop groups, but not significantly so. (\*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  compared with Spray nozzle)*

The groups Drop, Spray nozzle, and Duploject<sup>™</sup> have similar number of viable cells with p values close to 1, so not significantly different from each other (Figure 7).

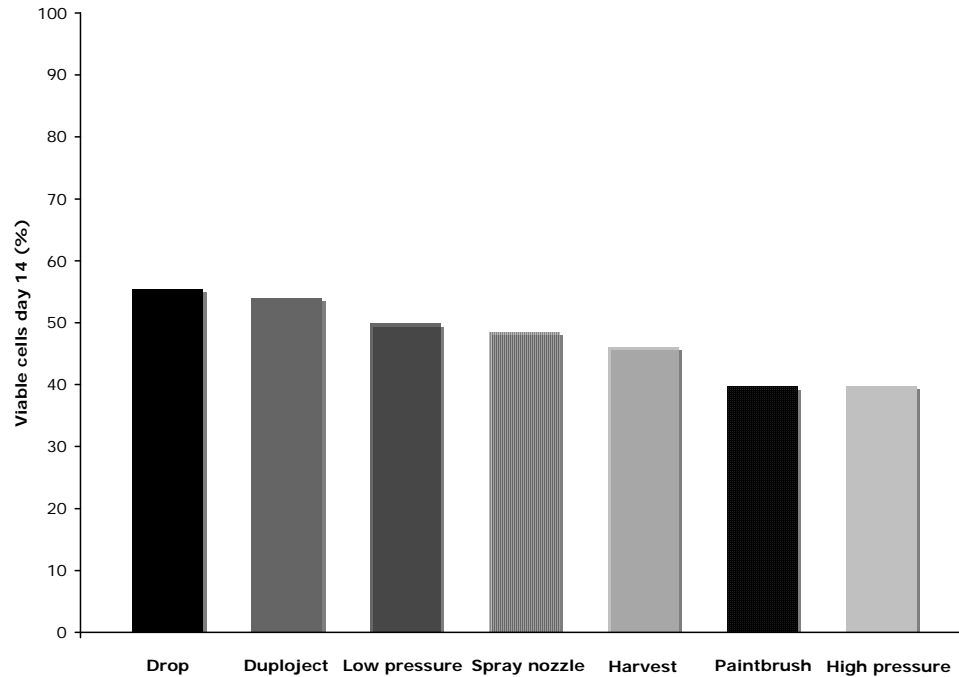
#### *Day 14*

By day 14 the numbers had levelled out to some extent, with the Drop group having the most viable cells, slightly more than the Duploject<sup>™</sup>, Low pressure, Spray nozzle, and Harvest<sup>®</sup> groups. All had significantly more surviving cells compared with the High

pressure and Paintbrush groups ( $p < 0.001$  in each case) but they did not differ significantly from them (Figure 8).



*Figure 7: Percentage (% of initial cell number) viable cells after 8 days of culturing. The cell count for proliferation assays on day 8 showed a shift in highest number of viable cells from Spray nozzle to Drop. If looking at clinically interesting techniques, the Drop, Spray nozzle, and Duploject<sup>TM</sup> groups show equal numbers of cells with a p-value close to 1.0, compared with High pressure, Low pressure, and Harvest<sup>®</sup> groups, which show significantly lower numbers than the Drop group. (\*\*\*,  $p < 0.001$  compared with Drop)*



*Figure 8: Percentage (% of initial cell number) viable cells after 14 days of culturing. At day 14 the numbers had levelled out to some extent, the Drop group showing the highest numbers of viable cells, close to the Duploject<sup>TM</sup>, Low pressure, Spray nozzle, and Harvest<sup>®</sup> groups. All of them are significantly higher in numbers compared with the High pressure and Paintbrush groups, but have no significant difference in relation to each other. (\*\*\*,  $p < 0.001$  compared with the Drop group).*

## ***Discussion***

Wound closure using autologous cultured keratinocytes in suspension will certainly continue to be a valuable tool in the future treatment of burns. We have shown that normal human primary keratinocytes, transplanted by different application techniques have a great variability in the viability and proliferative capacity of the cells, ranging from 47% (High pressure technique) to 93% (Spray nozzle technique), depending on the method of transplantation. One of the hypotheses of this study was that the use of a pressure device, such as the Tissomat applicator, damages the cells when delivering keratinocytes to a wound bed, and so less viable cells reach the wound to act in the

