Process Optimization for the *in vitro* Growth and Maintenance of Human Epidermal Keratinocytes

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

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Declaration

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "**Process Optimization for the *in vitro* Growth and Maintenance of Human Epidermal Keratinocytes"** submitted by the undersigned candidate in partial fulfillments of the requirements for the degree of Master of Science in Biotechnology under the department of Mathematics and Natural Sciences (MNS), BRAC University, Mohakhali, Dhaka, was carried out under the joint supervision of Professor Dr. Naiyyum Choudhury, Coordinator of Biotechnology Program, MNS Department, BRAC University and Dr. S. M. Asaduzzaman, Principal Scientific Officer and Director, Tissue Banking and Biomaterial Research Unit, Atomic Energy Research Establishment. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Abstract

In the past few decades, the *in vitro* cultivation of human epidermal keratinocytes has significantly been improved owing to several developments in terms of media, growth factors and overall culture conditions. This improvement has exhibited great applicability in the treatment of burn and ulcer patients as allografts and autografts, along with contributing in pharmacological tests, skin disorder study, and most recently, in induced pluripotent stem cell (iPSC) technology. However, human cell culture has not yet been advanced enough in Bangladesh, which urges extensive research here. The present study was therefore, aimed to establish an optimum culture condition for human epidermal keratinocytes (HEK). For this purpose, cell dissociation culture of HEK was comparatively analyzed with explant culture and the effects of substrate, donor age, serum concentration and growth factors on keratinocyte culture were evaluated. For cell dissociation culture, epidermal layer from human foreskin after circumcision was separated through cold trypsinization, which yielded 2.5×10^6 cells on an average from each foreskin with cell viability up to 90%. Compared to tissue culture plastic flasks and medium-conditioned flasks, gelatin-coated plate showed highest number of cell attachment (35-50%). The proliferation rate of keratinocytes from skin of newborn and infant (<3 years old) was found greater than that of middle childhood and teenaged donors (3-15 years old). Serum concentrations of 10-15% yielded 2-4 fold more cell proliferation than lower or higher levels. Additional insulin supplementation at 5 µg/ml gave better cell growth than serum (10%) or hydrocortisone supplement (0.4 µg/ml) alone. However, the most significant cell growth was obtained in serum containing medium supplemented with both insulin (5 µg/ml) and hydrocortisone (0.4 µg/ml). In context of cell growth, plating efficiency and development of confluent culture, cell dissociation method was found superior than that of explant culture. These findings will be helpful to the progress of optimizing human keratinocyte culture that may contribute in future application to diminish the pain of burn patients as well and can further help working with cell biology in our country.

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1. INTRODUCTION

1.1. BACKGROUND

Epidermis

Human skin is composed of three primary layers: epidermis, "epi" coming from the Greek meaning "over" or "upon", which is the outermost layer of skin and consists of stratified squamous epithelial tissue; along with dermis and hypodermis which are made up of collagenous tissue and fatty subcutaneous tissue, respectively (Fig. 1). Epidermis is a thin nonvascular layer and is nourished by diffusion from the dermis, which is separated from the epidermis by a basement membrane. Epidermis consists mainly of keratinocytes (95%) but also contains melanocytes, Langerhans cells, Merkel cells, and inflammatory cells.

