A COMPARATIVE IN-VITRO STUDY OF CULTURED PRIMARY NORMAL HUMAN EPIDERMAL KERATINOCYTE ON ARTIFICIAL DERMAL REGENERATION TEMPLATE AND FIBRIN SCAFFOLD

By

MOHAMAD FADZIRUL HADZLI BIN MOHAMAD MASRI

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ABSTRACT

Skin plays an important role in regulating our body homeostasis. Once the dermal layer is destroyed, it cannot heal by itself, a transplant or artificial dermal regeneration template is needed in order to restore its function. This study aims to explore the successfulness in the cultivation of primary normal human epidermal keratinocyte (pNHEK) within INTEGRA® dermal regeneration template (INTEGRA®-DRT) and fibrin. Keratinocytes were seeded on INTEGRA®-DRT and fibrin and observed on three day basis until one week. During the culture, cells were supplied with medium containing elevated calcium in order to promote cell differentiation. The cell viability analysis, growth pattern and three dimensional views were conducted using Laser Scanning Confocal Microscope (LSCM). From the analysis, pNHEK were grown on both scaffolds and the proliferation rate was better in fibrin compared to INTEGRA®-DRT ($p < 0.05$). However, the penetration rates are better in INTEGRA®-DRT scaffold compared to fibrin. In conclusion, both scaffolds are suitable to be developed as skin substitute depends on burn situation.

ABSTRAK

Kulit memainkan peranan yang penting dalam mengawal atur homeostasis badan manusia. Apabila lapisan dermal pada kulit musnah, ia tidak akan dapat sembuh dengan sendirinya. Trasnsplan atau acuan buatan pembaikan dermal diperlukan bagi mengembalikan fungsi asal kulit. Kajian ini dijalankan untuk menguji kebolehan sel keratin dari epidermal normal manusia (pNHEK) untuk hidup di atas INTEGRA® *dermal regeneration template* (INTEGRA®-DRT) dan fibrin. Sel keratin akan di masukkan ke atas material dan diperhatikan setiap tiga hari sehingga satu minggu. Ketika proses kultur, medium pertumbuhan sel akan digantikan dengan medium pertumbuhan yang mempunyai kandungan kalsium yang lebih tinggi, ini adalah bertujuan untuk menggalakkan proses pembezaan sel. Analisis sel hidup, corak pertumbuhan, dan pandangan tiga dimensi dijalankan menggunakan *Laser Scanning Confocal Microscope* (LSCM). Berdasarkan analisis pNHEK hidup di atas kedua-dua biomaterial dan kadar penyebaran sel adalah lebih baik di atas fibrin berbanding di atas INTEGRA®-DRT ($p < 0.05$). Walau bagaimanapun, kadar penembusan sel di atas INTEGRA®-DRT adalah lebih baik berbanding fibrin. Sebagai kesimpulan, kedua-dua biomaterial adalah sesuai untuk dibangunkan sebagai kulit gantian bergantung kepada tahap kecederaan disebabkan oleh kebakaran.

CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVATION

®	-	Registered
%	-	Percent
&	-	And
“”	-	Quotation mark
TM	-	Trademark
°C	-	Degree Celsius
dH_2O	-	Distilled water
mm	-	Millimeter
mL	-	Milliliter
µm	-	Micrometer
µM	-	Micromolar
µL	-	Microlitre
mM	-	Millimolar
nm	-	Nanometer
cm^2	-	Centimeter square
min	-	Minute
rpm	-	Rotation Per Minute
NHEK	-	Normal Human Epidermal Keratinocyte
pNHEK	-	Primary Normal Human Epidermal Keratinocyte
LSCM	-	Laser Scanning Confocal Microscope
HUSM	-	Hospital Universiti Sains Malaysia
INTEGRA®-DRT	-	INTEGRA® Dermal Regeneration Template
OT	-	Operation theatre

Kg	-	Kilogram
3D	-	Three Dimension
PBS	-	Phosphate Buffer Saline
ICT	-	Innovative Cell Technologies
PCTEK	-	Progenitor Cell Target Epidermal Keratinocyte
$CaCl_2$	-	Calcium Chloride
EDTA	-	Ethylenediaminetetraacetic Acid
DMEM	-	Dulbecco's Modified Eagle's Medium
FBS	-	Fetal Bovine Serum
KGM	-	Keratinocyte Growth Medium
DMSO	-	Dimethyl Sulfoxide
Ca^{2+}	-	Calcium
Fig.	-	Figure

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Introduction

Primary normal human epidermal keratinocyte (pNHEK) can provide a permanent coverage of large area from a skin biopsy. pNHEK is engineered from the biopsies of patient's own skin after getting the consent. The skin specimen will be cultivated in the culture medium that contains epidermal growth factor. During 18 to 25 days of culture, pNHEK might expand up to form a sheet that can be used as skin graft. Skin grafting is usually required for full thickness or deep partial thickness burn. pNHEK grafting is required to prevent further injury or infection. Successfulness of pNHEK application will be observed as thin, friable skin and contracture development on the wound site.

The application of pNHEK within INTEGRA® might help to restore the function of dermis and epidermis layer in which the INTEGRA® has the properties that resemble the actual dermis. Manipulation of these properties will help to reduce the risk of developing pain, scarring, infection and slow healing on full thickness or partial thickness burn patient.

1.2 Problem statement

Tissue-engineered skin is a significant advance in the field of wound healing and was developed due to limitations associated with the use of autologous skin grafts (autograft). These limitations include the creation of a donor site which is at risk of developing pain, scarring, infection and slow healing.

Application of tissue engineering principle will give a promising way in the management of massive burns. These developments may produce faster recovery with a shorter length of hospital stay and better long lasting quality of bioengineered skin to permit the start of rehabilitation programs sooner and to decrease the reconstructive needs.

Therefore, this research aims to explore the successfulness of pNHEK within INTEGRA®-DRT and fibrin scaffold so that it can be applied as another skin substitute.

1.3 Objectives

The main objectives of this project are:

1. To explore the successfulness in using pNHEK within INTEGRA®-DRT and fibrin scaffold.
2. To study the viability and growth pattern of pNHEK within INTEGRA®-DRT and fibrin scaffold by using LSCM with Live-dead cell staining.

1.4 Scope of works

For this project, the main focus is to explore the successfulness of using pNHEK within INTEGRA®-DRT and fibrin scaffold. pNHEK will be seeded onto the porous structure of INTEGRA®-DRT with the silicone layer facing down to serve as basement and onto fibrin scaffold. Observation will be carried out using LSCM with live dead cell staining technique every three days of culture until one week.

1.5 Study area

Keratinocyte cultivation is carried out by using optimized conditions from Rheinwald and Green methodology established in 1975. It is the most reliable procedure in obtaining relatively pure cultures of keratinocyte that can be maintained for long term.

This research will be conducted in Tissue Engineering Laboratory located in Burns Unit of Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan. All skin samples are obtained from patient biopsies after having their consent.

