

***In vitro* Co-Culture of Human Skin Keratinocytes and Fibroblasts on a Biocompatible and Biodegradable Scaffold**

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ABSTRACT

Background: Extensive full-thickness burns require replacement of both epidermis and dermis. In designing skin replacements, the goal has been to re-create this model and make a product which has both essential components. **Methods:** In the present study, we developed procedures for establishing confluent, stratified layers of cultured human keratinocytes on the surface of modified collagen-chitosan scaffold that contains fibroblasts. The culture methods for propagation of keratinocytes and fibroblasts isolated from human neonatal foreskin were developed. The growth and proliferation of normal human keratinocytes were evaluated in serum-free (keratinocyte growth medium) and our modified medium. Characterization of human keratinocytes was determined by using pan-keratin and anti-involucrin monoclonal antibodies. For fabrication of relevant biodegradable and biocompatible collagen-chitosan porous scaffold with improved biostability, modified method of freeze-gelation was used. In generating organotypic co-cultures, epidermal keratinocytes were plated onto the upper surface of scaffold containing embedded fibroblasts. **Results:** The results showed that the growth of isolated human skin fibroblasts and keratinocytes in our modified medium was more than that in the serum-free medium. The different evaluations of collagen-chitosan scaffold showed that it is relevant to growth of cells (fibroblast and keratinocyte) and has a good flexibility in manipulation of the living skin equivalents. **Conclusion:** These findings indicate that the integration of collagen-chitosan scaffold with co-cultured keratinocyte and fibroblast *in vitro* provides a potential source of living skin for grafting *in vivo*. *Iran. Biomed. J. 13 (3): 169-177, 2009*

Keywords: Co-culture, Keratinocyte, Scaffold, Human skin

INTRODUCTION

The integument, the largest organ in the body, has long been the subject of biological and medical interest as a tissue for transplantation, reconstruction, and pharmacologic manipulation, and as a tool to access properties of environmental toxins, cosmetic, and other formulations. The skin is divided into two anatomically distinct regions, dermis and epidermis. The structure and function of normal integument depend on an intact epidermis anchored to its vascular, elastic dermis. In designing skin replacements, the goal has been to re-create this model and make a product which has both essential components [1].

The fibroblast is the cell type which is the most

prevalent in dermis and is responsible for synthesizing and depositing collagen fibers in continuous networks that form the structural scaffold. During connective tissue repair, fibroblasts exhibit several different activities. At first, they migrate from adjacent tissues into the wound region. In the wound region, they proliferate and synthesize a collagen-rich extracellular matrix that fills the wound defect. Fibroblasts produce different growth factors that induce proliferation of keratinocytes *in vivo* (normal skin) and *in vitro* (artificial living skin) [2].

The epidermis is a complex tissue that develops a stratum corneum at the interface of the skin and atmosphere, affording the primary barrier to the environment. The principal cell type of the epidermis is keratinocytes, which makes up the

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stratified epidermis and stratum corneum [3].

The keratinocytes grow and stratify to form a continuous epithelial layer over the surface of the dermal membranes. The latter serves as mechanical support for the cell sheets and as a biodegradable connective tissue matrix into which vascular supply and non-inflammatory connective tissue can develop from the wound bed [4]. The keratinocyte has been the subject of considerable basic research because it undergoes many of the normal processes of differentiation *in vitro*. Keratinocytes enlarge as they differentiate, much as they do *in vivo* and also terminally differentiate and form cornified envelopes. Terminal differentiation of the keratinocyte *in vitro* requires calcium. Calcium allows stratification by assisting the formation of desmosomal contacts and promotes differentiation [5, 6]. Two different substrates for culturing keratinocytes are: dead de-epidermized dermis [7] which originally developed by Pruniéras *et al.* [8], and the dermal equivalent (collagen-fibroblast lattice) developed by Bell *et al.* [9]. However, there are many kinds of synthetic substrates such as those described by Boyce and Hansbrough [10], Tiollier *et al.* [11], Shahabeddin *et al.* [12], Rosdy and Clauss [13], Lee *et al.* [14], and Mazlyzam *et al.* [15].