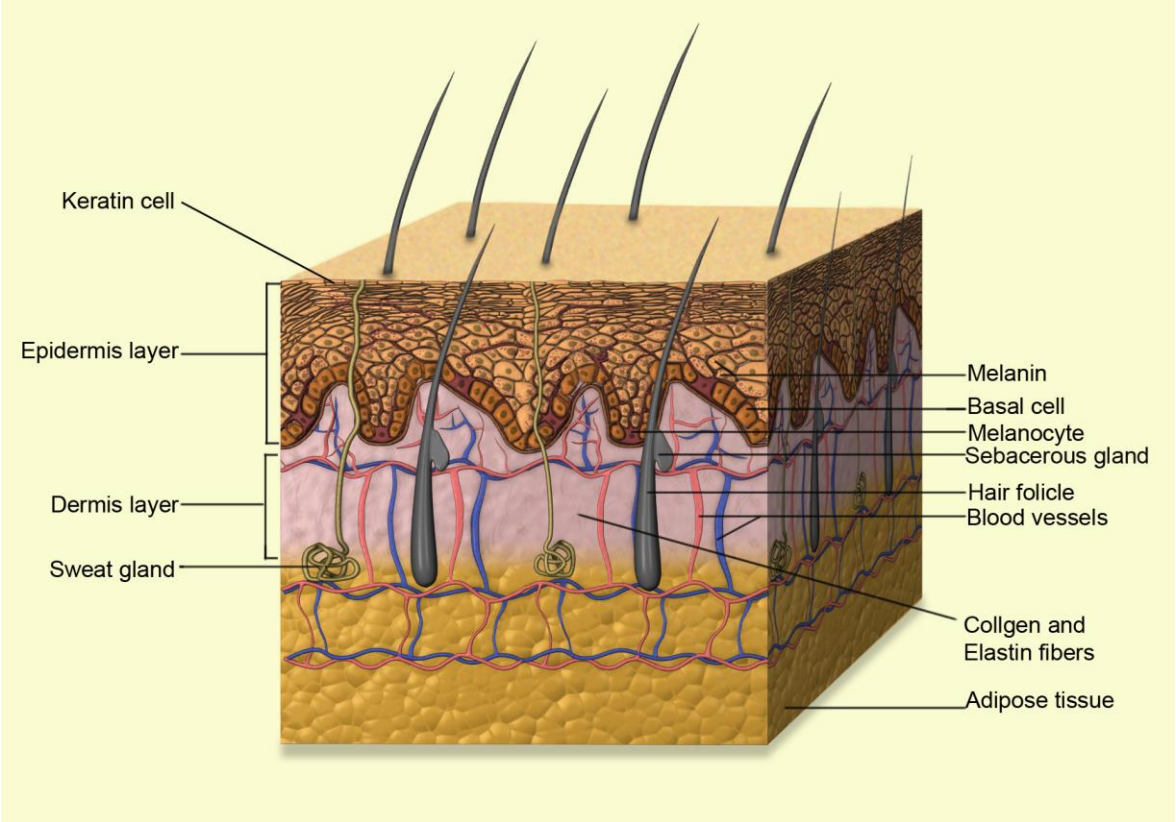

Figure 1: Schematic diagram of human skin.

Epidermis is divided into several layers or strata (Stratum corneum, Stratum lucidum, Stratum granulosum, Stratum spinosum, and Stratum germinativum or Stratum basale) where cells are formed through mitosis at the innermost layers and move up the strata changing shape and composition as they differentiate and become filled with keratin (Fig. 2).

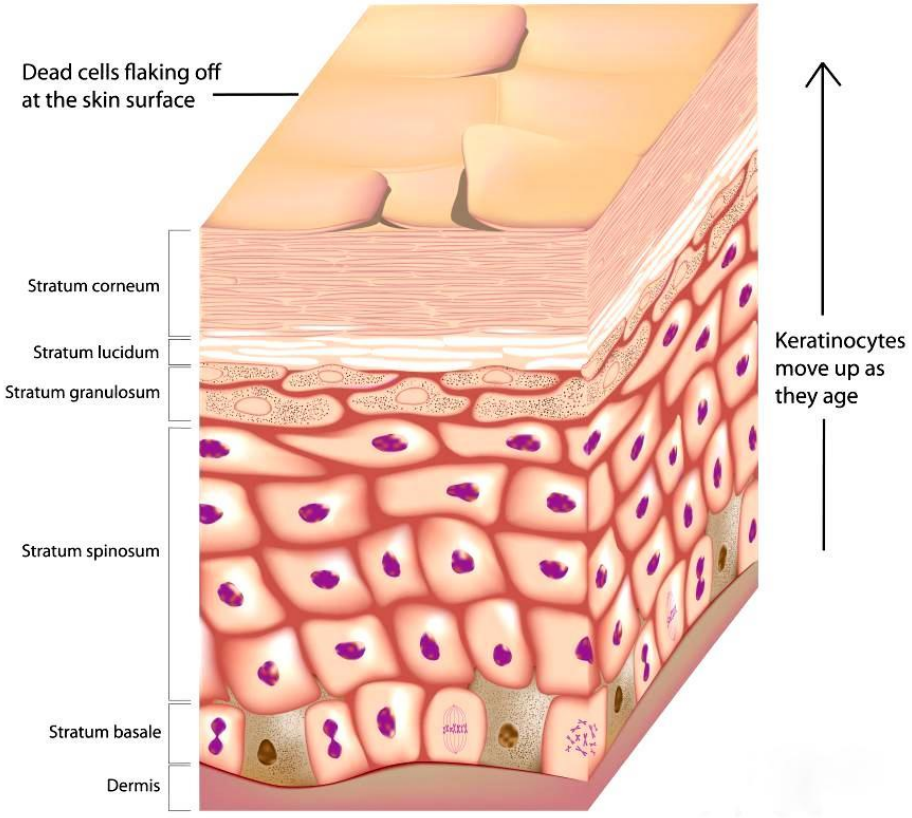

Figure 2: Layers of epidermis.