CHAPTER 2

LITERATURE REVIEW

2.1 Human skin

Skin is the largest organ of our body in terms of its size and weight (Church *et al.*, 2006, Metcalfe and Ferguson, 2006). Skin weighs between 6 and 10 kg in adult male and has 1.5 to 2.0 square meters of surface area. In comparison, newborn have only 0.2 to 0.3 square meter of skin surface area (Church *et al.*, 2006). Skin plays an important role in maintaining body homeostasis (Boyce *et al.*, 2002), thermoregulation, protection from infection, immunological, neurosensory, and metabolic function such as vitamin D metabolism (Church *et al.*, 2006, Boyce *et al.*, 2002).

Skin is divided into three anatomical regions that is epidermis, dermis and hypodermis. Epidermis is the outer layer of the skin, it is thin and totally cellular but sufficient to provide vital barrier function to our body. Keratinocyte is the most predominant cell present on the epidermal layer in which it occupies about 95% to 97% (Boyce *et al.*, 2002). Epidermal cells are constantly being sloughed in order to repair and replace damage and old cell (Church *et al.*, 2006, Metcalfe and Ferguson, 2006).

Dermis is the inner layer beneath the epidermis that made up of collagen and elastic fibers. It is the location where nerves, blood vessels, sweat glands and hair follicle are present. Dermal cell component consists of endothelium, smooth muscle, fibroblasts, nerve cells, immune response cell and pilo-erector muscles (Boyce *et al.*, 2002). Fibroblast is the major cell present in this area of skin which is capable of producing remodeling enzymes such as proteases and collagenases that play an important role in

wound healing process. Hypodermis is the region beneath the dermis or also known as subcutaneous tissue that is where larger blood vessels and nerves are located. This region of the skin contain considerable amount of adipose tissue that is well vascularized contributing to the mechanical and thermoregulatory of the skin (Metcalfe and Ferguson, 2006).

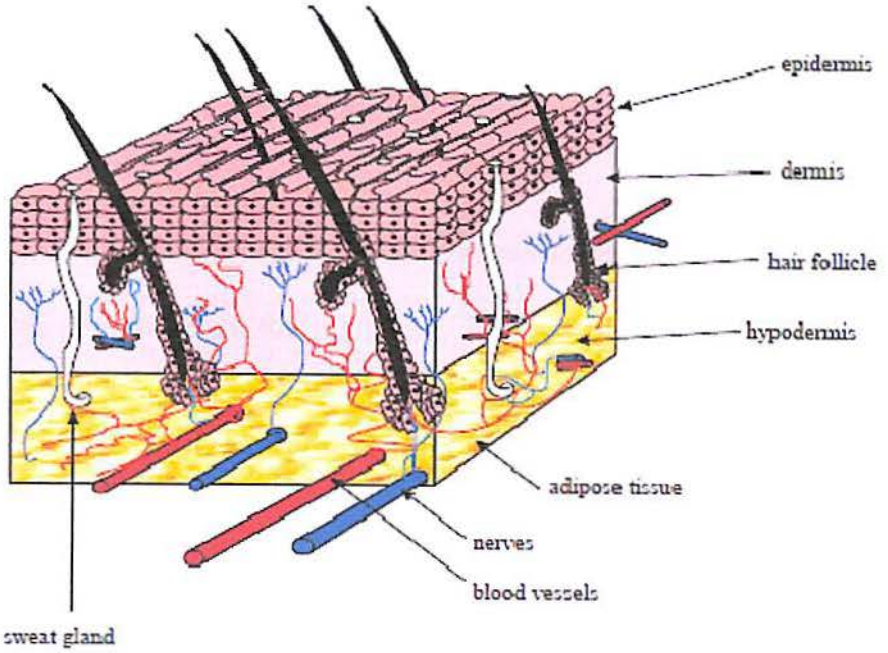


Figure 2.1: Schematic structure of skin (Metcalfe and Ferguson, 2006).

Human epidermis is an important tissue that provides a sturdy, flexible and self repairing barrier between our environment and internal body. One of it function is to help in homeostasis regulation of our bodies such as protection from dehydration and loss of nutrients. Apart from that, it forms a physical barrier that provide protection against the living organisms around us and also toward protection against potentially harmful physical, chemical and biological hazard (Poumay and Coquette, 2007).

2.2 Burn injury

A burn injury implies damage or destruction of skin and/or its contents by thermal, chemical, electrical or radiation energies or combination. Burns are one of the most problematic traumas encountered in medicine. It can happen to everybody from babies to elderly, it is also a problem that can occur in either developing or developed countries (Hettiaratchy and Dziewulski, 2004). Burn injury can be classified into three stages based on their depth.

First degree burns are superficial and it cause local inflammation of the skin. These types of injury affect only the epidermis and could be due to sunburn (Papini, 2004). The inflammation is characterized by pain, redness, and a mild amount of swelling.

Second degree burns are deeper that is between upper dermis and the epidermis (Papini, 2004) and it has manifestation like first degree but with additional blistering of the skin.

Third degree burns are much deeper compared to second degree as it involves all layers of the skin where all the regenerative elements are being destroyed (Papini, 2004). Blood vessels and nerves are damaged and this cause them to appear white leathery and tend to be relatively painless.

2.3 Skin tissue engineering

According to Ikada (2006), tissue engineering approach for organ and tissue reconstruction has started approximately three decades ago. By definition, tissue engineering is a relationship between engineering and life sciences in order to provide

living substitutes for medical and biologic applications (Rani Samsudin, 2001). The objectives of tissue engineering is to regenerate patient own tissue and organ that is biocompatible, high biofunctionality, and free of immune rejection when applied to the patient. Three most important components in tissue regeneration are the cells, scaffold, and growth factor (Ikada, 2006).

In this study, we will be focusing on the skin substitute that will be used in full thickness burn injury. Full thickness burn injury that involve 30% of total body surface area can be covered with autologous skin but full thickness injury more than 90% will exhaust the autograft donor site. According to Loss *et al.*, (2000) most patient before 1940 with burn covering more than 40% of total body surface area die due to limited availability of autologous donor site. Due to various development and production of biomaterial, this problem can be overcome and rate of survival for full thickness burn injury patient has increased up to 50% including in children (Barret *et al.*, 2000, Walthall *et al.*, 2002a).

Scaffold is one of the important components in tissue-engineering. Among the requirement for a scaffold is that it is reproducible into well defined three-dimensional (3D) structures designed in order to enhance cell attachment and tissue development, high porosity and large pores to provide unrestricted and spatially uniform cell distribution, chemical properties that allow surface modifications in order to promote cell attachment, differentiation and biodegradable at controlled rate for it to achieved long term biocompatibility *in vivo* (Metcalfe and Ferguson, 2006, Vunjak-Novakovic and Radisic, 2003).

