Dynamic Biochemistry, Process Biotechnology and Molecular Biology ©2010 Global Science Books

Multifunctional Implantable Chitosan for Skin Tissue Engineering and Wound Healing

Waleed Nazmy El Mazney[1] • Mohamed Reda Mohamed Diab[1] • Yasmine Mamdouh William[2*]

[1] Research and Development Sector, Vacsera, El-Agouza, Giza, Egypt
[2] Faculty of Biotechnology MSA and Greenwich Universities.6 October City, Egypt. Old Royal Naval College, Park Row, Greenwich, London SE10 9LS, UK

Corresponding author: * **yasmine-mamdouh@hotmail.com**

ABSTRACT

Chitosan, the deacetylated derivative of chitin, is considered as a new technology for treatment of skin wounds. In this study, primary cell cultures of baby mice (1-2 days) skin tissue, supplemented with 10% fetal calf serum were seeded onto air-dried chitosan. In a mice model, the cells developed to epithelial tissues can be used for implantation for skin regeneration and wound healing. Under anesthesia, wounds of the same size were induced in each tested mouse and their chitosan-free control counterpart. Tested mice included two chitosan-treated groups, one with a suture wound while the other was left with an open wound. Assessment of the treatment efficacy revealed the following. On day 9 the wound closure of the suture chitosan group was better than the suture control group. Despite this, the closure rate of the suture control group continued to increase and became nearly equal at day 11 to that of the suture chitosan group, showing a significant increase on days 13 and 16 compared to the test group. For the open wound groups, on days 3 and 9 the wound closure of both the chitosan and control groups were similar, but on days 11 and 13 the chitosan group significantly accelerated wound closure and completely healed on day 16 compared to the control and sewn groups. Results clarify the ability of using a biodegradable implement in transplanting culture substrates for skin regeneration in an open wound.

Keywords: anesthesia, biodegradable implements, cell culture, skin regeneration, skin substitutes

INTRODUCTION

Skin injury is one of most common problems in the surgical field and still not totally resolved (Ma *et al*. 2003). There are different types of treatments for wounds, which mainly depend on the wound location, injury depth, tissue type, and degree of infection.

Treatments are categorized into local care and different dressing. Wound dressing are generally classified as passive products, interactive products and bioactive products. Traditional dressings like gauze and tulle dressing that account for the largest market are passive products. Interactive products, comprised of polymeric films and forms, are mostly transparent, permeable to water vapor and oxygen but impermeable to bacteria. Bioactive dressing delivers active substances in a wound for its healing: either by delivery of bioactive compounds or dressings is constructed from material having endogenous activity (Paul and Sharma 2004).

In the past decades, many biological skin substitutes such as auto-, allo- and xenograft have been applied for wound healing. Autograft cells are obtained from the same individual's body, allograft cells are taken from the body of a different organism of the same species, while xenograft cells are isolated from individuals of different species (Atiyeh *et al*. 2005a). Autograft is considered to be the best grafting method, but may not be enough to repair massive burns and full thickness wounds in which the epidermis and dermis have been lost (Huang *et al*. 2008).

For these reasons, many studies turned toward tissue engineering. During the last few years, chitins and chitosans and their derivatives (bioactive products) extracted from crustacean exoskeletons (Atiyeh *et al*. 2005b) have been used for tissue and organ regeneration. This is primarily due to the many advantages they inherently offer such as increased healing stimulation and accelerated hemostasis. Their inhibition effects on matrix metallproteinases enhances the functions of polymorphonuclear leukocytes (PMN) promoting granulation and organization as well as impacting growth factors (Ueno *et al*. 2001b; Atiyeh *et al*. 2005a; Muzzarilli 2009). Therefore this work will involve *in vitro* seeding cells obtained from skin tissues of neonatal mice of the same species (allograft) onto air-dried chitosan as a simple, cheap and available multifunctional material to track their effect on skin regeneration when implanted into mature mice.

MATERIALS AND METHODS

Chitosan scaffold preparation

Chitosan was extracted from exoskeletons of giant tiger prawn shrimps, *Penaeus monodon*, present in the sea of Suez Gulf, Egypt. The production of chitosan from crustacean shells obtained as a food waste is cheap, readily available and economically feasible.

Chitosan was prepared according to Entesar *et al*. (2008) with some modification to obtain more pure chitosan: small shrimp peels collected, washed, cut into small pieces and boiled in a solution of 1% sodium bicarbonate, (Sigma-Aldrich, US). 5% hydrochloric acid (HCL), (Surchem Products Ltd., England), was added to the mixture then filtered after the formation of foam. The resulting solid was boiled for a second time in a 1% sodium bicarbonate solution (Sigma-Aldrich), for 4 hrs before being treated with 50% sodium hydroxide (NaOH) (Sigma-Aldrich), to transform the chitin to chitosan. Finally the mixture was poured in ethanol for 6 hrs to eliminate any proteins and coloring traces.

1 g of chitosan was washed with 100 ml of distilled water, stirred, heated and filtered. The resulting mixture was washed again several times to bring it up to neutrality before being treated with a solution of 1% acetic acid. The treated mixture was then heated, stirred and filtered 5 times for more purities. After cooling, it was placed in an incubator to dry for 2 days. The resulting dried chitosan scaffold was cut into circles 0.7 cm in diameter.

Received: 30 December, 2009. Accepted: 21 June, 2010.

Original Research Paper

Primary cell culture of mice skin tissues

Before being inoculated onto cells, chitosan scaffold circles were immersed into three different ethanol concentrations (20 min each): 95, 85 and 75%. To confirm sterilization, each scaffold was transferred into tissue culture flasks dispersed with Hank's medium (Eagle 1955) and 10% fetal calf serum (FCS; GibcoBrL, Germany), before being incubated at 37°C for 48 hrs.