Keratinocyte

Keratinocytes, the predominant cell type in the epidermis, form an effective barrier to the entry of protein antigens, chemical irritants, and infectious agents into the body, all while resisting environmental stress, external pressure, and sheer force (Fuchs, 1995). V. Lulevich *et al*. (2010) described that a typical keratinocyte has a relatively symmetrical shape with a 12 µm nucleus in the center. Immunofluorescent staining revealed that high-density keratin filament networks are homogeneously distributed in the cytosol, while actin filaments are mostly concentrated at the membrane periphery (Fig. 3) (Lulevich *et al*., 2010).

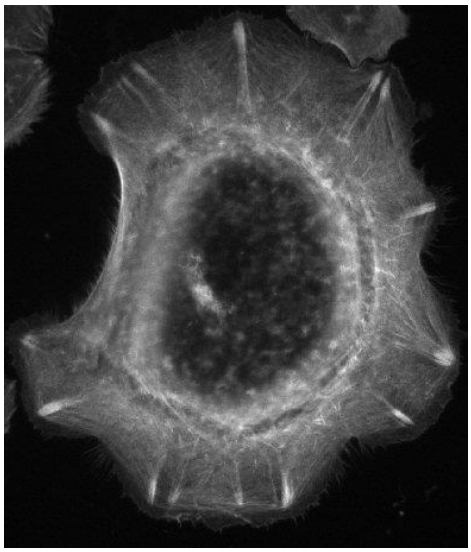

Figure 3: Structure of a typical keratinocyte.

Human epidermal keratinocytes undergo continuous and rapid proliferation and are thought to be continually regenerated from a pool of multipotent stem cells (Fuchs, 2007) which reside in the lower part of the epidermis (stratum basale) and are attached to the basement membrane through hemidesmosomes. These stem cells divide in a stochastic manner yielding either more stem cells or transit amplifying cells (Houben *et al*., 2007) (Fig. 4). Some of the transit amplifying cells continue to proliferate then commit to differentiate and migrate upward to stratum spinosum where keratinization begins (Gilbert, 2000). The polyhedral keratinocytes in this layer have large pale-staining nuclei as they are active in synthesizing fibrilar proteins, known as cytokeratin, which build up within the cells aggregating together forming tonofibrils. The tonofibrils go on to form the desmosomes, which allow for strong connections to form between adjacent keratinocytes. Moving further to upper layer, stratum granulosum, keratinocytes lose their nuclei and their cytoplasm appears granular as they contain keratohyalin granules, which are filled with histidine- and cysteine-rich proteins that appear to bind the keratin filaments together. Lipids, contained into those keratinocytes within lamellar bodies, are released into the extracellular space through exocytosis to form a lipid barrier. Those polar lipids are then converted into non-polar lipids and arranged parallel to the cell surface. Eventually the keratinocytes reach the top layer called stratum corneum which is composed of 10 to 30 layers of polyhedral, anucleated corneocytes (final step of keratinocyte differentiation) that have completed their differentiation program and have lost their nucleus and cytoplasmic organelles. Corneocytes are surrounded by a protein envelope

(cornified envelope proteins), filled with water-retaining keratin proteins, attached together through corneodesmosomes and surrounded in the extracellular space by stacked layers of lipids, which are eventually shed from the surface (desquamation). In normal skin, the rate of keratinocyte production equals the rate of loss (McGrath *et al*., 2004) taking about two weeks for a cell to journey from the stratum basale to the top of the stratum granulosum, and an additional four weeks to cross the stratum corneum (Marks *et al*., 2006). The entire epidermis is replaced by new cell growth over a period of about 48 days (Lizuka, 1994)