In this study, INTEGRA®-DRT is used as the scaffold. INTEGRA®-DRT is an artificial skin consisting of bilayer membrane system. This template consists of a specific three dimensional (3D) porous collagen-glycosaminoglycan (chondroitin-6-sulfate)

matrix that serves as a scaffold for dermal regeneration and covered by temporary epidermal substitute made of thin polysiloxane (silicone) (Loss *et al.*, 2000, Moiemen *et al.*, 2001, Walthall *et al.*, 2002b, Bansky, 2005, Palao *et al.*, 2003). A few characteristics regarding the silicone layer in this template is that it help in immediate wound closure, prevents fluid loss from the wound (Loss *et al.*, 2000), provide mechanical protection, protective barrier against the invasion of bacteria (Palao *et al.*, 2003) and has water vapor permeability similar to our normal skin. Meanwhile, the three dimensional matrix consists of cross-linked collagen and glycosaminoglycan which acts as the extracellular matrix and serves as a template for ingrowth of host fibroblast, macrophage, an endothelial cell synthesizing a "neodermis" (Palao *et al.*, 2003, Bansky, 2005) which then allowed formation of a full thickness epidermis-dermis complex (Jeng *et al.*, 2007). Many studies have been done on INTEGRA®-DRT and the results have shown that this artificial skin is the only treatment in order to restore the loss of function of dermal layer. Despite all it function and advantage, INTEGRA®-DRT also has its disadvantage. According to a study, INTEGRA®-DRT require healthy and non-infected wound base and for severe burn treatment, autograft is needed only after 3-4 weeks for epithelial cover (Enoch *et al.*, 2006). Another researcher stated that the use of INTEGRA®-DRT involve two stages that is the application of the INTEGRA®-DRT to resurfaced the wound site and the second stage that is the removal of the silicone layer and application of split skin autograft (Ghattaura and Potokar, 2009).

Autograft that are used for this stages are the keratinocyte. It is the first epidermal normal cell type that has been grown successfully in culture. Keratinocyte are culture in a medium containing their growth factor which will help to facilitate tissue regeneration (Ikada, 2006). The culture of keratinocyte has been studied by Rheinwald and Green on teratogenic cell line (Rheinwald and Green, 1975, Inoue *et al.*, 2006, Hancock and Leigh,

1989). Through this study, Rheinwald and Green (1975) have found that condition in culturing those cell line mimics the epidermal keratinisation and from there, normal human keratinocyte were grown easily and often used for grafting patient with extensive burn (Barret *et al.*, 2000).

2.4 Fibrin glue

In 1909, fibrin was identified to have an effect in treating wounds. It has been proved that the use of fibrin as skin graft can promote cell adherence and hemostasis by reducing hemorrhage on burn excision. The combination of fibrin and fibronectin can support the growth and proliferation of keratinocyte and fibroblast (Currie *et al.*, 2001). The hemostasis process also is favored by the presence of aprotinin in the fibrin glue which play an important role in preventing fibrinolysis of the fibrin polymer by inhibiting the action of plasmin. This also on the other hand helps to maintain the structural integrity of the matrix form (Ronfard *et al.*, 1991).

2.5 Laser Scanning Confocal Microscope (LSCM)

Confocal microscope was introduced by Marvin Minsky in 1950s. This microscope is capable of revealing single molecule, multiple target molecules simultaneously by using different probes and able to distinguish between live and dead cells. Apart from that, LSCM is also capable to control depth of field, reduction of background information away from the focal plane and collect serial optical sections from thick section meaning that it can give three dimensional view from the specimen (Claxton *et al.*, 2008).

CHAPTER 3

MATERIALS & METHODOLOGY

The first step in the methodology is to understand the research topic. In order to ensure this project is successful, a few aspect and criteria has been taken into consideration. Studies and discussion has been done to choose the best possible steps to gathering information about the project, analyse the data, produce the model simulation and also how the report has to be presented. Appendices 3.1 show the flow chart of methodology for this project.

3.1 Materials & Equipments

3.1.1 Skin sample

For this study, sample was obtained from donor skin biopsy with their consent. This procedure was performed by licensed medical surgeon in Operation Theater (OT) of HUSM.

3.1.2 Biomaterials

INTEGRA®-DRT (Integra Life Science Corporation) and fibrin glue (Tisseel®).

3.1.3 Reagents

The reagents used in this study are Dulbecco's Phosphate Buffer Saline (DPBS) (GIBCO®), 70% denatured ethanol, dispase (GIBCO®), coating matrix (Cascade Biologics™), Accutase (Innovative Cell Technologies), TrypLE™ Express (GIBCO®), progenitor cell target epidermal keratinocyte medium CnT-07(PCTEK medium) (CELLnTEC), 1.2 mM calcium chloride ($CaCl_2$), 0.2% trypsin-EDTA, Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO®), fetal bovine serum (FBS) (GIBCO®), trypan blue, live-dead cell staining kit (cat no: MP 03224) (Invitrogen™), fibrin glue (Tisseel®), and fluorescent mounting medium (Dako)

3.1.3.1 Preparation of elevated $CaCl_2$ (1.2 mM) concentration into medium.

According to CELLnTEC, the manufacturer of PCTEK Culture Medium, the concentration of $CaCl_2$ in CnT-07 is 0.7 mM. In order to have 1.2 mM $CaCl_2$, 0.5 mM of $CaCl_2$ stock solution were prepared. In order to do that, 0.056 g of $CaCl_2$ is weighed and mixed with 500 µL of PBS in a centrifuge tube. Mixture is then mixed using vortex and filtered into a new centrifuge tube. Next, 10 mL of PCT is pipetted into a 50 mL centrifuge tube, followed by 5 µL of the stock solution.

3.1.4 Equipments

The equipments used in this study are 70 µm cell strainer (BD Falcon™), centrifuge (Eppendorf 5810 R), 50 ml centrifuge tube (nunc™), T/ 75 flask (nunc™),

biohazard safety cabinet, blade, petri dish, LSCM (Leica MICROSYSTEMS), haemocytometer, forceps, and scalpel.

3.2 Methodology

3.2.1 Skin preparation

Skin sample was placed in sterile DPBS at 4 °C immediately after circumcision for safe transportation. The cutaneous fat layer was then removed by using blade if necessary. Next, skin was incubated at room temperature for 2 minutes in 70% denatured ethanol solution followed by rinsing 3 times in sterile PBS. Skin sample is then cut by using blade into small pieces at approximately 3 X 3 mm before incubating in dispase solution at 4 °C for overnight.

3.2.2 Keratinocyte cell culture

This experiment was performed according to the methods described by Lim *et al.*, (2007).

3.2.2.1 Isolation of human epidermal keratinocyte

After overnight incubation in dispase, skin piece was placed in a sterile petri dish containing PCTEK medium supplemented with growth factors in order to prevent drying. The epidermis layers were then be detached or separated from dermis by using sterile forceps with the skin and epidermis remain submerged in PCTEK medium. Separated

epidermis layer later was incubated in 5 mL 0.25% trypsin-EDTA solution for 15 min at 37 °C. Suspension is agitated every 5 min to increase the amount of basal keratinocytes dissociation. Ten mL of DMEM containing FBS was then added to the suspension. Suspension was then filtered through cell strainer before centrifuged for 5 min at 800 rpm. After centrifugation, pellet is re-suspended with the growth medium. Next, keratinocyte were counted on haemocytometer using Trypan blue solution in order to determine the cell viability. A 2 x 10^5 viable cells/ ml will then be seeded in 75 cm^2 culture flask containing 15 mL PCTEK medium. For every 2 to 3 days, culture medium was changed in order to promote the growth of the cell.

3.2.2.2 Subcultivation of human epidermal keratinocyte

A highly proliferative keratinocyte clone (70% of confluence) was rinsed twice with sterile PBS and incubates with 0.25% trypsin-EDTA solution at 37 °C for 5 min. Once the cells get dislodged from the flask, DMEM containing FBS was added and centrifuged at 800 rpm for 5 min. Pelleted cells was then re-suspended in fresh PCTEK medium at split ratio 1: 3 or 6 X 10^4 viable cells/ ml. The medium later was changed for every 2 days.