The fabrication of tissue engineering scaffolds for skin replacement is an important topic in biomaterial research [16]. The three-dimensional structure of scaffolds provides physical support and acts as a substrate regulating cell growth, adhesion and differentiation. Recently, much work has been done on constructing tissue-engineered bilayer skin equivalents [17]. Scaffold fabricated by Huang *et al.* [18] contained 2 layers, dermis and epidermis. Epidermis was made from gelatine hydrogel which contained epidermal growth factor (EGF)-loaded microspheres. Their results indicated that this bilayer skin equivalent was a proper template for skin tissue engineering. Liu *et al.* [19] used bone marrow mesenchymal stem cells in their scaffolds and their satisfactory results showed a better healing and keratinization, less wound contraction, and more vascularization for their scaffolds. Chitosan and collagen are among the most used natural biodegradable polymers as tissue scaffolds [20]. Chitosan is a polysaccharide constituted of N-glucosamine and N-acetyl-glucosamine units, in which the number of N-glucosamine units exceeds 50%. It displays interesting properties such as biocompatibility and biodegradability and its degradation products are non-toxic, non-immunogenic and non-carcinogenic. Therefore,

chitosan has prospective applications in many fields such as tissue engineering. Disadvantages of chitosan are its poor physical property of being brittle at the dried stage and restriction of cell spreading and cytoskeletal actin distribution [21, 22].

Physical and biological properties of chitosan can be modified by cross-linking chitosan with other materials such as gelatin, tricalcium phosphate and collagen. Collagen is a major component of extra cellular matrix, which has the disadvantages of a fast degradation rate and low mechanical strength. The specific properties of chitosan and collagen can be combined to create new materials with unique structural and mechanical properties appropriate for tissue regeneration [20, 23]. In human keratinocyte cultures, bovine pituitary extract is commonly used because of its recognized ability to promote proliferation of keratinocytes [6]. There are a wide range of materials which might possibly be used as agents to stimulate keratinocyte growth such as oligosaccharides (alginate oligosaccharides) [24] and paracrine growth factors for keratinocytes secreted by fibroblasts such as keratinocyte growth factor [25, 26], insulin-like growth factor II [27], and hepatocyte growth factor/scatter factor [28].

In the present study, living skin equivalents (containing derm and epiderm) were prepared by combining cultured human fibroblasts with a collagen-chitosan matrix (derm) and overlaying this lattice with human keratinocytes (epiderm). For this purpose, we applied a new developing freeze-gelation technique [29, 30] for both chitosan and collagen to fabricate a three-dimensional scaffold and chemical cross-linking between collagen and chitosan performed by glutaraldehyde.

MATERIALS AND METHODS

Culture media. Several different combinations of hormones and growth factors were employed in our studies. For the purpose of clarity and the following three media designations were employed throughout this paper. For cultivation of fibroblasts we used RPMI1640 medium plus 10% (v/v) fetal bovine serum (FBS) and penicillin (100 IU/ml)-streptomycin (100 µg/ml, pH 7.2, all from Gibco-BRL, Germany). For cultivation of keratinocytes, the following media were used: Keratinocyte growth medium (KGM, serum-free medium) with its supplement and modified Green's medium consists of DMEM and Ham's F12 media in a 3:1 ratio

supplemented with 10% (v/v) FBS, EGF (10 ng/ml), hydrocortisone (0.4 µg/ml), insulin (5 µg/ml), penicillin (100 IU/ml)-streptomycin (100 µg/ml), epinephrine (0.4 µg/ml), prostaglandin E2 (10 ng/ml) and L-glutamine (2 mM), (pH 7.2, all from Gibco-BRL, Germany).

Chemical reagents. Collagenase type II, Trypsin/EDTA and PBS (0.15 M) were obtained from Gibco-BRL, Germany. Calcium, nystatin, mitomycin C, glutaraldehyde, collagen type I from bovine skin and chitosan (medical grade) were purchased from Sigma, USA.