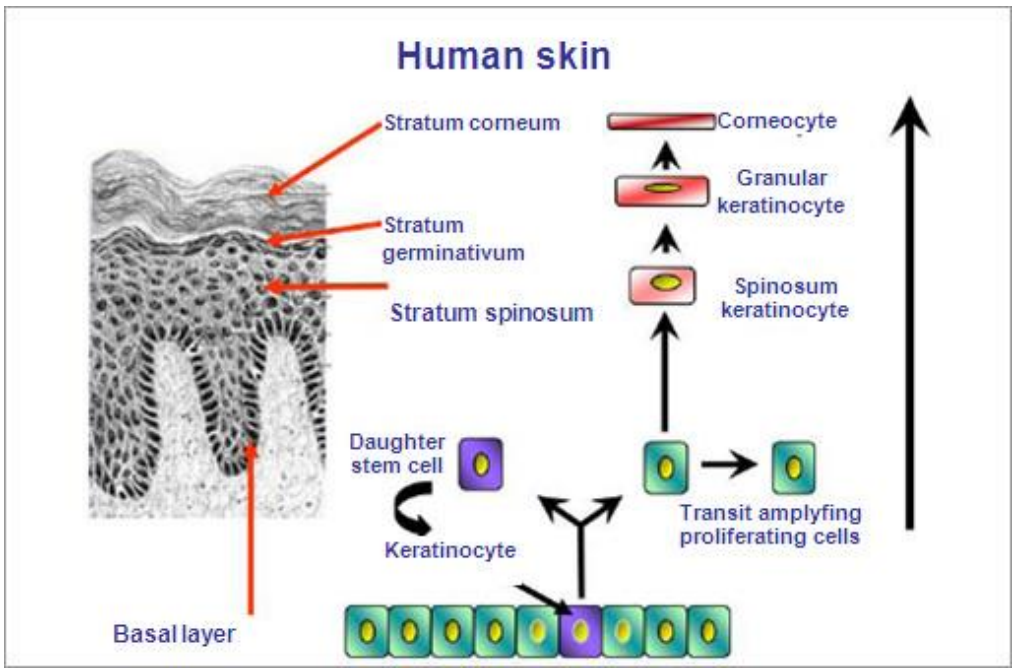

Figure 4: Schematic presentation of life cycle of keratinocyte.

1.2. LITERATURE REVIEW

1.2.1. BASIC TECHNIQUES OF KERATINOCYTE CULTURE

Culturing human skin is among the oldest of tissue culture techniques. The first attempts to culture skin was made in 1898 by Ljunggren (Matoltsy, 1960) who reported that fragments of human skin were able to persist in a living state in ascitic fluid at room temperature for many days and subsequently it was possible to graft these explants to a human patient. Since then several laboratories have reported techniques for in vitro cultivation of human epidermal keratinocytes

either as whole skin explant outgrowths or as dispersed cells after treating skin with a protease (Fig. 5); both strategies have their own advantages and disadvantages.

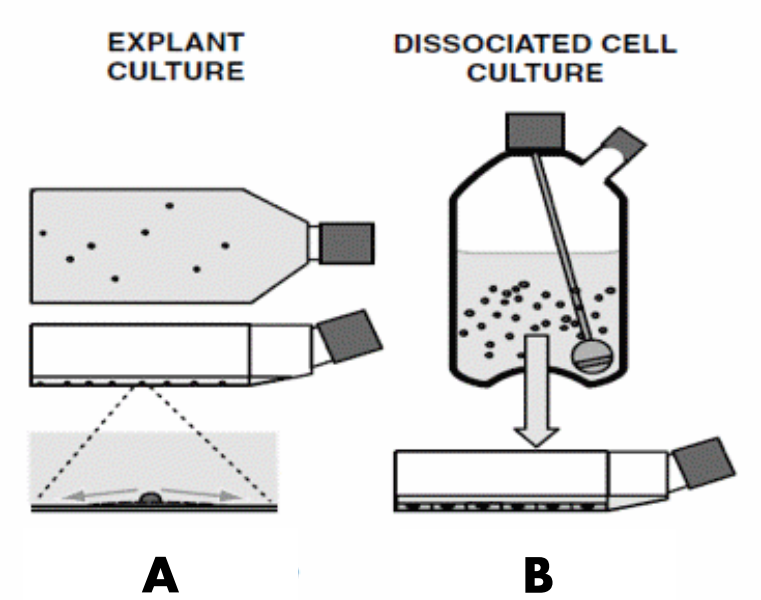

Figure 5: Types of keratinocyte culture; (A) Explant culture; skin tissue explanted on culture flask; cells migrate from tissue to form outgrowth; (B) Dissociated cell culture; disaggregated tissue in suspension; cells form monolayer on culture flask.