3.2.3 Preparation of fibrin

Briefly, fibrin glue powdered (TISSEEL®) was diluted with 1 ml of aprotinin. Solution was then subjected into two fold dilution by adding 3 mL of dH_2O. Next, Thrombin 4 U was mixed with 1 mL of $CaCl_2$ (4 μM/ mL) before being further diluted two times by the additional of 3 mL of $CaCl_2$ (4 μM/ mL). Diluted fibrin glue and

thrombin was then mix together in 1:1 ratio by pipetting 250 μL of fibrin and 250 μL of thrombin into the well. Mixture was then left for a few minutes until it is set to be used.

3.2.4 Differentiation of highly proliferative normal human epidermal keratinocyte (NHEK) within INTEGRA®-DRT and fibrin.

NHEK was harvested from flask T-75, once achieved approximately 70-80% of cell confluence is achieved. INTEGRA®-DRT was then placed into a 96-well plate prior to cell seeding. Cell seeding density was adjusted accordingly at 2.0 X 10^5 viable cells/ mL and seeded within INTEGRA®-DRT 3D structure and fibrin by micropipette technique. The keratinocytes will be differentiated into a cellular multilayer (3-dimensional reconstructed epidermis) by implementing elevated $CaCl_2$ concentration (1.2 mM) in the culture medium. Control in this study consists of INTEGRA®-DRT and fibrin scaffold that is without cell and cell seeded on flask.

3.2.5 Preparation of live/dead cell staining dye.

This preparation was conducted according to the protocols stated in the instruction manual of Invitrogen Live/ Dead® Viability/ Cytotoxicity kit for mammalian cells. Briefly, in order to prepare 4 μM assay solution, 4 μL from 2 mM of Ethidium homodimer-1 in Dimethyl sulfoxide (DMSO)/ H_2O was pipette into 2 mL of PBS in a test tube. Later, 1 μL from 4 mM of Calcein AM in anhydrous DMSO was pipette into the test tube. Solution was then mixes by using vortex or shake using hand. Test tube was then covered with aluminium foil in order to protect the dye from light.

3.2.6 Assessment of NHEK growth and invading pattern within INTEGRA®-DRT and fibrin *via* Live-dead cell staining.

Culture growth medium for the seeded material was first removed by using pipette. Next, the material was rinsed twice using PBS. After rinsing, 100 µL of dye were loaded into the well by using pipette. After the well is filled with dye, the plate was incubated for 30 minute at 37 °C in a dark compartment. Later, the dye was removed from the well before washed out two times with PBS. Finally, seeded materials were transferred onto a clean slide, mounted using anti mounted medium and covered with covered slip. Slide was then observed under LSCM.

3.2.7 Statistical analysis

In this study, both scaffolds were scanned at approximately 80 µm from the entire material using LSCM. From there, each 10 µm layer were analyzed in order to determine the viability of the cells within the material using this formula:

$$\frac{\text{Number of live cells}}{\text{Total number of cells}} \times 100\,\% =$$

From the cell viability obtained from each observation, statistical analysis was conducted to identify if there was a significant differenced in cell viability between each layers and materials from each hours of observation. The statistical analysis was conducted using the Student's t test with value of $p < 0.05$ was regarded to be statistically significant.

CHAPTER 4

RESULTS

4.1 pNHEK culture

In this study, the culture of keratinocyte was performed through modified Rheinwald and Green method. By this method we have able to successfully culture the keratinocyte from human skin. The cell takes about 1 week to reach 80 – 90% of confluence in this primary culture (Figure 4.1).

After the cells reached confluence, it is trypsinized in order to detach the cell from the flask (Figure 4.1 (D)) so that we can proceed to the process of subculturing the keratinocyte.

4.2 Cell seeding on INTEGRA®-DRT and fibrin scaffold

In this section, once the cells have reached 80 – 90% of confluence on passage 1, they were trypsinized and seeded onto the INTEGRA®-DRT (Figure 4.2) and fibrin. On the next day of culture, plate was check for the proliferation of keratinocyte followed by assessment using LSCM. The next observation was conducted on the next three and six day.

LSCM was used in this these study for the purpose of generating three dimensional (3D) view for the keratinocyte depth of penetration within the scaffold. LSCM was also capable in distinguishing between live and dead cells within the scaffold.