Isolation and culture of human dermal fibroblasts. Human dermal fibroblasts were isolated from neonatal foreskin obtained at the time of circumcision, with consent from parents. Samples were carried to the laboratory in RPMI medium containing 10% (v/v) FBS and penicillin (100 IU/ml)-streptomycin (100 µg/ml) and can be stored 1 week at 4°C. Before working with samples they were washed three times in washing solution, RPMI containing penicillin-streptomycin and nystatin for remove of probably infection. Samples were rinsed several times with PBS to remove any blood and serum, and then were placed in a 9-cm dish to remove most of the subcutaneous fat and membranous material with either a scalpel blade or a sharp pair of curved scissors. Skin was cut into strips 1 cm wide using scalpel and epidermis was separated from dermis using two pairs of forceps. For separation of keratinocytes, the strips were placed, epidermis side up, in 0.25% trypsin and then incubated at 4°C overnight or at 37°C for 1 h. The dermis was cut into very small pieces (5mm²) using curved scissors and these pieces were transferred into a collagenase Type II (200 IU/ml) solution in an incubator at 37°C for 2 h. Then, the suspension was centrifuged at 500 ×g for 5 min. The pellet was resuspended in culture medium. The isolated cells were counted using hemocytometer and then seeded into culture dishes at cell density of 2 × 10⁵ cells/cm². The cells were cultured in a CO₂ incubator at 37°C and the culture medium was changed twice a week. The cells were subcultured before they reached confluence by treating them with Trypsin-EDTA for 5 min and splitted 1:3 into new dishes [9, 14].

Isolation and culture of human epidermal keratinocytes. The epidermis obtained from previous section was incubated in 0.25% trypsin

solution at 4°C overnight or at 37°C for 1 h. Then, it was transferred into a 15-ml centrifuge tube and was vortexed gently for 2 min, and then the same volume of FBS was added in order to inhibit the action of the trypsin. The suspension was centrifuged at 500 ×g for 5 min, the supernatant was discarded and the cell pellet was resuspended in culture medium. After counting the cell and determining their viability by using trypan blue exclusion, the cells were seeded on tissue culture dished in three media protocols: 1) KGM medium, 2) modified Green's condition by using 3T3 cells and 3) modified Green's condition by using foreskin human fibroblast cells. In Green's method, the cells were seeded alongside growth arrested 3T3 cells (obtained from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran, NCBI-C162) or foreskin human fibroblast cells (obtained from neonatal foreskin samples) at a cell density of 1.5 × 10⁵ cells/cm². For this purpose, 3T3 cells were incubated with 10 µg/ml mitomycin C at 37°C for 2 h and then mitomycin C solution was discarded and the cells were rinsed 3 times with culture medium. Approximately 40% of the epidermal cells were attached and the cells began to spread out on dish within 24-48 h. The culture medium was changed daily and the dishes were confluent by 10-15 days [3, 10, 13].

Characterization of keratinocyte cells. For characterization of keratinocytes, two common types of monoclonal antibodies were used: anti-human involucrin produced in mouse (Sigma, USA) and anti-pan-keratin produced by C11 hybrid cells (NCBI, H-189). The keratinocytes were fixed using 4% formaldehyde (Sigma, USA) and after 3 times of washing with PBS, anti-involucrin and anti-pankeratin were incubated on the cells for 1.5 h. After washing 3 times with PBS, anti-mouse-Ig-FITC (Sigma, USA) was used as secondary conjugate antibody. The positive cells were detected using fluorescence microscope (Zeiss, Germany) [4, 31, 32].

Fabrication of a novel collagen-chitosan scaffolds by using freeze-gelation method. Freeze-gelation was presented by Hsieh *et al.* [29] and Ho *et al.* [30] which can be used to prepare highly porous scaffolds without using the time and energy consuming freeze-drying process. The porous structure was generated during the freeze of a polymer solution, following which either the solvent was extracted by a non-solvent or the polymer was gelled under the freezing condition. This method

was used for bonding of chitosan with collagen by glutaraldehyde (GA) which enhances mechanical properties and biostability of collagen. Chitosan was dissolved in acetic-acid aqueous solution (1 M) to form a 2 wt% polymer solution and collagen was dissolved in 0.1% acetic-acid aqueous solution with concentration of 4 mg/ml; GA was used in concentration of 0.25%. Chitosan and collagen solutions in a 2:1 ratio were mixed and GA was added to polymer solution then the polymer solution was placed in a glass Petri dish and frozen at -20°C . The frozen solution was immersed in NaOH/ethanol aqueous solution to adjust its pH to allow for gelation of polymer mixture. The NaOH/ethanol aqueous solution was pre-cooled to -20°C so that the gelation occurred below the freezing point of chitosan solution and drying at room temperature was performed after gelation to obtain our scaffolds. After washing with double distilled water (10 min \times 5 times), the scaffolds were immersed in 70% ethanol for sterilization. Porous formation in scaffold was evaluated by scanning electron microscopy.