Three neonatal mice were asphyxiated then sterilized with 70% ethanol (Sigma-Aldrich) both limbs and tail was removed from each mouse. The mice skin was isolated, and cut into small pieces 3 mm long in 100 ml warm 0.25% Trypsin-EDTA solution (Sigma-Aldrich) then stirred for 45 min. After complete turbidity, the trypsin was cooled, filtered and centrifuged after adding equal volume of Hank's medium supplemented with 10% FCS. The supernatant was discarded and the pellet was left at 4°C then washed twice with the same supplemented media. The pellet was re-suspended in a small volume (counted using improved Neubauer haemocytometer, Boeco, Germany) before being seeded into the chitosan scaffold, which was present in sterile plates of 24 wells and incubated at 37°C. Following the formation of a complete sheet, chitosan films were implanted onto the mice for skin regeneration.

Mice anesthesia

To determine the optimum dose of anesthesia for best duration and recovery, four different doses (40, 45, 50 and 55 mg/kg) were injected intraperitoneally (IP) in all mice. 10 ml of saline was added to 100 mg thiopental to get a concentration of 0.1 ml/mg thiopental (Sandoz, Egypt). The accurate thiopental concentration used for anesthesia was calculated according to Clifford's (1984) equation:

The thiopental concentration (mg) = (average mice weight (g) × 1.25 mg of thiopental) / Maximum mice weight (g).

Chitosan implantation to mice wounds for skin regeneration

20 mice ranging from 21-25 g were divided into 4 groups of 5 mice each. Mice were anesthetized (IP).With optimal thiopental concentrations of 50 mg/kg.

In all groups, a circle of full skin (thickness = ~ 0.8 cm diameter) was completely removed from each mouse by using sterile scissors and forceps after disinfecting the mices' dorsal skin with 70% ethanol.

For the first group, the pieces of chitosan 0.7 cm in diameter containing a complete sheet of neonatal mice skin cells were implanted on the wound area. During implantation, the chitosan scaffolds were inverted so cells faced down while the free surface faced up. Implants were fixed to the edges of the wound without using silk sutures, and the wound was covered with gauze for 30 min.

For the second group, nothing was applied in order, i.e. the control group. No wounds were left open in both first and second groups.

For the third and fourth groups, a tie was applied to the wound area with a 6-0 silk suture. The third group received the implant while the fourth one did not and was used as the control group. The average wound size was measured with a micro ruler 4 days after transplantation and extended over a period of 16 days.

The animal experiment was conducted according to the committee guidelines of Vacsera, which met the NIH guidelines. The animals were fed with a standard diet and housed in the animal care facilities of Vacsera.

Statistics

Data points are given as mean and ±standard. Differences between means were analyzed for statistical significance assuming equal or unequal differences in the standard deviation as appropriate. The closure percentage was calculated according to the formula:

Wound closure percentage = (healed area/total wound area) × 100.

RESULTS AND DISCUSSION

General characteristics of air-dried chitosan

The chitosan prepared with evaporation at 37°C showed a rigid transparent corrugated surface (**Fig. 1**, center). With phosphate buffer saline or media, it became jelly-like and stable. The transparency facilitated the film detection of the cell morphology seeded on the chitosan during inoculation, therefore identifying the best time and cell state for implantation into the mouse. This is supported by Khan and Peh (2003) who reported that the film transparency is crucial for treatment with chitosan films as the film clarity in particular makes it possible to visualize the wound.

In vitro culture

Five days after inoculating the cells onto the scaffolds, fibroblast was homogenously dispersed into the chitosan to develop a complete sheet across the plastic surface (**Fig. 1**, center).

Cells are either anchorage-dependent or need to be formed in a suspension culture. Anchorage-dependent cells need to attach themselves to an extracellular matrix (ECM) in order to grow and proliferate (Dai *et al*. 2004). This ECM can be produced via the cells themselves or, alternatively, with a suitable biomatrix to which the cell binds (Maurer 1992) the effects of surface features such as pores, pits and random surface roughness on cell behavior are important for the adherence of anchorage-dependent cells on the matrix and their consequent spread on it (Brunette 1988; Curtis and Wilkinson 1997).

Air-dried chitosan showed that a corrugated surface has the potential of increasing the particle surface area and facilitate cell adhesion. The increasing number of adhering cells to air-dried chitosan compared to those adhering to the dish surface confirmed previous studies (Himes and Hu 1987; Ng *et al*. 2004) showing the higher efficiency of particle cultivation technique compared to cell culture on a flat substrate, such as culture dishes. Ranucci *et al*. (2001) and Posonnet *et al*. (2002) showed that when the surface is randomly modified, submicron-scale roughness significantly affects cells adhesion. It also agrees with the results of Deutch *et al*. (2000), who revealed that the number of neonatal rat primary cardiac myocytes adhered to micro-pegged membranes increased 4-fold in comparison with untextured membranes. On the other hand, such findings differed from those reported by Wang *et al.* (2003) who mentioned that the percentage of living fibroblast cells on a polystyrene surface and plain chitosan films after 48 hr seeding were 99 and 85%, respectively. The same difference was obtained by Ng *et al*. (2004) in which 3D chitosan scaffolds did not support cell attachment and proliferation. The difference in cell adhesion to chitosan can be affected *in vitro* by the degree of deacetylation (DD). The higher the % DD of chitosan, the higher amount of free amino group on the surface of chitosan producing a positively charged surface which allows the interaction points between the negatively charged cells and the culture surface (Prasitsilp *et al*. 2000). Thus, chitosan shows very good cytocompatibility *in vitro*, but this property not only depends on the DD but also on the characteristics of sample-like natural sources, the method of preparation, and molecular weigh (MW) (Arana *et al*. 2009). Chatelet *et al*. (2001) proved that a variety of cell line-like myocardial, endothelial and epithelial cells, fibroblasts, hepatocytes, condrocytes and keratinocytes were cytocompatible with chitosan *in vitro*. However, the type of cell may be a factor that also affects adhesion as fibroblasts exhibit a more negative charge surface than keratinocytes (Aranaz *et al*. 2009).

Mice anesthesia

There was a direct and proportional relationship between thiopental concentrations and mice weight (**Fig 2**, center).