a) Explant culture

The culture of cells adopting explant method is one of the simplest procedures, which involves finely chopping the tissue into fragments of no larger than 1-2 mm^3. The tissue fragments (explants) are placed to a glass or treated plastic culture flask and once attached, further media is added to cover the explants completely (Wolf and Ahne, 1982). If successful, cells will migrate out from the explants and cover the growth surface eventually forming a culture.

Cultures of human skin explants have been used from long before to model adult skin epidermal growth and behavior. Bornstein in 1930, and Pinkus in 1932, observed cultures of normal human skin over periods of several weeks and noted migration and growth of true epithelial cells (Everett *et al*, 1951). Parshley and Simms have studied the nutritional requirements of skin explants in tissue culture (Parshley and Simms, 1946). Up to 1950s, skin explants were essentially cultured on plasma clot (Fig. 6), but the duration of culture was short and biochemical analysis was not

possible due to the complexity of the medium. Later the breakthrough of chemically defined medium for cell culture and use of trypsin for subculturing cells grown from explants made conspicuous progress in cell culture technique.

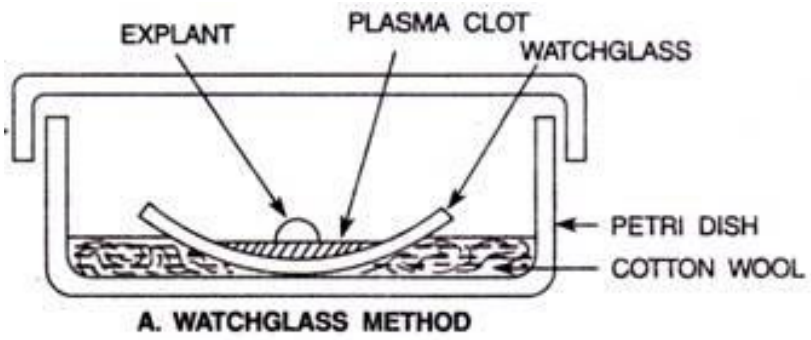

Figure 6: Explant culture on plasma clot

Epithelial cell outgrowth preparations from whole skin explants are suitable for many investigations, such as, drug toxicity (Everett *et al*, 1951; Livingood, 1953), delayed hypersensitivity (Everett, 1952), toxic effects of antibiotics (Funan, 1952), cytological examination (Harnden, 1960), wound healing (Matoltsy, 1955), and keratinization (Matoltsy, 1957; Reinertson, 1961; Pullar, 1964). One of the principal advantages of explant culture is that some aspects of the tissue's architecture can be preserved within the explants and the biochemical functions and hormonal responses as closely as possible to those *in vivo* (Dils, 1994), which makes them more suitable than dissociation cell cultures for physiological studies. Moreover, this often reduces considerably the number of experiments necessary with whole animals to investigate a given problem like skin psoriasis, allergenic reaction etc.

On the other hand, the explant cultures have several disadvantages too: (1) Dermis and dermal cells are intimately associated with epithelial cells in explant cultures, so obtaining pure culture of epithelial cells is difficult. (2) The size of the explanted tissue is sharply limited so that only small quantities of epithelial cells are usually present in the outgrowths. (3) Quantitation of outgrowth cells is difficult and inaccurate. (4) Subculture is difficult. (5) The outgrowth of epidermal cells varies in thickness. Close to the explant, 5 or more layers of cells can be seen on vertical sections (Prunieras *et al*., 1976), whereas only a single one-cell-thick layer is visible at the edge of the outgrowing sheet. Because of these variations in thickness, accurate count of mitosis cannot be obtained. (6) The biology of epidermal cells growing out of the explants can only be grossly

evaluated. Since dermal cells and intracellular matrix are introduced into the culture vessel along with epidermal elements, both can release various macromolecules which can strongly affect epidermal cell metabolism *in vitro* as they do *in vivo*.

b) Dissociated cell culture

In dissociation method, suspension of cells is obtained by chopping the tissue or organs into small fragments followed by mechanical disaggregation or treatment with proteolytic enzymes, which may then be cultured as a monolayer on a solid surface, or as a suspension in a culture medium (Unchern, 1999). The most obvious advantage of cell culture and of tissue dissociation in particular is that it makes individual living cells accessible and can be expanded to replicate cultures (Swain *et al*., 2014), making it possible to overcome the shortcomings existing in explant cultures.