Tensile testing. Static monotonic tensile test was performed to evaluate the mechanical properties of the specimens, according to the ASTM D638 type IV protocols. The specimens were dumbbell-shaped and subjected to tensile monotonic test at 25°C with stroke rate of 1 mm/min. This test was performed utilizing a dynamic servohydraulic testing machine (HCT 25-400, Zwick/Roell, Germany). Data was analyzed using Toolkit 98 software (Zwick/Roell, Germany).

Scanning electron microscopy. Samples of the scaffolds were fixed in 4% GA at 48°C for 12-24 h. They were then washed in 0.1 M sodium cacodylate buffer and dehydrated stepwise in a series of acetone (30-100%). Afterwards, the samples were dried using critical point dryer (BALTEC CPD030, Switzerland), sputtered with gold and analyzed with scanning electron microscope (JEOL 6400, Japan).

Fabrication of a bilayer skin. Fibroblasts isolated from dermis were cultured in the scaffolds with cell density of 5×10^5 cells/ml in a 6-well plate cell culture dishes. After 10 min, culture medium was added to each well. The scaffolds containing fibroblasts were carried to a designed insert, made by Teflon and stainless steel grid, and then

keratinocytes were added to the dermal part of reconstructed skin with cell density of 1×10^6 to cover the scaffolds. The medium used inside the inserts was modified Green's medium and RPMI was used outside of the insert. Keratinocytes attached within 12-24 h and formed a nearly confluent layer on top of the scaffold. After the medium level lowered to the lower part of the scaffold, the cultures were raised to the air-liquid interphase thus nourishment restricted to diffusion from below. This air-lift procedure was defined as the start of the culture time of organotypic cultures.

Differentiation and multi-layer formation of keratinocytes were observed in high concentration of Ca^{2+} (1.5 mM) added to media. Living skin equivalents containing derm (scaffold and fibroblast cells) and epiderm (multi-layer of keratinocytes) was evaluated by hematoxylin and eosin (H and E) staining method.

Hand estaining. The biopsy and specimens were conventionally fixed in 10% neutral formalin, grossly examined by an experienced, professional pathologist, using a standardized protocol [33], selecting relevant areas for paraffin embedding and subsequent routine histopathological examinations.

RESULTS

Cell isolation and culture. The dermal fibroblast cells from dermis and keratinocytes from epidermis of human foreskin were in good growth condition and showed a relevant morphology cytoplasm under the inverted microscope.

Dermal fibroblasts. As mentioned later, the seeded fibroblasts (2×10^5 cells/cm²) reached confluence within 3 days. The cells had high proliferation ability and showed spindle cytoplasm with thin and long projections, a typical fibroblasts-like morphology (Fig. 1a).

Keratinocytes. At first day, the cells showed a short and wide spindle shape and at day 3, proliferated cell colonies could be seen. The cells showed epithelial morphology like a "pavement stone" (Fig. 1b). Cultures reached confluence and ready to passage within 2 weeks. The growth and proliferation of keratinocytes in our modified medium and onto mitomycin C-treated 3T3 mouse