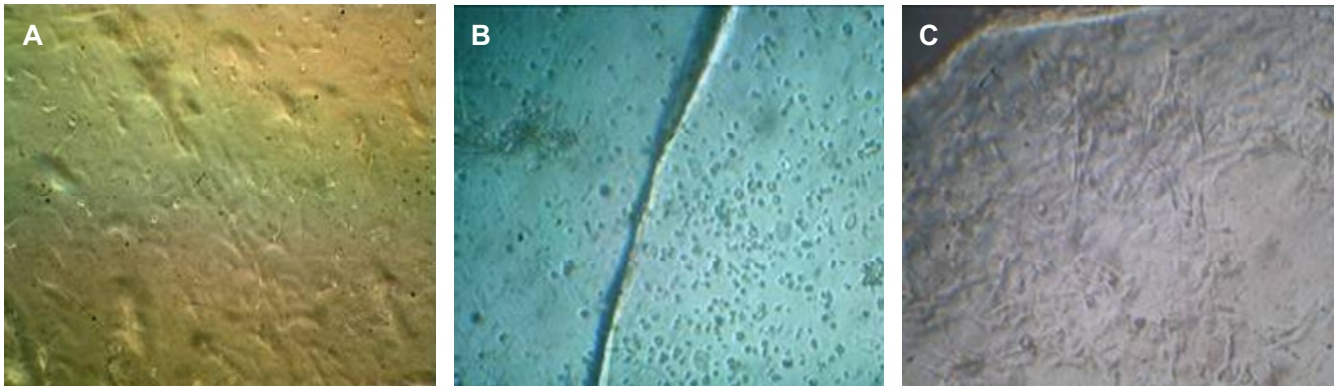

Fig. 1 Cell multiplication and proliferation on chitosan scaffold. (**A**) Air-dried chitosan corrugated surface in Hank's medium (X 400). (**B**) Cell adhesion to chitosan is greater than to the flat surface of dish after 8 hrs (X 100). (**C**) Cells adhere uniformly to the chitosan after 3 days (X 100).

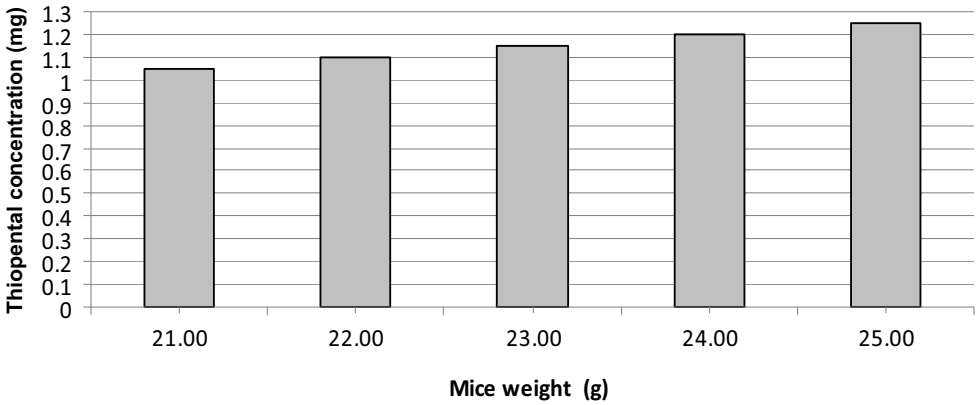

Fig. 2 Direct proportional relationship between thiopental concentration (mg) and mice weight.

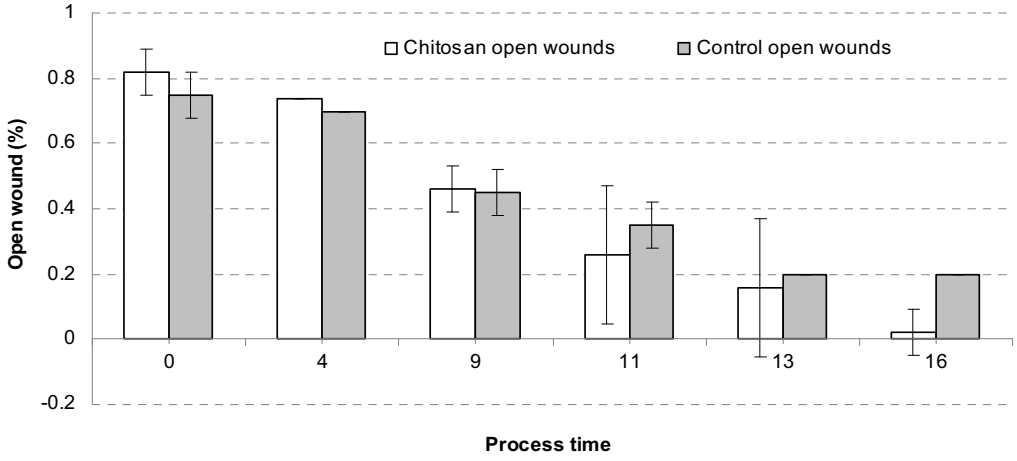

Fig. 3 Open wound percentage in both chitosan and control groups.

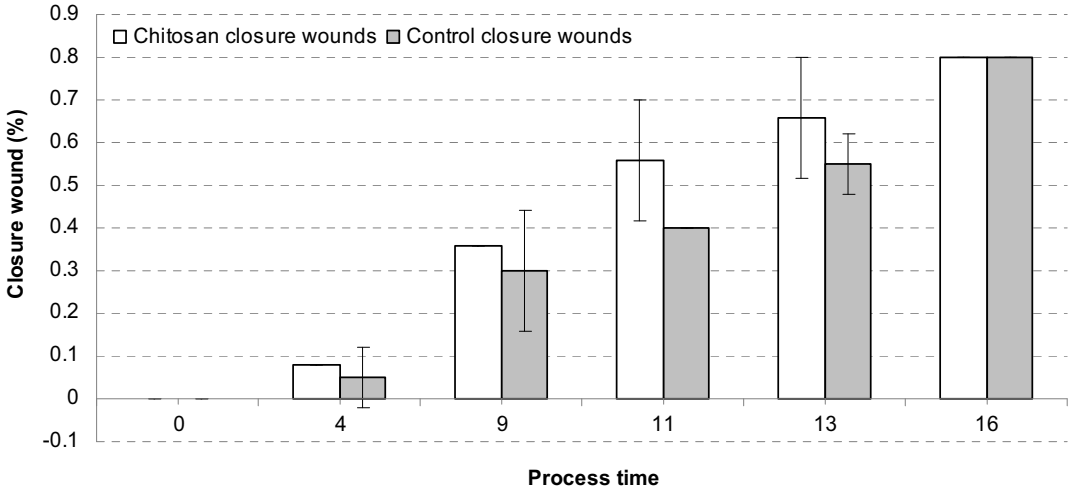

Fig. 4 Closure wound percentage in both chitosan and control groups.