The most common method for cell isolation in dissociation culture is the enzymatic digestion. Proteolytic enzymes are capable of hydrolyzing peptide bonds in protein (Motyan *et al*., 2013). The purpose is that these enzymes digest connective tissue and the components of the surrounding extracellular matrix facilitating the release of cells from a wide variety of tissues. Several enzymes viz. trypsin, collagenase, dispase, protease, pronase E, elastase, hyaluronidase are available in the market for the detachment of cultured cells, cell dissociation and cell component or membrane-associated protein isolation (Unchern, 1999). Among these, trypsin is the most suitable and commonly used enzyme which, on the basis of temperature, can be applied in two ways, either warm trypsinization or cold trypsinization. Warm trypsinization is comparatively faster which involves treatment of tissue with trypsin at 37^0C for 3-5 hours. Cells which are dissociated should be collected after every half hour, enabling minimum exposure of cells to warm enzyme (Pathak, 2007), because cell surface proteins can be cleaved by over-trypsinization which leads to dysregulation of the cell functions. In contrast, cold trypsinization implicates overnight tissue digestion at 4^0C which may be more beneficial than warm trypsinization in the sense that cell damage is likely to be less at low temperature, resulting in higher yields. Other enzymes like collagenase and dispase digest tissue less aggressively than trypsin (Shenoy, 2007), but require very long time to disperse tissue.

The technique of dissociated cell culture for human epidermal cells has a timeworn history. As Medawar in 1941 successfully separated a pure epidermal sheet from human skin by trypsinization, it has been possible to readily obtain dissociated epidermal cells for expansion in tissue culture. In the subsequent years several attempts were taken to culture disaggregated epidermal keratinocytes as monolayers (Cruickshank *et al*., 1960; Briggaman *et al*., 1967; Yuspa *et al*., 1970; Karasek and Charlton, 1971; Fusenig, 1971; Fusenig and Worst, 1974). But these studies showed that the cultures grew only to a limited extent and could not be satisfactorily subcultured. Then a cutting-edge technique was developed by Rheinwald and Green (1975) who used lethally irradiated mouse 3T3 cells as feeder layer to support long-term clonal growth (20-50 cell generations) of keratinocytes. This 3T3 feeder technique permitted the isolation of pure keratinocyte clones uncontaminated with viable fibroblasts and has been widely used to study keratinocyte biology. In this method, however, the activation of 3T3 cells must be kept under control, and there is a risk of contamination of human cells with mouse retroviruses or mouse genetic material**.** Moreover, the presence of living cells from another species (the feeder layer) make this model somewhat complicated for certain pharmacological and biological experiments, such as *in vitro* carcinogenesis. The complications in using 3T3 feeder layer was omitted by Boyce and Ham (1983), who adopted a serum-free medium for primary keratinocyte culture, in which the 3T3 feeder layer is no longer needed and therefore has benefits for use in clinical applications.

1.2.2. FACTORS AFFECTING KERATINOCYTE CULTURE

Mammalian cells *in vivo* are in a carefully balanced homeostatic environment and most cell types in *in vitro* culture also have stringent requirements as to the range of physical factors (e.g., temperature, pH, humidity, CO_2 etc.) which are for growth and performance. Some factors are crucial enough to influence the cell growth, cell viability and cell metabolism that both increase or decrease from their optimum level seem to be detrimental for cell culture, or even fatal. For best results, therefore, culture systems have to be developed in which physical environment matches the cells' requirements, and in which the changes induced in the environment by cell growth and metabolism can be corrected or compensated to maintain homeostasis.

a) Physical factors

Temperature

Temperature is a factor of overriding importance, having an effect on all aspects of cells' metabolism and protein synthesis and degradation. Therefore, the observed parameters in cell culture like proliferation, adhesion, colony formation, protein expression, and others are also temperature-dependent. That is why cell culture requires constant maintenance of optimum temperature throughout the incubation period with the help of incubator.