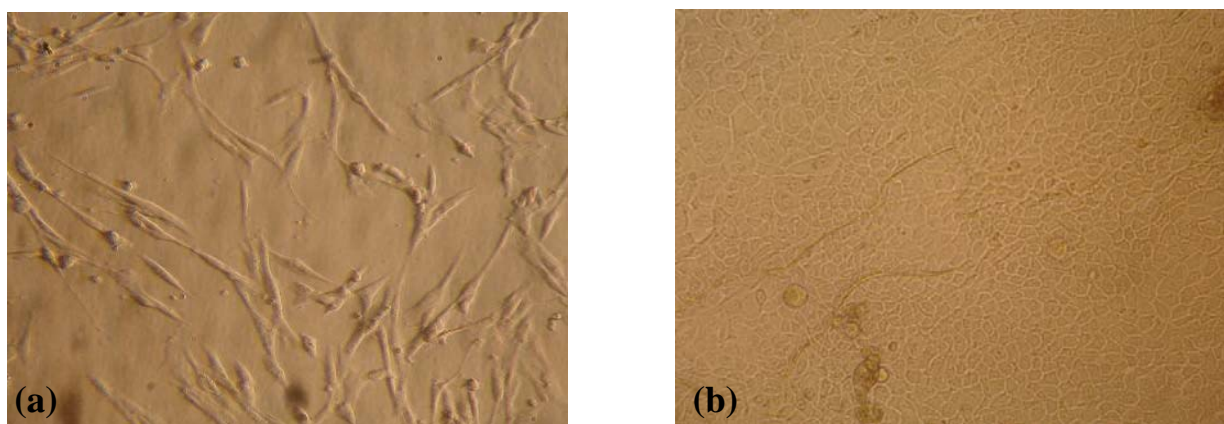


Fig. 1. Representative fibroblast and keratinocyte isolated from human foreskin. (a) fibroblasts (100 \times) and (b) keratinocytes (100 \times).

embryo fibroblasts were more significant than in purchased serum-free media in liquid form which may be the result of a bad condition of its transport.

Characterization of human keratinocytes. Keratinocytes were characterized by using anti-pankeratin and involucrin monoclonal antibodies and FITC-conjugated antibody (Fig. 2). The expressions of differentiation markers such as keratins and involucrin showed a similar pattern observed in normal skin.

Fabrication of scaffold. A novel collagen-chitosan was fabricated by freeze-gelation method (Fig. 3a) and porous formation in scaffold was evaluated by scanning electron microscopy (Fig. 3b). The average size of scaffold pores was appropriate for fibroblast proliferation and rehabilitation of skin defects ($300 \pm 50 \mu\text{m}$). This scaffold showed a good flexibility, so we can

manipulate the skin equivalents easily during the culture and the grafting.

Tensile testing. Figure 3c shows the ultimate load needed to rupture the specimens. After normalizing the load and stroke data, the ultimate stress was occurred about $16.3 \pm 0.9 \text{ MPa}$ at $313.15 \pm 26\%$ elongation.

Co-culture of fibroblasts and keratinocytes in scaffold. Fibroblast cultured in scaffold and keratinocyte cultured on scaffold were evaluated by inverted microscope. The air-exposed cultures of epidermal keratinocytes on the top of the sheet (containing fibroblast cells) as a dermal equivalent (Fig. 4a) for 2 weeks gave rise to the H and E. It consisted of both epidermal and dermal components. In the H and E, a multi-layered epidermis with a horny layer was formed on a dermis-like tissue, similarly to native epidermis (Fig. 4b).

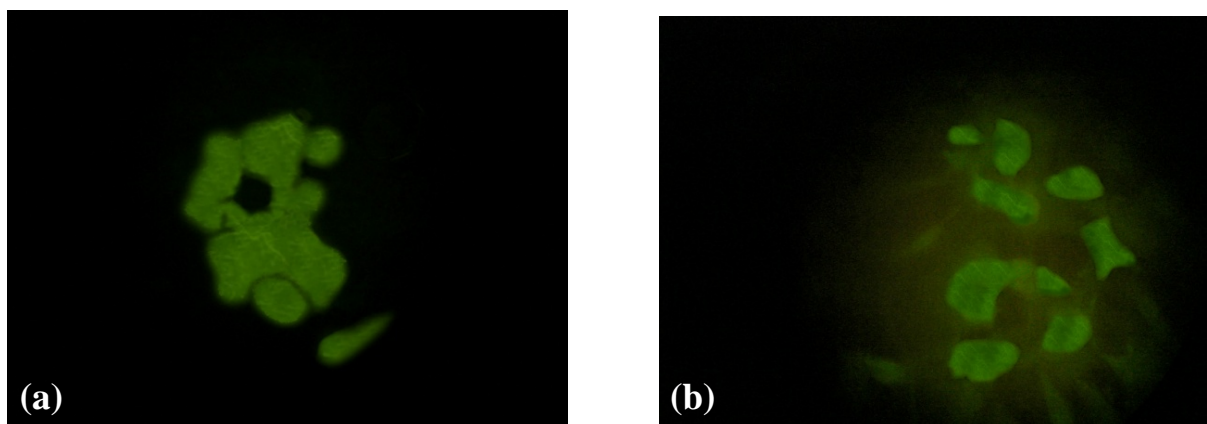


Fig. 2. Characterization of human keratinocyte. (a) anti-pan-keratin (keratinocyte cells, 500 \times) and (b) anti-involucrin (keratinocyte cells, 500 \times).