The shortest recovery duration after biomaterial implantation was when the optimum concentration dose of 50 mg/kg of thiopental was used.

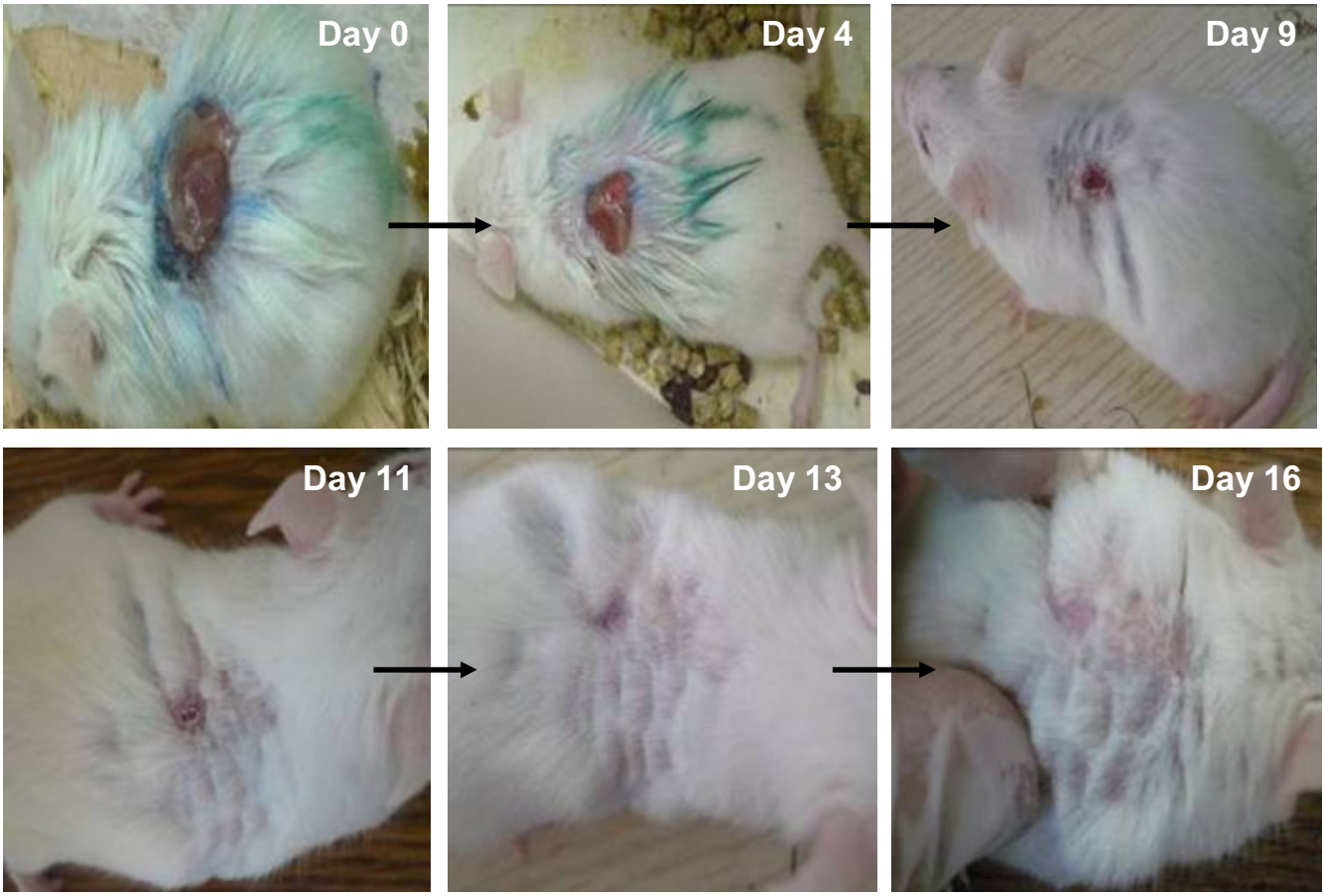

Fig. 5 Steps of skin regeneration of mice group treated with chitosan scaffold only (open wounds).