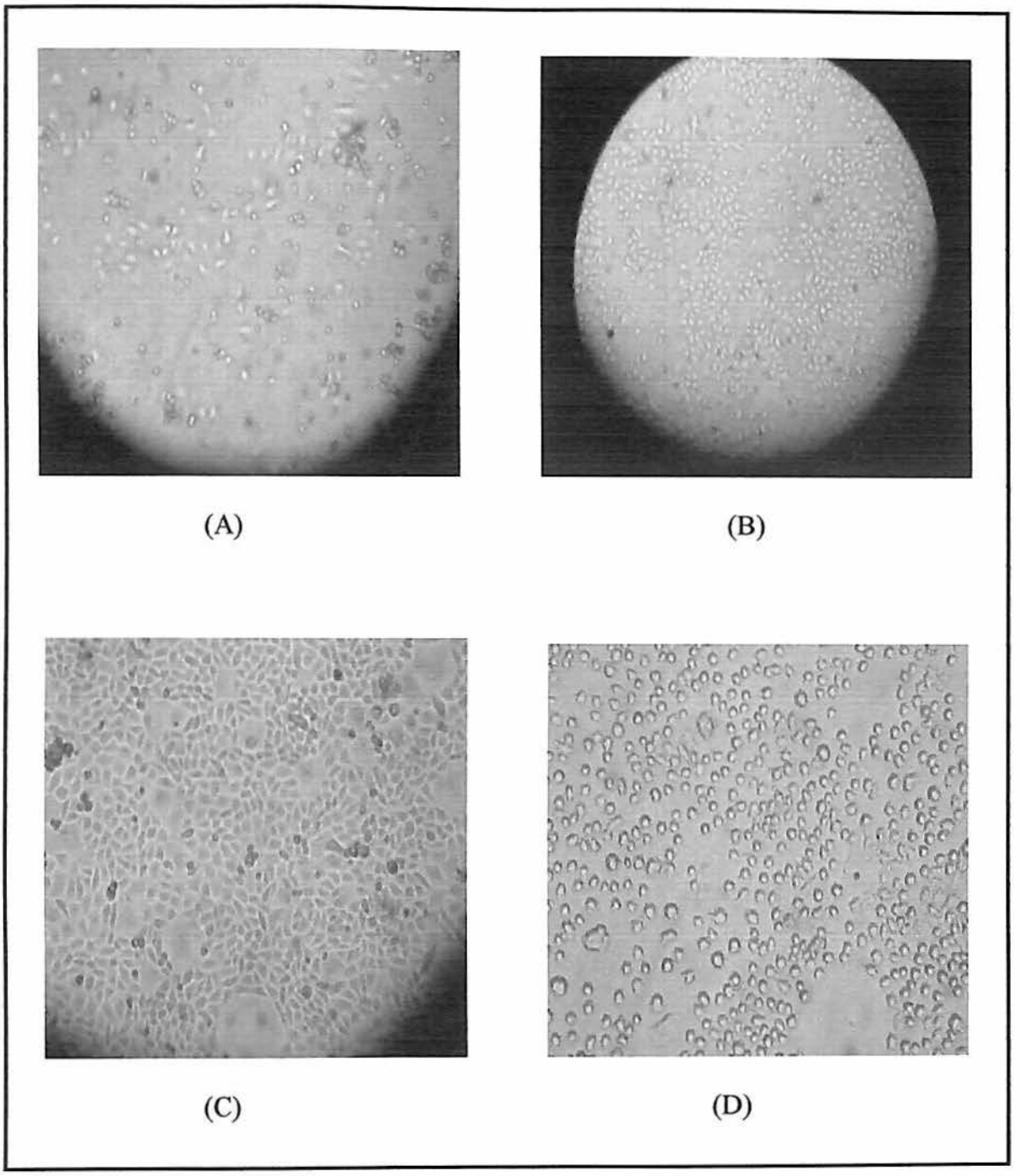


Figure 4.1: Keratinocyte growth in primary culture.

A) Day 2 after primary culture

B) Day 3 after primary culture

C) Day 7 after primary culture

D) After trypsinization

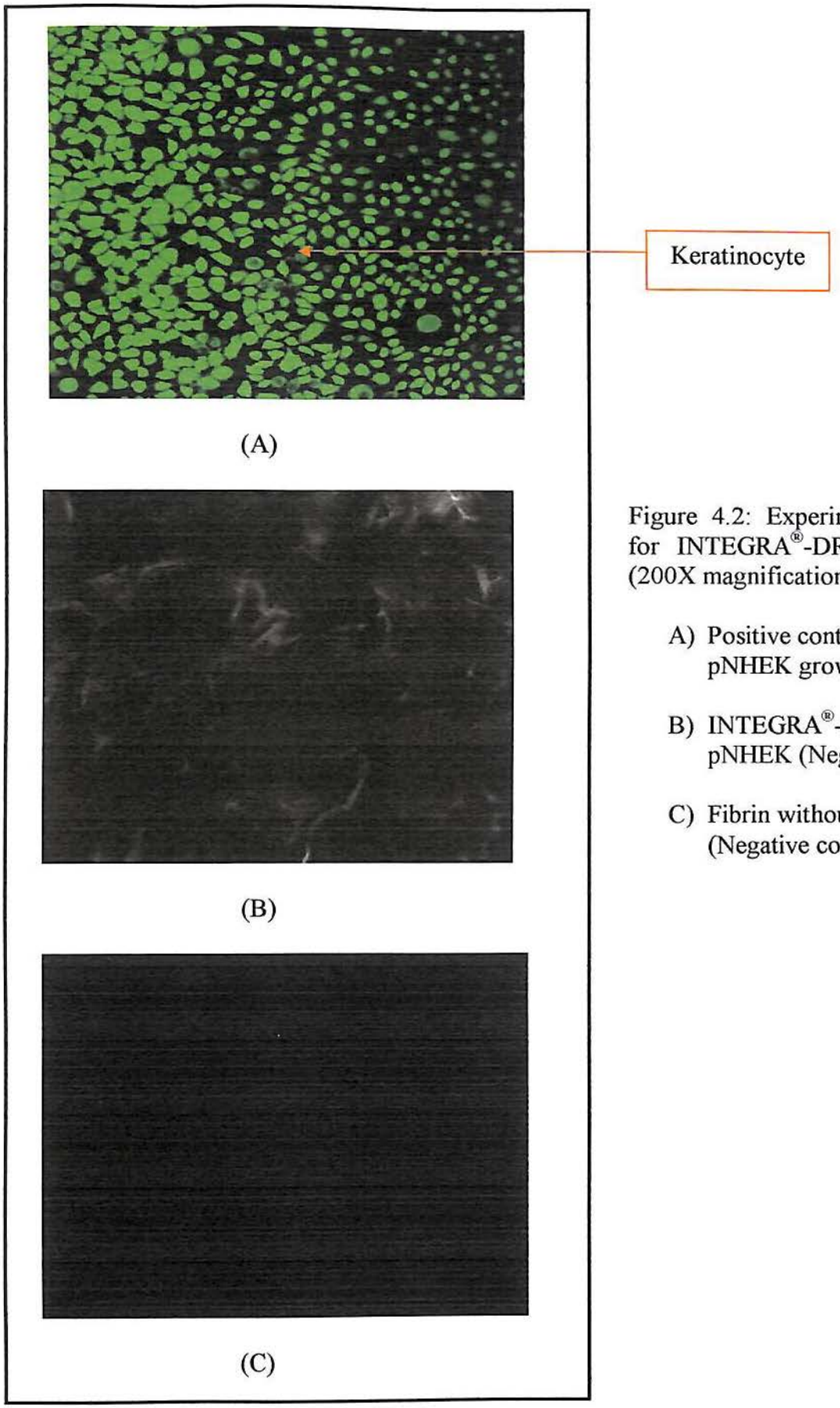
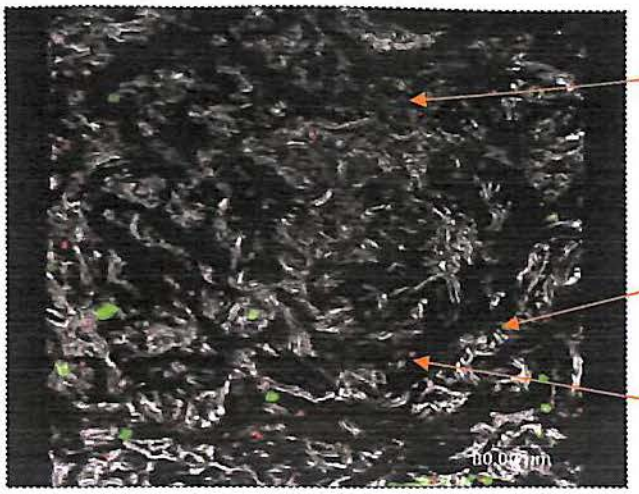


Figure 4.2: Experimental control for INTEGRA®-DRT and fibrin (200X magnification).