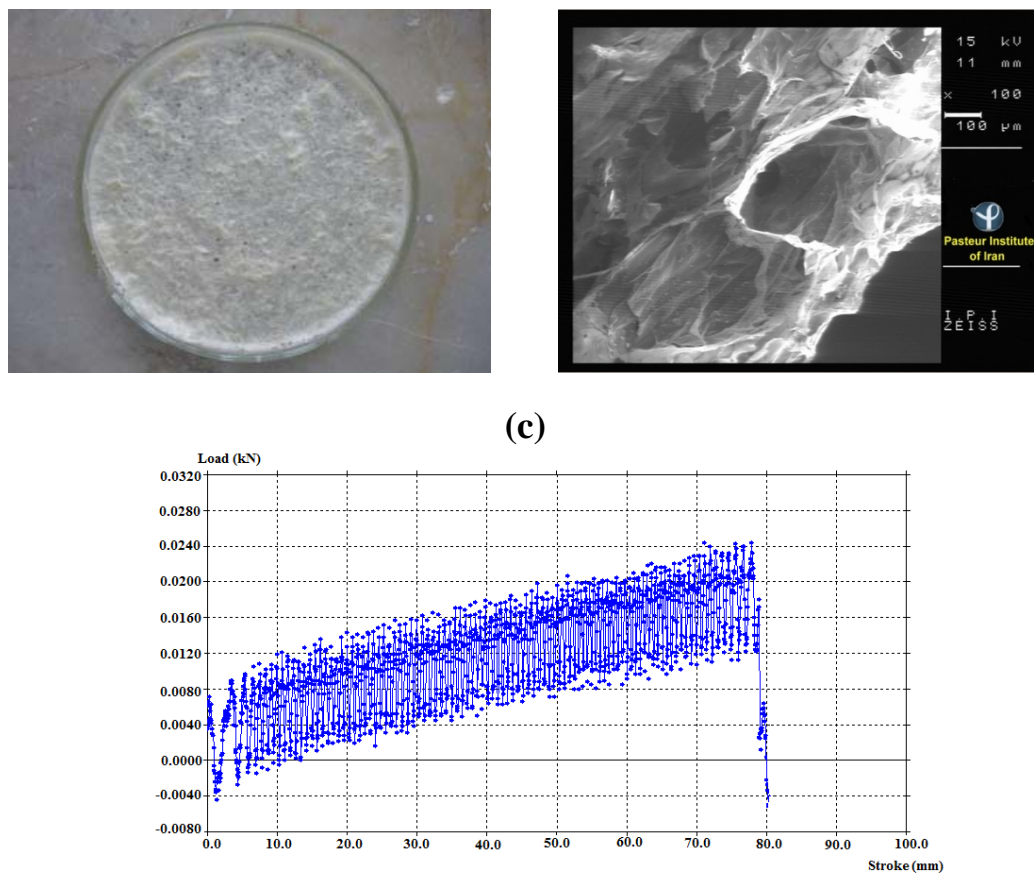


Fig. 3. Collagen-chitosan evaluation fabricated by freeze-gelation method. (a) direct observation; (b) scanning electron microscopy and (c) tensile test.