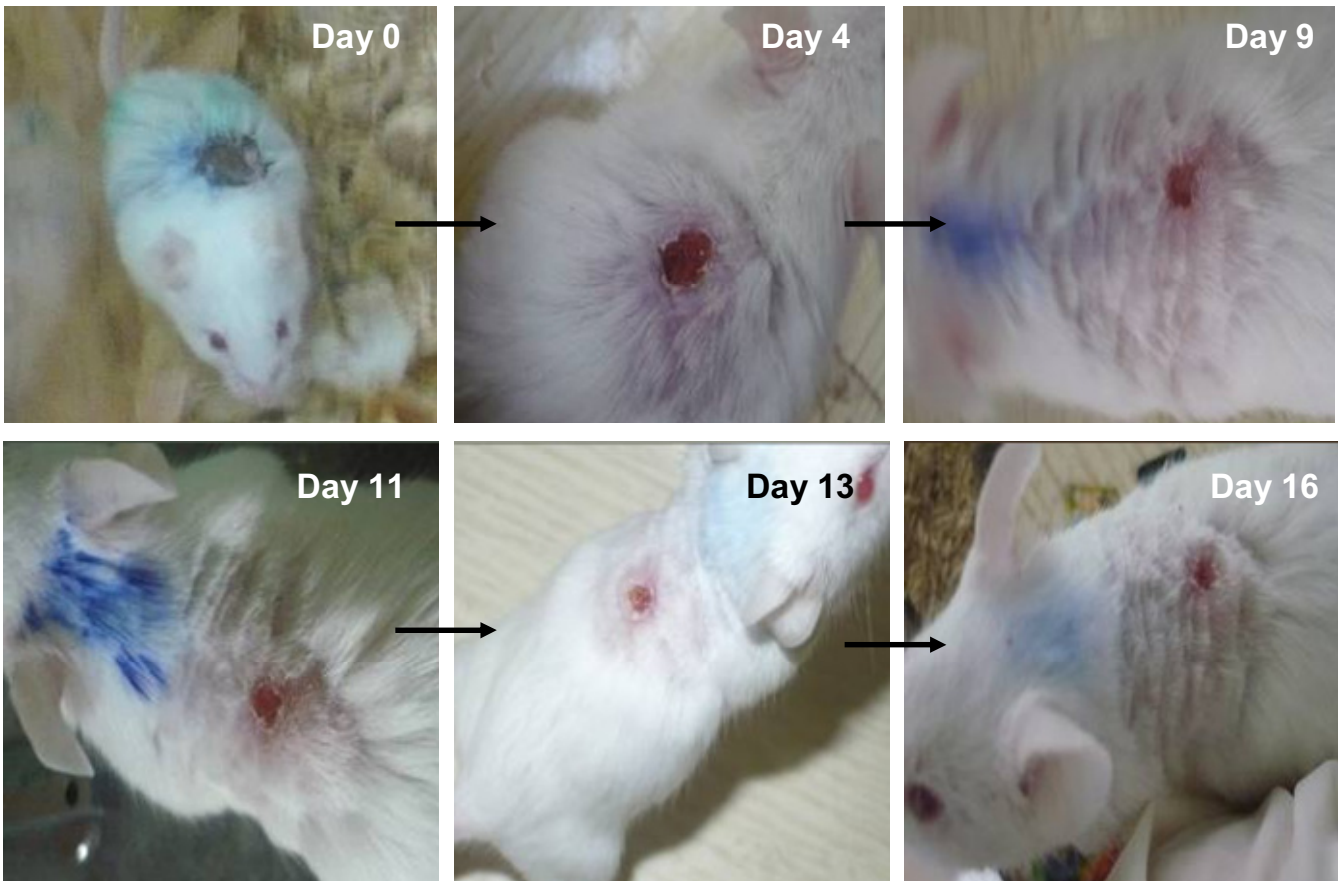

Fig. 6 Steps of skin regeneration of the control group (open wounds).

Skin regeneration

1. The chitosan group

On day 4, the percentage closure of this group improved slightly more than the control group, but on day 9, the percentage decreased compared to the control group. Improvement of both groups was in general insignificant. On day 11, there was a significant increase of closure reaching 68%, a 24% improvement. On the contrary, the control group in which the closure percentage increased to 52% only improved by 7.5%. On day 13, wound closure of the chitosan group reached 81% compared to the control group, which only reached 70%. On day 16, there was complete healing for the chitosan group while the control group only healed 75% (**Fig. 4**, center).

The average healing time was 14.4 days and 18.6 days for the chitosan open wound and control groups, respectively. The chitosan group consequently showed an average healing time 4 days shorter than the control group (**Fig. 3**, center; **Figs. 5, 6**, left).

2. The suture group

On day 9, the percentage closure in suture chitosan group exceeded four times that of the control group. On day 11, the percentage healing of the suture chitosan group and the control group were similar, But on day 13, the closure percentage of the control group significantly increase more than the suture chitosan group. On day 16, the closure area of both groups increased but wounds were not completely healed.

It was found that the healing time for the suture chitosan group and the suture control groups were respectively of 20 days and 18.2 days. Both groups results were non significant in comparison to the group of chitosan open wound (**Figs.7, 8**, left). Healing time depends on a variety of factors, such as wound size and location, pressure on the wound, swelling, circulation, blood glucose levels, wound care, and what is being applied to the wound. Healing may occur within days, weeks or require several months.

Chitosan acetate bandage is strongly bactericidal: it reduced the inflammatory cells in the wound area and had beneficial effect on wound healing especially during the early period (Burkatovskaya *et al.* 2008); this explains the increasing in closure area at early days.

Chitosan promotes granulation and organization and therefore it is beneficial for open wounds where certain polymorphonuclear leukocytes (PMN) function are enhanced, such as phagocytosis and the production of chemical mediators (Ueno *et al.* 2001a). This agrees with our experiment as it was found preferable to apply the chitosan to the open wound and not the suture one. The suture used on mice increased the healing time for both open wound and control groups, which may be due to the stretching of the two sides of skin that may lead to the bending of the chitosan and the loss of its function. This somewhat loss of pressure over the wound agrees with Gustafson *et al.* (2007) who reported that the dressing application included applying pressure over the wound by the use of fingers (as recommended by manufacturers). This form of pressure was observed to be uneven and associated with inadequate adhesion to the wound surface depending on the application techniques and models used; such loss of pressure may partially explain as well the results variation within the suture wound. A study by Lee *et al*. (2003) explained that gelatin-based scaffolds incorporated with fibroblasts isolated from a child's foreskin enhanced the wound-healing rate and re-epithelialization of a full-thickness skin defect rather than the acellular scaffold after one week. Hima Bindu (2010) indicated that chitosan and gelatin have proven wound healing properties individually, and the combination of these two polymers and incorporation of ciprofloxacin, a drug, into the composite films may show improvement in wound-healing activity. He found that drug-loaded chitosan-gelatin composite films showed more wound-healing properties than chitosan-gelatin blank composite film and blank chitosan film without interfering with the strength of the film. In acute burn wounds and in chronic wounds the clinical application of cultured human autologous keratinocytes in a fibrin sealant matrix as tissue substitute resulted in adherence to wound beds, attachment and spreading over the wound (Kopp *et al*. 2004).