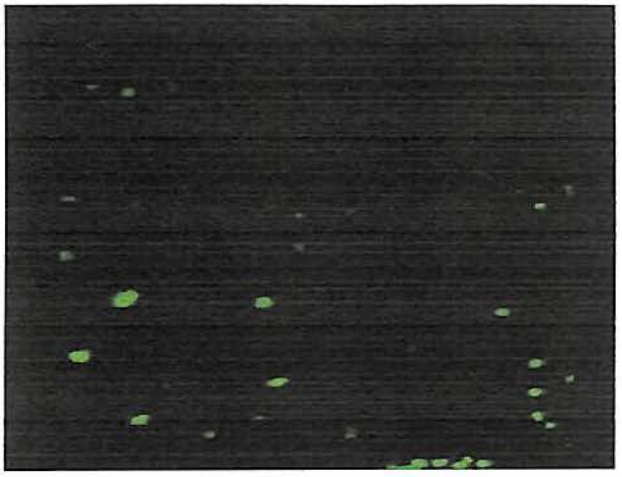
A) Positive control for pNHEK grown on plate

B) INTEGRA®-DRT without pNHEK (Negative control)

C) Fibrin without pNHEK (Negative control)



(A)



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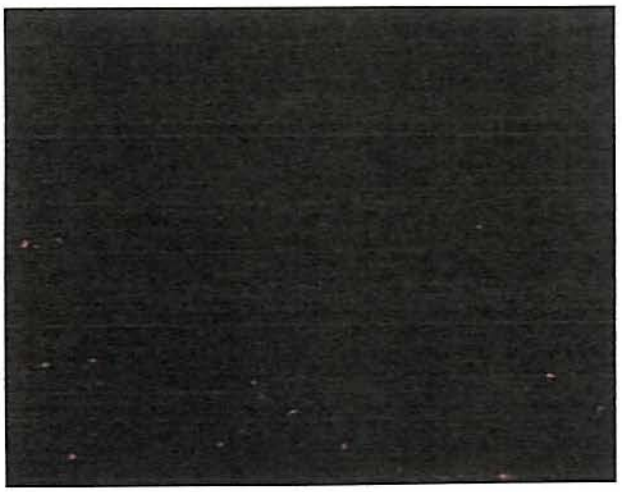

(C)

Figure 4.3: 24-hour post-seeding on INTEGRA®-DRT (200X magnification).

A) Overlay image of Live/Dead stain

B) Image of live pNHEK

C) Image of dead pNHEK

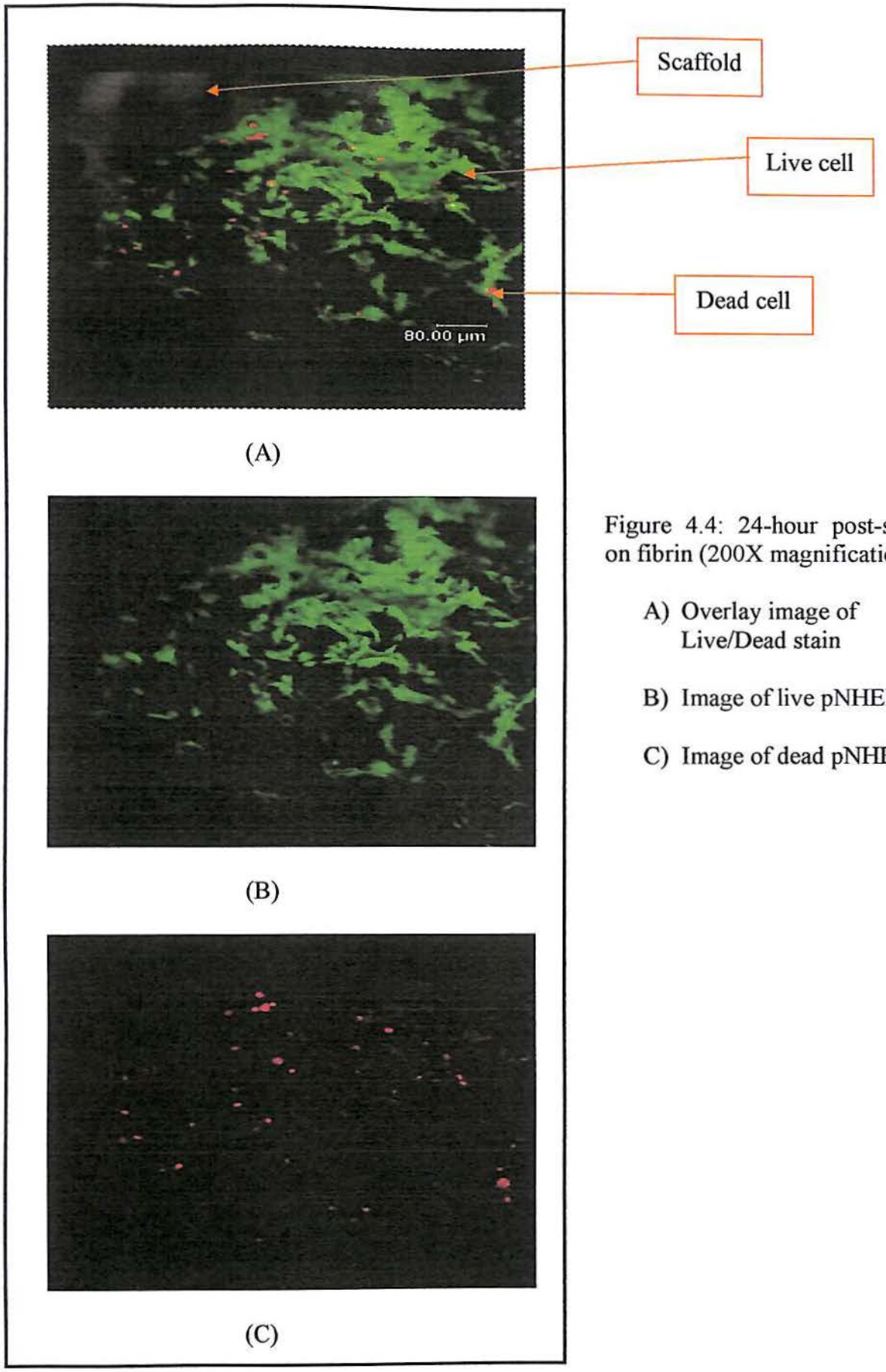

Figure 4.4: 24-hour post-seeding on fibrin (200X magnification).