DISCUSSION

The integration of scaffold and cultured keratinocytes *in vitro* provides a potential source of skin for grafting. For generating organotypic co-cultures, epidermal keratinocytes were plated onto upper surface of collagen-chitosan scaffolds containing embedded fibroblasts, where they attached rapidly and formed confluent layers within

1-2 days. In the absence of fibroblasts, only thin epithelia developed with rapid loss of proliferation within 2-3 days. It could be demonstrated by means of this *in vitro* system that the co-cultured fibroblasts produce growth factors which are essential for epidermal morphogenesis [2]. There are many studies which have focused on the development of nutritionally optimized and readily defined and reproducible media and culture conditions for

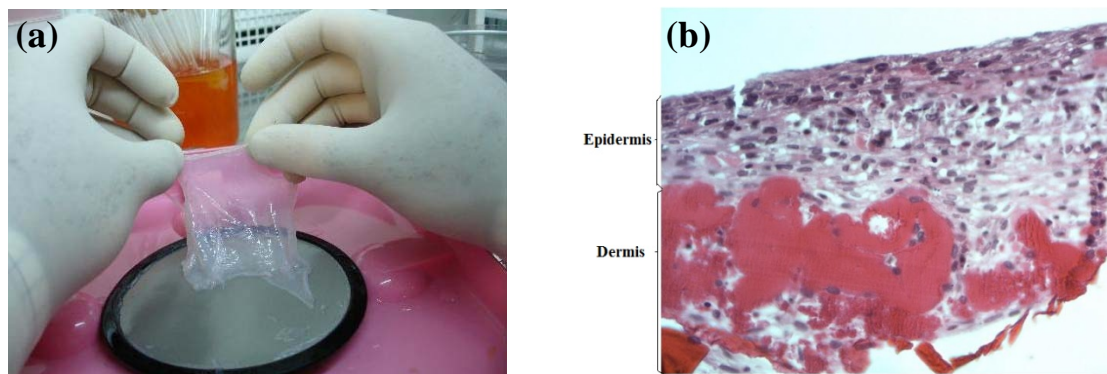


Fig. 4. Skin equivalent fabricated with human skin fibroblasts and keratinocytes and biocompatible, biodegradable collagen-chitosan scaffold. (a) representative of skin equivalent and (b) histology view of bilayer skin produced (photograph of H and E staining (100 \times)).

keratinocyte cells [13, 27, 28 and 36]. Our aims were not to establish a new medium but we used some components of two different protocols [34, 35]. Our results showed that new combination was performed well. Keratinocytes were plated onto tissue culture dishes using two different protocols: a) with no feeder layer in KGM and b) onto mitomycin C-treated 3T3 mouse embryo fibroblasts. The later protocol utilized DMEM supplemented with growth factors which mentioned in material part of this article. Although Coolen *et al.* [36] showed that keratinocytes can be cultured without the need of a fibroblast feeder layer and FCS, our results demonstrated that keratinocyte cell growth was greater in our modified medium than in serum-free media without feeder cells. Because of insecure transport of the KGM medium, quantitative analysis of cell proliferation using dimethyl thiazol diphenyl tetrazolium bromide (MTT) or other protocols was not meaningful.

One of the important purposes of adding chitosan is providing additional amino groups which function as binding sites to increase the GA cross-linking efficiency. Therefore, the interpenetration of collagen and chitosan in the scaffold is crucial [20, 37]. So, our combination is a good replacement of collagen gels. GA was used to treat the scaffolds to improve their biostability. The fabricated scaffold showed suitable tensile properties for skin grafting applications within the range of normal human skin (15-150 MPa) [38].

Freeze-gelation scaffolds used in this article are compared with the freeze-drying method; the presented method is time and energy-saving, with less residual solvent, and easier to be scaled up [29, 30]. Besides, the problem of formation of surface skin can be resolved and the limitation of using solvent with low boiling point can be lifted by the presented methods. H and E staining was performed for air-exposed cultures of epidermal keratinocytes on the top of the scaffold sheet as a dermal equivalent after 2 weeks. Results indicated that it consisted of both epidermal and dermal components. These findings indicate that our model may be useful as a dermal equivalent for grafting *in vivo*. However, a long-term follow-up study will be needed to more precisely evaluate its fate after grafting. In conclusion, the findings from this study demonstrated that a new dermal equivalent, that closely resembles a dermis *in vivo*, could be constructed by culturing dermal fibroblasts alone in a special culture medium. In addition, the dermal equivalent may be useful for experimental and

clinical purposes, such as the reconstruction of a skin equivalent *in vitro* and grafting *in vivo*.

All these results suggest that collagen-chitosan scaffold, cross-linked by GA using freeze-gelation method, is a potential candidate for dermal equivalent with enhanced biostability and good biocompatibility.

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