Natural biopolymers such as collagen and fibronectin have been investigated as potential sources of material to which cells can attach (Metcalfe and Ferguson 2007). On studying allograft effect of rat oral mucosa fibroblasts as new seed cells incorporated into collagen and chitosan-collagen to construct the tissue engineered oral mucosa lamina. It was found that both the fibroblast-populated collagen lattices and the fibroblast-populated chitosan collagen lattices can repair skin defects on Wistar rats effectively and feasible (Wu *et al*. 2006). Cultured epidermal allografts (CEAl) in the treatment of deep dermal burns obtained from young healthy donors and fixed on tulle grass carrier (Grasolind) were compared with empty Grasolind. The reduction of the non-epithelialized wound area was 86.5% when covered through CEAl and only 71.2% when covered with tulle grass (Grasolind) only. This difference was statistically significant (Brychta *et al*. 2002). Delivering cultured autologous keratinocytes to human burns and non-healing wounds on a medical grade polymer coated with a chemically defined plasma polymerised functional surface containing 20% carboxylic acid (referred to as PPS), revealed that this type of tissue engineered skin substitute facilitated the healing of grafted burns wounds and for patients with chronic wounds resulted in a complete healing for approximately half the number of ulcers and a major reduction in ulcer size for all other cases, but these results required repeated applications of cells (Zhu *et al*. 2005). Dressing changes should be minimized and the ulcer should be kept moist and the surrounding skin dry (Hansson 1997). Wood *et al*. (2007) tried to improve the application of silicone pseudo-epidermis, Integra®, a skin substitute, of clinical practice consists of two procedures by the combination of Integra® with a non-cultured autologous suspension of cells to achieve a one-step skin repair of full-thickness skin wound on porcine, he found that the cells remain viable, migrate through the Integra® template and self-organize into differentiated epidermis. This supports our work that the high cost of dressings is a potential disadvantage of their use and chosen of dressing should be on the basis of cost effective and associated with less pain.

CONCLUSION

From the results obtained in our work, we concluded that skin grafting is crucial for wound healing and tissue regeneration and that chitosan is considered as one of the most promising biopolymers for tissue engineering and wound healing. This said, it remains that, improving the mechanical properties of chitosan as biomaterials (such as the construction of complex tissues and organs) is essential for this application.

This new technology attracted lately much attention for having the potential to replace damaged tissues and organs in severe burns while overcoming the drawbacks of biological skin substitutes. Such technology could replace down the road the traditional artificial organ transplantation methods, avoiding all together immune system rejection complications and risk of infections.

In the present study, we performed chitosan scaffolds in the special air drying form for tissue engineering. In addition we evaluated and identified the effect of implantable particles loaded with cell culture (isolated from neonatal mice skin tissues for delivery) into skin tissue defects. This was done with the intention to overcome the drawbacks of traditional biological skin substitutes. We demonstrated as well the accelerated wounds healing when implanted with implantable scaffolds. Therefore we conclude that chitosan

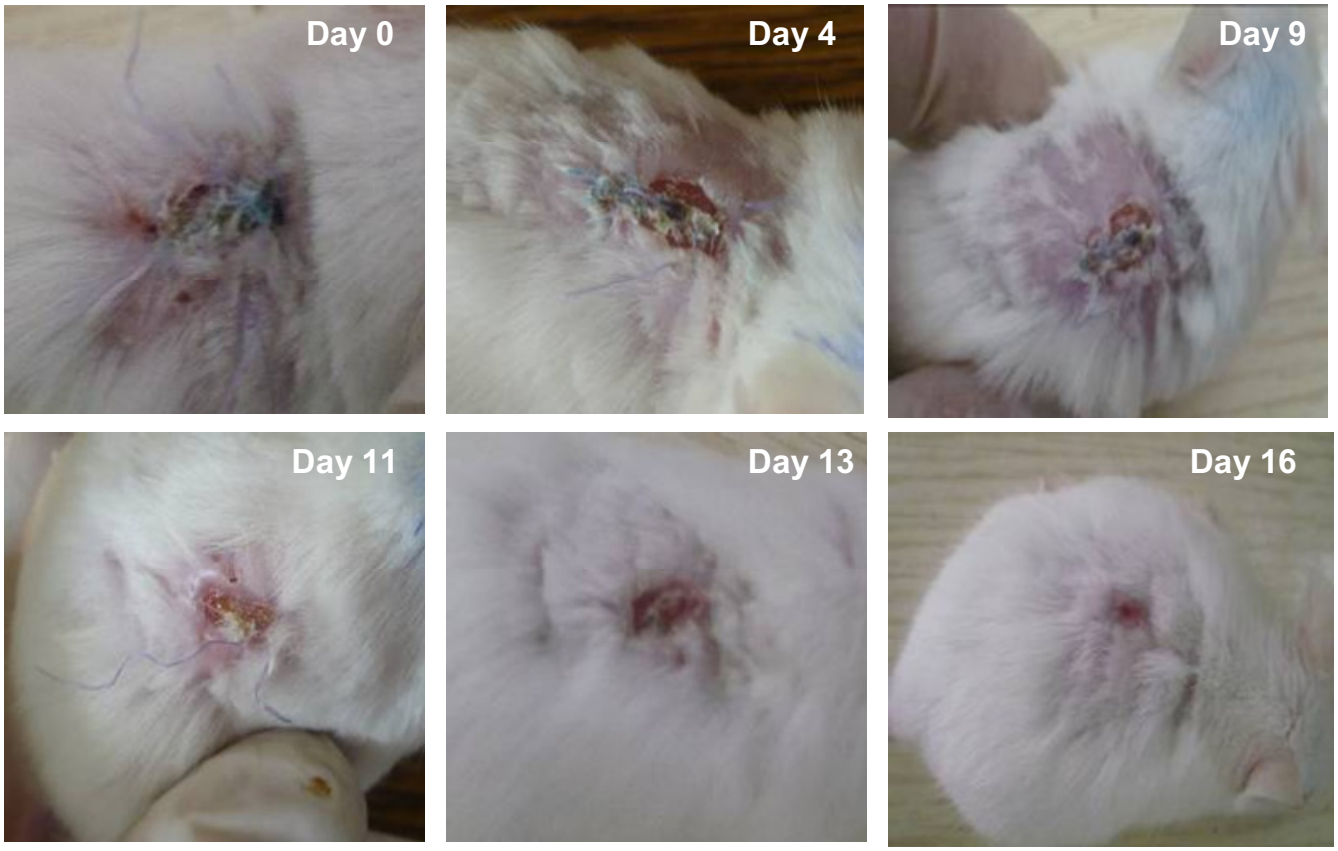

Fig. 7 Steps of skin regeneration of mice suture group with chitosan scaffolds.