wound closure. Duncan et al [12] studied the effect of aerosol transfer of keratinocytes compared with delivery by keratinocytes on a fibrin membrane to a de-epidermalised dermis. They found that even though cells in suspension experience different types of stresses (hydrostatic, shear, and elongation) when being sprayed, a total of 20% more cells were present on the de-epidermalised dermis than in the fibrin membrane. Harkin et al [11] used the Tissomat applicator and found that the viability after transplantation, using pressures ranging from 70-138 kPa, was similar to that of cells just recently detached from the culture dish. When they adjusted the Tissomat applicator to deliver 207 kPa however, the percentage (about 70%) of viable cells was lower. This is closer to, but still much higher than what we found in this study. The contradictory results we present are in the region of 50% viable cells after transplantation delivered at the pressure of 200 kPa, as opposed to around 70% found by Harkin et al, who also presented a considerably higher overall survival of cells. The reasons for this are not obvious, but the fact that they used a sealant for co-transplantation might have increased their yield of viable cells. We think that a combination of cells-carrier, -vehicle or, -soluble matrix used when transplanting cells in suspension both lessens the physical impact and damage to the cells and provides better starting conditions for the cells by providing a three dimensional scaffold, which leads to a quicker and better proliferation of cells or regeneration of tissue at hand. This theory is supported by a recent study [13] that was conducted in our laboratory. In this present study however, so that we could evaluate the actual survival of the cells using different techniques, we excluded sealants and carriers. In continuum, even though Harkin et al showed unaffected cell viability after transplantation with low pressures, they noted a significant reduction in the metabolic

activity of the cells. This corresponds quite well to our results, and might be the answer as to why the keratinocytes, after transplantation, had a diminished capacity to proliferate compared with what is usually seen when passing keratinocytes in culture. The results of the Drop, Duploject™ nozzle, and the Spray nozzle techniques, showed that at least half of the cells adhered and started to proliferate. However, when we studied the surface of the culture dishes after transplantation with the Drop, and Duploject™ nozzle we saw large areas in complete loss of cells and suspension (Figure 2B and 2C). In consequence, the question arises: can these methods fully cover a large sized wound without leaving major areas uncovered? As burns or other large wounds often are substantially irregular, and far from smooth, one can imagine that cells pool in cavities or are washed off the wound and end up in the bed, instead of covering the wound area. We find these methods more suitable for smaller burns and ulcers. The Spray nozzle technique, in comparison, covered the whole surface of the culture dish, using the same volume of suspension. The Paintbrush technique showed surprisingly high cell survival of around 60% after transplantation. However, as predicted, a number of brush fibres contaminated the surface (data not shown). The plating efficacy was good, only roughly 40% of the cells failed to adhere, but the proliferation capacity seemed poor and the cultures never recovered to reach the cell yield of the other methods even after 14 days of culture. This could be the result of different stresses, presented by Duncan et al [12], as it is likely that the cells sustained at least elongation stress by being smeared across the surface and that this in turn would lead to reduced cellular metabolism and proliferative capacity, which was also described by Harkin et al [11]. The Harvest® technique gave a cell survival of about 70% after transplantation, and a proliferation profile that matched the other low pressure

techniques. The system is small and easy to operate, compared with the Tissomat applicator that requires additional tubes, medical air and a good hand-foot co-ordination. However, a new and improved pressure regulator (EasySpray<sup>®</sup>, Baxter Medical AB, Kista, Sweden) is about to replace the older Tissomat pressure device. The High pressure technique, frequently used in various clinics, showed only 25% cell adherence and the cells did not seem to fully recover in proliferative capacity, compared with other regimes. When we counted the cells sprayed with the High pressure technique in a haemocytometer, we also noted subjectively a higher number of fragmented cells and a number of the cells, even though they had not turned blue from trypan blue, looked morphologically disturbed and gave the impression of unhealthy cells (data not shown). The fact that the Duploject<sup>™</sup> nozzle resulted in almost the same cell survival as the Drop technique made us think, even more, that pressure alone can cause substantial cell damage. When evaluating the Low pressure Tissomat method, we noticed that the pressure, 50 kPa was just enough to cause the suspension to drip on to the culture dish, making it comparable to the Drop technique. Wood et al [14] presented in 2003 a study in which they evaluated the clinical potential of keratinocytes in suspension. The device used to transplant the keratinocytes in that study was an ordinary spray nozzle attached to a standard syringe, similar to the one we used in this study. The spray nozzle used in our study had in a previous pilot study (data not shown), in which we compared several nozzles on the market, proved suitable for this application. We wanted the channel in the spray cap to be wide enough to let the cell suspension pass through the nozzle but narrow enough to create a fine mist when distributing the suspension with only minor pressure being applied. It also had to fit a standard syringe and permit being sterilised through an

autoclave without losing its properties. The spray nozzle chosen provided a good distribution of suspension over the surface, and showed similar cell viability to the Drop technique when compared.

The fact that single cell suspensions can be transported to the clinic frozen in small vials, stored and then thawed in amounts suitable for the occasion just before transplantation makes it an appealing option to cultured epithelial sheets and other measures. Together with straightforward equipments like a spray nozzle attached to a syringe, these features would undoubtedly facilitate the future use of single cell suspension in transplantations of burns and other cutaneous wounds.

This in vitro study is not to be compared to in vivo or situ conditions, where the wound bed is a hostile environment with different factors that affect survival and adherence of cells.

However, it does provide valuable information about different measures to be used when transplanting autologous keratinocytes in a single cell suspension, and we hope that, with further studies, advances in this field will lead to the development of an equipment that is fairly cheap and easy to operate and allow cells to be sent to the clinic, loaded in a spray device and ready to use. We think that investing time and effort in developing the methods further will decrease both time and costs of treatment, and provide better healing and less pain and discomfort for patients.

### ***Conflict of interest statement***

There are no conflicts of interest concerning this study.

### ***Acknowledgements***

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