Under *in vivo* conditions, the uppermost layer of the human epidermis, the stratum corneum, is exposed to the environment, the temperature of which varies with the season of the year and the geographic position. The thermoregulatory processes within human body maintain the surface skin temperature generally between 30^0C and 33^0C. This temperature is lower than the temperature of the underlying vital epidermal layers (37^0C). The temperature of 37^0C has been demonstrated as optimum for the *in vitro* cultivation of epidermal keratinocytes from human skin by the most of the researchers (Funan et al, 1952; Karasek, 1966; Liu and Karasek, 1978; Boyce and Ham, 1983; Staiano-Coico *et al*, 1985; Papini *et al*, 2003; Guo and Jahoda, 2009; De Corte *et al*, 2011; Lamb *et al*, 2012). In agreement with other researchers, the experiment of Pamela Hawley-Nelson (1983) exhibited that lowering the culture temperature from 37^0C to 34^0C or 31^0C reduced colony number to 50% and 10% respectively. The number of cell passaging also decreased with the declining of temperature (Hawley-Nelson *et al*., 1983). However, a contradictory result was reported by Peter K. A. Jensen (1981) who claimed to grow epithelial cells successfully from human skin explants at temperature as low as 32-33^0C and showed this low temperature to be effective in preventing fibroblast contamination. A few report have also been published on cultivation of human epidermal keratinocytes keeping incubation temperature below 37^0C, for example, 35^0C (Briggaman *et al*, 1967) and 32^0C (Marcelo *et al*, 1983).

pH and CO_2 level

Mammalian cells can survive over a wide pH range (6.6-7.8), but the optimal growth of keratinocytes is obtained at pH 7.2-7.4 (Karasek, 1966; Briggaman, 1967; Karasek and Charlton, 1971; Moore and Karasek, 1971; E. Kenneth Parkinson 2002). At a more alkaline pH (>7.8) or

acidic pH (<7.0), growth is markedly decreased, or does not occur, although other cell types (fibroblasts) continue to multiply and divide under these conditions (Karasek, 1966). However, a slightly lower pH (7.0-7.2) was reported to be optimal by Pamela Hawley-Nelson (1983) whose experimental results showed that the colony formation and number of cell passages at pH 7.4 was less than that at the pH 7.0-7.2.

The pH of the cell culture medium needs to be maintained at a constant level, otherwise high or low pH can be detrimental. The main substances causing pH changes are lactic acid and carbonic acid produced as byproducts by cellular metabolism which make the cell culture medium acidic. To compensate this, the culture medium should have some buffer capacity which can be achieved by including an organic (e.g., HEPES) or bicarbonate- CO_2 based buffer system.

Traditionally, Basal Cell Culture media have been buffered by HCO_3- (bicarbonate). As cells grow in culture, CO_2 evolves; the dissolved CO_2 forms a buffering system with the bicarbonate. However, if cell density is low or the cells have entered into the so-called "Lag Phase," they may not produce sufficient CO_2 to maintain optimal pH and to counter these potential problems, bicarbonate-buffered media require the use of incubators with a 5-10% CO_2 atmosphere. 7-10% CO_2 is optimal but many researchers successfully use it in 5% CO_2. The addition of sodium bicarbonate in the presence of CO_2 will drive the following equation to the left to allow for an equilibrium to be achieved and the pH maintained at 7.2-7.4.

$$NaHCO3 + H2O \leftrightarrow Na+ + HCO3- + H2O \leftrightarrow Na+ + H2CO3 + OH- \leftrightarrow Na+ + H2O + OH- + [CO2(aq) \leftrightarrow CO2(g)]$$

The bicarbonate- CO_2 buffer system is most commonly used to maintain physiological pH of a culture, but it has at least two major drawbacks: (1) Bicarbonate is a weak buffer with a pKa of 6.1, which is far removed from the desired pH range of cell culture media (7.0-7.4) making it difficult to prevent rapid pH changes. (2) Although bicarbonate is cheap, supplying a constant level of CO_2 to cell cultures is definitely not, as it requires expensive CO_2 incubators or fermentors. Bicarbonate, however, is non-toxic and has nutritional value, and hence is commonly used.

Another buffering agent, HEPES, is a zwitterionic organic chemical buffer that can maintain physiological pH despite changes in carbon dioxide concentration (produced by cellular

respiration), and provides an alternative to the use of bicarbonate for buffering because it does not require elevated levels of CO_2. Unlike the low pKa of bicarbonate, HEPES has a pKa of 7.66 making it a much stronger buffer in the pH 7.2-7.6 range. The disadvantages of using this buffer, however, are toxicity and lack of nutritional value to the cells.