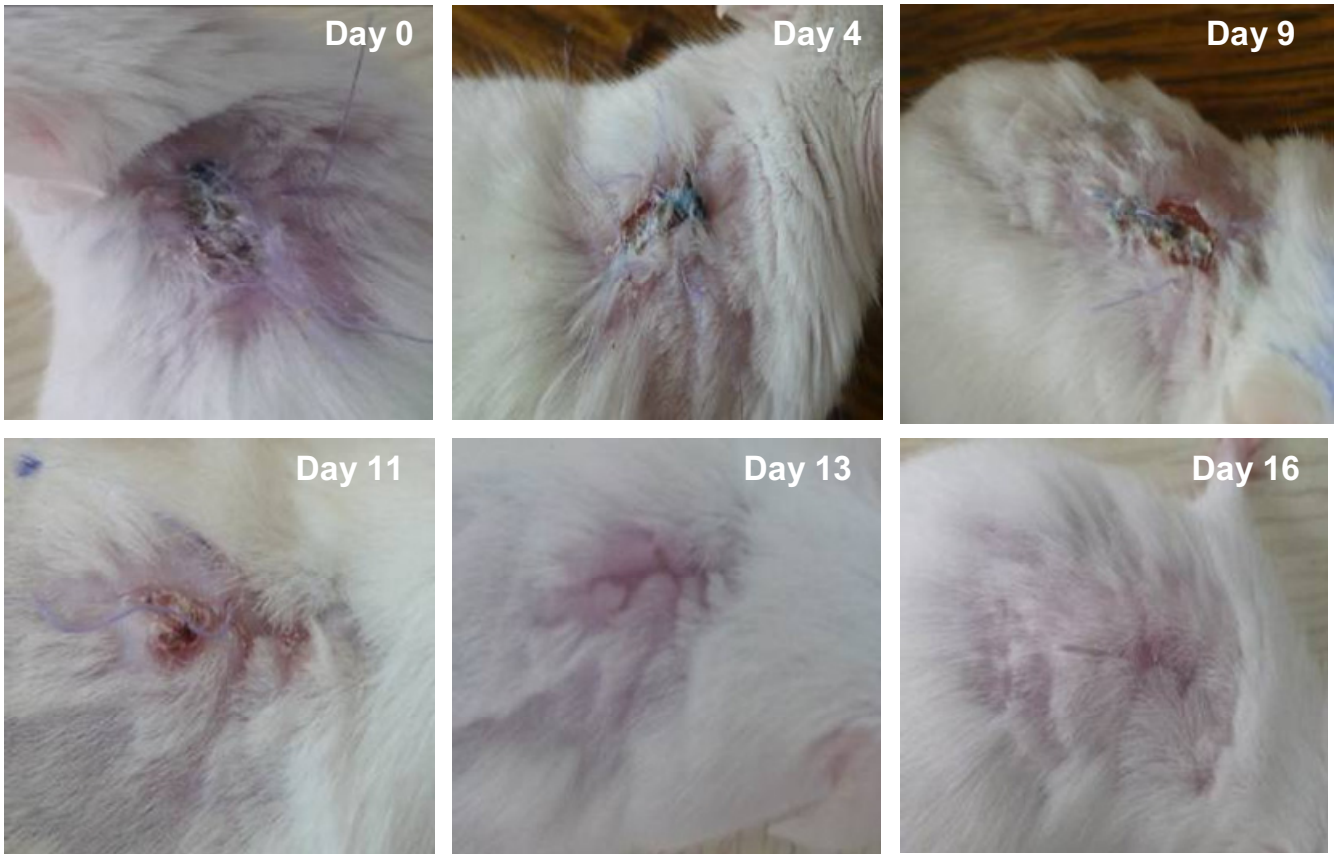

Fig. 8 Steps of skin regeneration of mice suture without chitosan (control group).

should be considered as an effective biomaterial scaffold for skin regeneration and tissue engineering applications due to its ability to possess implantable properties and biocompatibility.

ACKNOWLEDGEMENTS

This work has been partially supported and funded by the U.S-Egypt Joint Fund. We would also like to thank MSA University, Dr.

Aida Jihad, Dr. Ahmed Atef, Dr. Ossama Saad and Dr. Ahmed Nada.

REFERENCES

Aranaz I, Mengibar M, Harris R, Panos I, Miralles B, Acosta N, Galed G, Heras A (2009) Functional characterization of chitin and chitosan. *Current Chemical Biology* **3**, 203-230

Atiyeh BS, Gunn SW, Hayek SN (2005a) State of the art in burn treatment. *World Journal of Surgery* **29**, 131-148

Atiyeh BS, Hayek SN, Gunn SW (2005b) New technologies for burn wound closure and healing. *Journal of the International Society for Burn Injuries* **31**, 944-956

Brunette DM (1988) The effect of implant surface topography on the behavior of cells. *International Journal of Oral and Maxillofacial Implants* **3**, 231-246

Brychta P, Adler J, Řihová H, Suchánek I, Kaloudová Y, Koupil J (2002) Cultured epidermal allografts: Quantitative evaluation of their healing effect in deep dermal burns. *Cell and Tissue Banking* **3**, 15-23