A) Overlay image of Live/Dead stain

B) Image of live pNHEK

C) Image of dead pNHEK

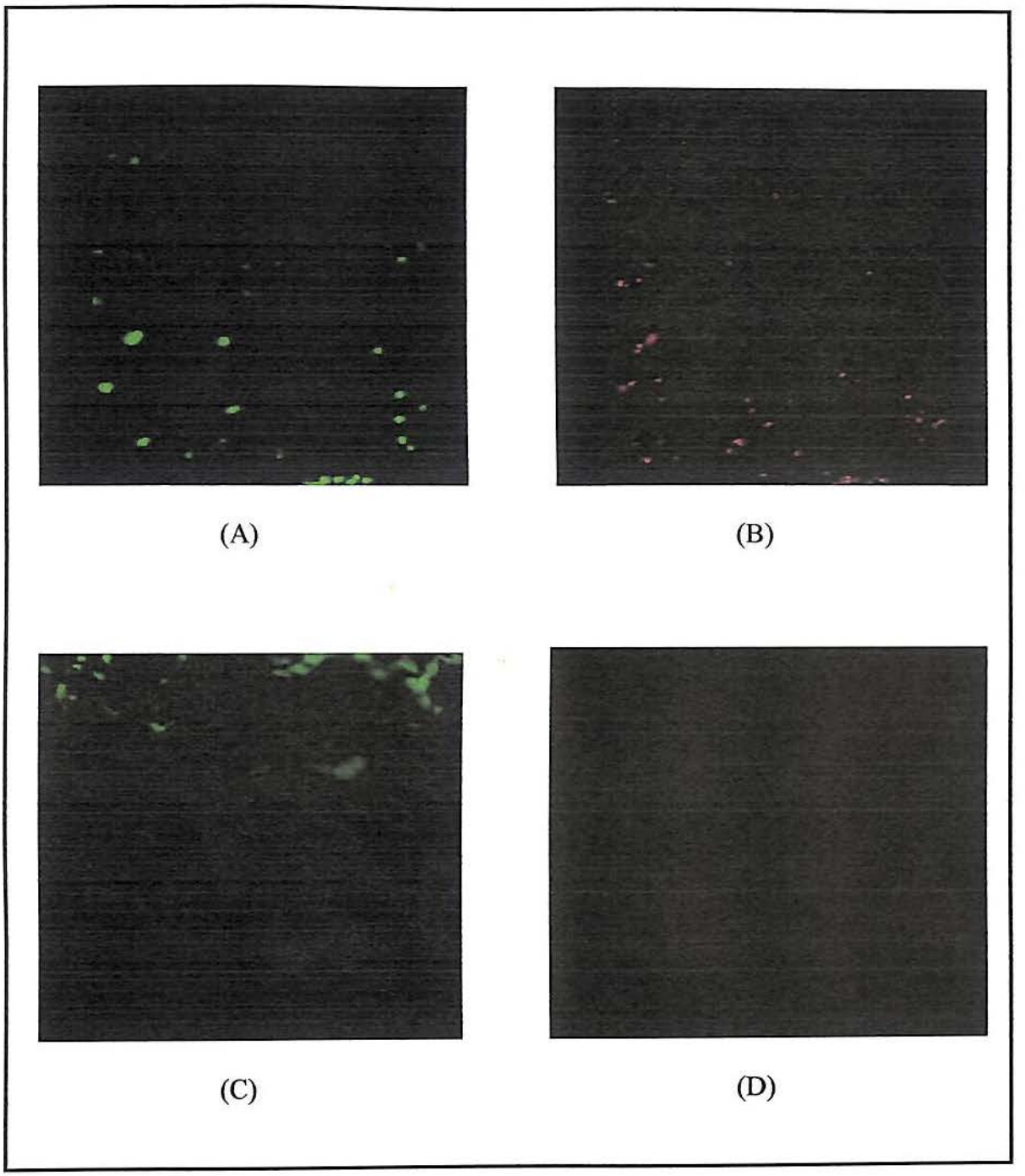

Figure 4.5: 10 μm from top of material 24-hour post-seeding on INTEGRA®-DRT (A & B) and fibrin (C & D) at 200X magnification.

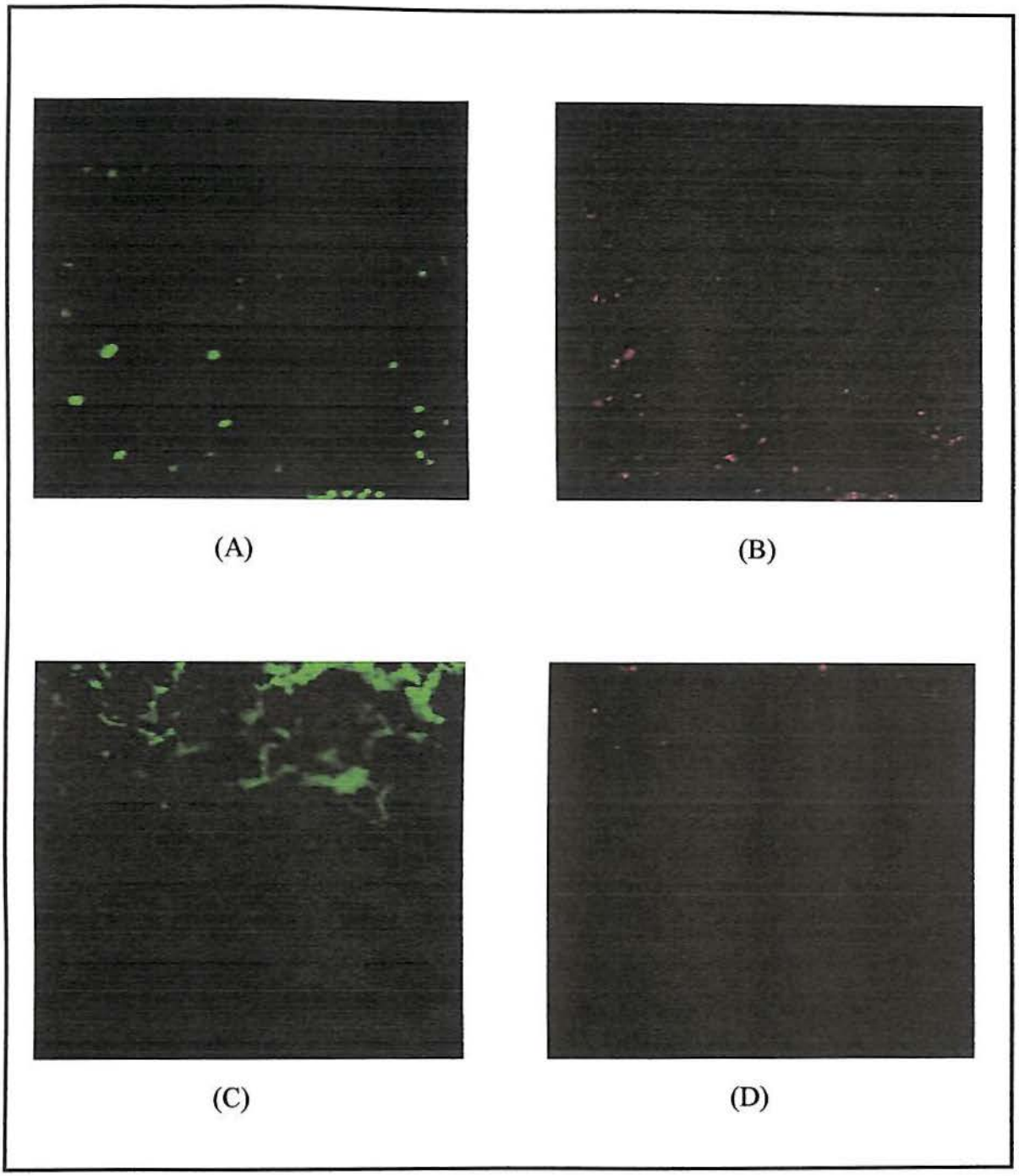

Figure 4.6: 20 μm from top of material 24-hour post-seeding on INTEGRA®-DRT (A & B) and fibrin (C & D) at 200X magnification.