Burkatovskaya M, Castano AP, Demidova- Rice TN, Tegos GP, Hamblin MR (2008) Effect of chitosan acetate bandage on wound healing in infected and non-infected wounds in mice. *Wound Repair and Regeneration* **16**, 425-431

Chatelet C, Damour O, Domard A (2001) Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials* **22**, 261-268

Clifford DH (1984) Preanesthesia, anesthesia, analgesia, and euthanasia In: Fox JG, Gohen BJ, Loew FM (Eds) *Laboratory Animal Medicine*, Academic Press, Orlando, pp 530-562

Curtis A, Wilkinson C (1997) Topographical control of cells. *Biomaterials* **18**, 1573-1583

Dai NT, Williamson MR, Khammo N, Adams EF, Coombes AG (2004) Composite cell support membrane based on collagen and polycaprolactone for tissue engineering of skin *Biomaterials* **25**, 4263-4271

Deutsch J, Motlagh D, Russell B, Desai TA (2000) Fabrication of microtextured membranes for cardiac myocyte attachment and orientation. *Journal of Biomedical Material Research* **53**, 267-275

Eagle H (1955) Nutrition needs of mammalian cells in cell. *Science* **122**, 501-504

Entsar SA, Khaled SA, Maher ZE (2008) Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology* **99**, 1359-1367

Gustafson BS, Fulkerson P, Bildfell R, Aguilera L, Hazzard MT (2007) Chitosan dressing provides hemostasis in swine femoral arterial injury model. *Prehospital Emergency Care* **11**, 172-178

Hansson C (1997) Interactive wound dressings. A practical guide to their use in older patients. *Drugs Aging* **11**, 271- 84

Himes VB, Hu WS (1987) Attachment and growth of mammalian cells on microcarriers with different ion exchange capacities. *Biotechnology and Bioengineering* **24**, 1155-1163

Hima Bindu TVL, Vidyavathi M, Kavitha K, Sastry TP, Suresh Kumar RV (2010) Preparation and evaluation of ciprofloxacin loaded chitosan-gelatin composite films for wound healing activity. *International Journal of Drug Delivery* **2**, 173-182

Huang S, Deng T, Wang Y, Deng Z, He L, Liu S, Yang J, Jin Y (2008) Multifunctional implantable particles for skin tissue regeneration: Preparation, characterization, *in vitro* and *in vivo* studies. *Acta Biomaterialia* **4**, 1057-1066

Khan TA, Peh KK (2003) A preliminary investigation of chitosan film as dressing for punch biopsy wounds in rats. *Journal of Pharmacy and Pharmaceutical Science* **6**, 20-26

Kopp J, Jeschke M, Bach A, Kneser U, Horch R (2004) Applied tissue engineering in the closure of severe burns and chronic wounds using cultured human autologous keratinocytes in a natural fibrin matrix. *Cell and Tissue Banking* **5**, 89-96

Lee SB, Jeon HW, Lee YW, Cho SSK, Lee YM (2003) Artificial dermis composed of gelatin, hyaluronic acid and (133),(136)-glucan. *Macromolecular Research* **11**, 368-374

Ma L, Gao C, Mao Z, Zhou J, Shen J, Hu X, Han C (2003) Collagen/chitosan porous scaffolds with improved biostability for skin tissue. *Biomaterials* **24**, 4833-4841

Maurer XYZ (1992) Cellular responses to chitosan *in vitro*. In: Freshney RI (Ed) *Animal Cell Culture*, IRL Press, Oxford, UK, pp 773-778

Metcalfe AD, Ferguson MW (2007) Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *Journal of the Royal Society Interface* **22**, 413-37

Muzzarilli RA (2009) Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrates Polymers* **76**, 167-182

Ng KW, Khor HL, Hutmacher DW (2004) *In vitro* characterization of natural and synthetic dermal matrices cultured with human dermal fibroblasts. *Biomaterials* **25**, 2807-2818

Ponsonnet L, Comte V, Othmane A, Lagneau C, Charbonnier M, Lissac M, Jaffrezic N (2002) Effect of surface topography and chemistry on adhesion, orientation and growth of fibroblasts on nickel-titanium substrates. *Materials Science and Engineering C. Biomimetic and Supramolecular Systems* **21**, 157-165

Prasitslip M, Jenwithisuk K, Kongsuwan k, Damrongchai N, Watts P (2000) Cellular responses to chitosan *in vitro*: The importance of deacetylation. *Journal of Material Science: Materials in Medicine* **11**, 773-776

Ranucci CS, Moghe P V (2001) Substrate microtopography can enhance cell adhesive and migratory responsiveness to matrix ligand density. *Journal of Biomedical Material Research* **54**, 149-161

Ueno H, Mori T, Fujinaga T (2001a) Topical formulations and wound healing applications of chitosan. *Advanced Drug Delivery Reviews* **52**, 105-115

Ueno H, Nakamura F, Murakami M, Okumura M, Kadosawa T, Fujinaga T (2001b) Evaluation effects of chitosan for the extracellular matrix production by fibroblast and the growth factors production by macrophages. *Biomaterials* **22**, 2125-2130

Wang YC, Kao SH, Hsieh HJ (2003) A chemical surface modification of chitosan by glycoconjugates to enhance the cell biomaterial interaction. *Biomacromolecules* **4**, 224-231

Wood FM, Stoner ML, Fowler BV, Fear MW (2007) The use of a non-cultured autologous cell suspension and Integra dermal regeneration template to repair full-thickness skin wounds in a porcine model: a one-step process. *Burns* **33**, 693-700

Wu Z, Ding Y, Zhang L, Zhong S, Jiang T (2006) Primary grafting research of tissue engineered oral mucosa lamina propria on skin full thickness wounds. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **20**, 172-176

Zhu N, Warner RM, Simpson C, Glover M, Hernon CA, Kelly J, Fraser S, Brotherston TM, Ralston DR, MacNeil S (2005) Treatment of burns and chronic wounds using a new cell transfer dressing or delivery of autologous keratinocytes. *European Journal of Plastic Surgery* **28**, 319-330