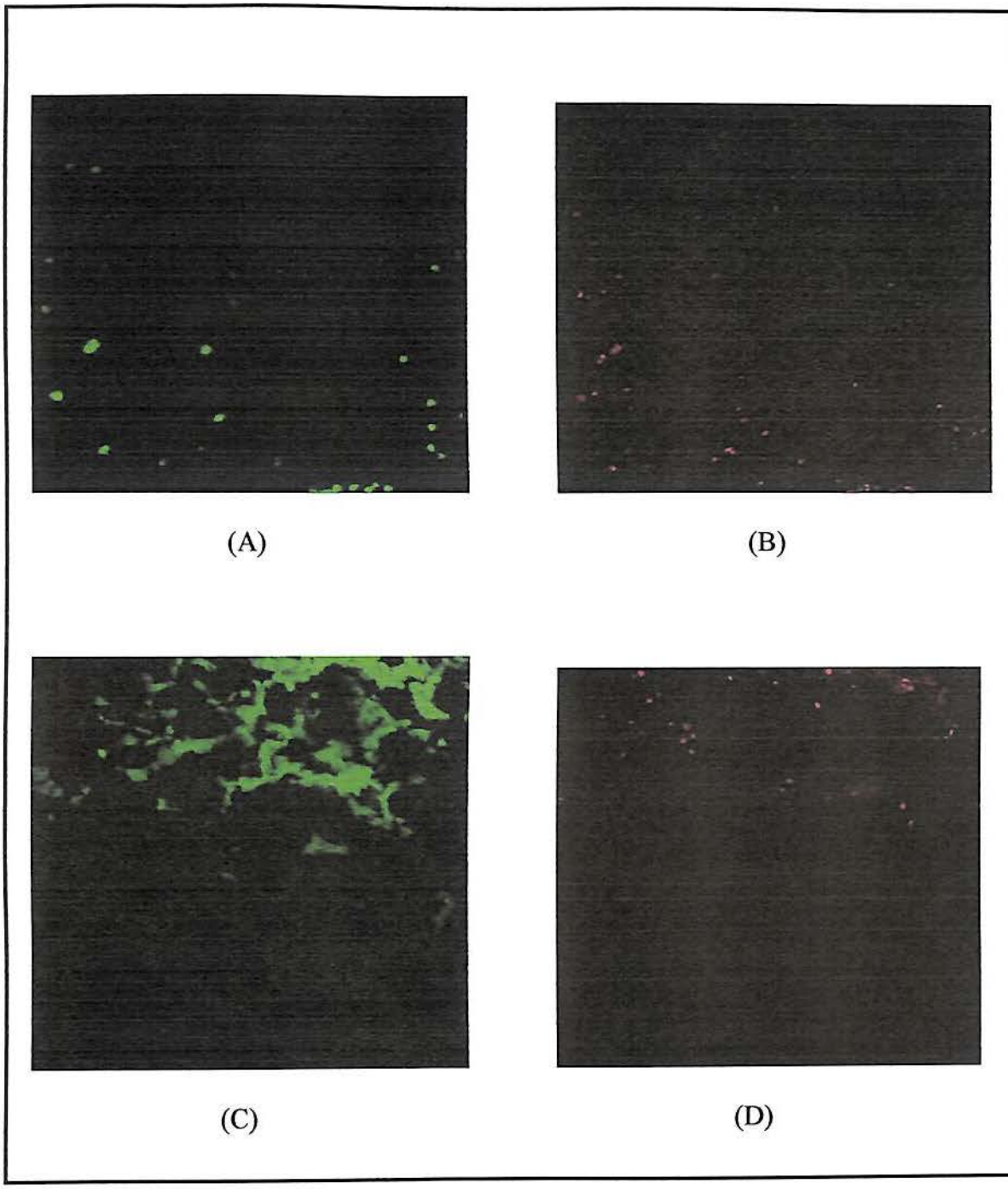

Figure 4.7: 30 μm from top of material 24-hour post-seeding on INTEGRA®-DRT (A & B) and fibrin (C & D) at 200X magnification.

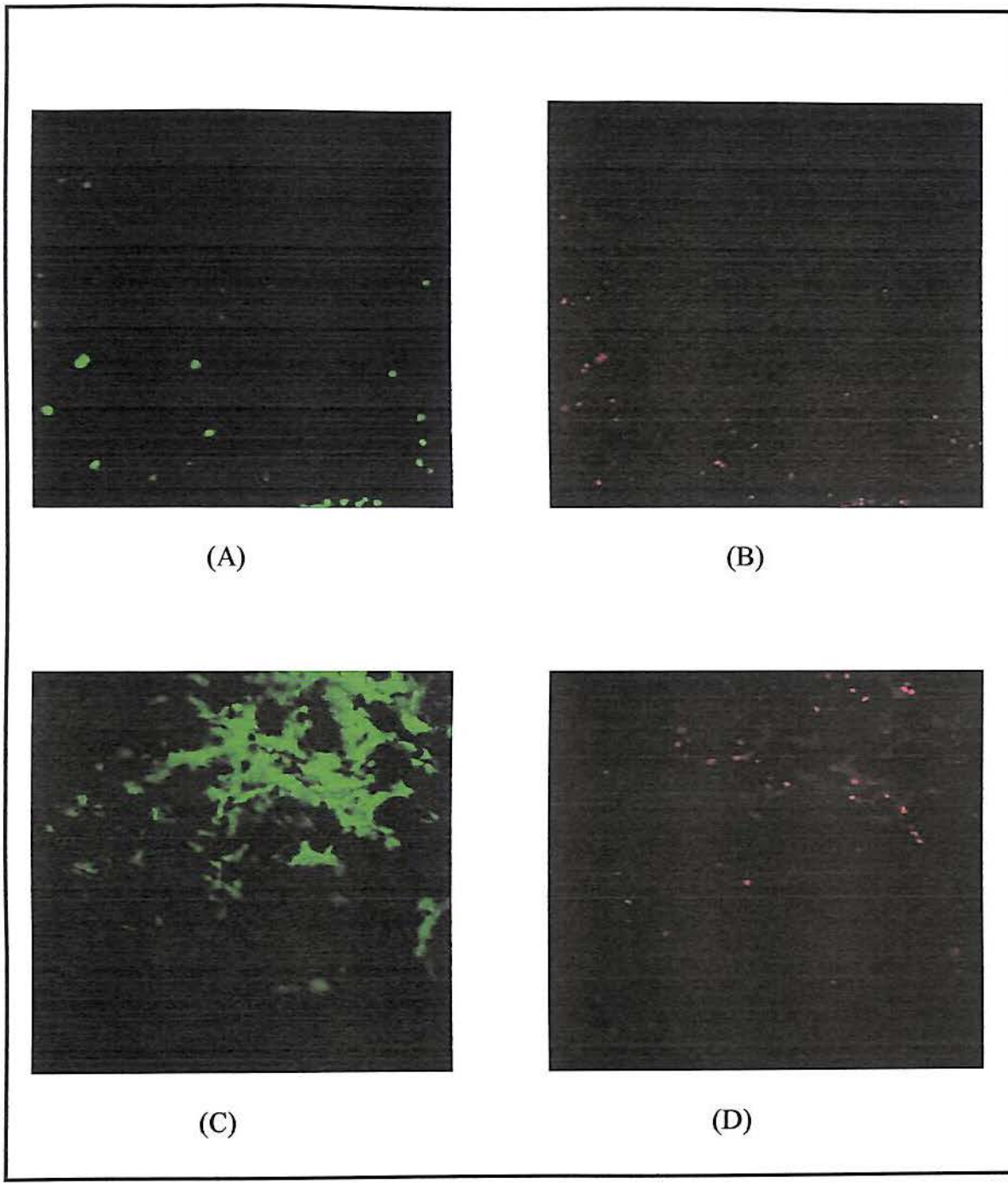

Figure 4.8: 40 μm from top of material 24-hour post-seeding on INTEGRA®-DRT (A & B) and fibrin (C & D) at 200X magnification.