The pH of the cell culture media is usually monitored by phenol red. During cell growth, the medium changes color as it changes pH due to metabolites released by the cells. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple (Fig. 6). For most tissue culture work (pH 7.4), the medium should be bright red.

b) Chemical factors

Serum

Serum is the blood fraction remaining after the natural coagulation of blood, followed by centrifugation to remove any remaining red blood cells. Serum is a complex mix of albumins, growth factors and growth inhibitors. It is one of the most important components of cell culture media and serves as a source for amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture (von Seefried, 1976). Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. The optimum concentration of serum supplementation in growth media is 10-20% (Pamela, 1980). Supplementation of media with serum serves the following functions:

- Serum provides the basic nutrients (both in the solution as well as bound to the proteins) for cells.
- Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.
- It provides several binding proteins like albumin, transferrin, which can carry other molecules into the cell. For example: albumin carries lipids, vitamins, hormones, etc. into cells.

- It also supplies proteins, like fibronectin, which promote attachment of cells to the substrate.
- It also provides spreading factors that help the cells to spread out before they begin to divide.
- It provides protease inhibitors which protect cells from proteolysis.
- It also provides minerals, like Na^{+}, K^{+}, Zn^{2+}, Fe^{2+}, etc.
- It increases the viscosity of medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts as a buffering agent which helps in maintaining the pH of the culture media.

Due to the presence of both growth factors and inhibitors, the role of serum in cell culture is very complex. Unfortunately, in addition to serving various functions, the use of serum in tissue culture applications has several drawbacks:

- Lack of uniformity in the composition of serum.
- Testing needs to be done to maintain the quality of each batch before using.
- May contain some of the growth inhibiting factors.
- Presence of serum in media may interfere with the purification and isolation of cell culture products.
- Increase the risk of contamination, as serum may contain adventitious viruses such as bovine vial diarrhea virus (BVDV), bovine parvovirus, bovine adenovirus, and blue tongue virus.

To avoid the complications of using serum-containing media for cell culture, numerous commercial companies have developed serum-free media that support keratinocyte propagation and expansion in 2-dimensional tissue culture. However, according to R. Lamb and C. A. Ambler (2012), in this growth condition keratinocytes fail to form into a stratified, mature epidermis in a skin equivalent model (Lamb and Ambler, 2012).

c) Growth factors

Epidermal growth factor

Epidermal growth factor (EGF), a polypeptide (Fig.7) with molecular weight of 6045-Da and 53 amino acid residues, was first isolated from the male mouse submaxillary gland by Stanley Cohen, and found to enhance growth and maturation of epidermis in newborn mice (De Cohen and Elliot, 1963) and found to be an extremely potent mitogen for fibroblasts (Armelin, 1973; Kohen *et al*, 1975).

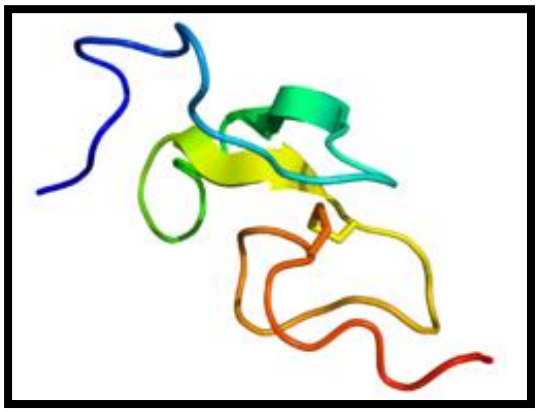

Figure 7: NMR structure of mouse epidermal growth factor.

In 1977, Reinwald and Green found larger colonies of human keratinocytes by the addition of mouse EGF at concentration as low as 0.3 ng/ml, which increased in magnitude up to concentrations of 10 or 30 ng/ml. At the same time, EGF also increased colony forming efficiency and the culture lifetime was amplified from 50 cell generations to over 140 cell generations when compared to keratinocyte cultivation without EGF (Reinwald and Green, 1977). In 1982, Edward O'Keefe proposed that epidermal cells possess a functional receptor for EGF which binds and responds to EGF contributing to keratinocyte proliferation (O'Keefe, 1982).

We know today that EGF acts by binding with high affinity to epidermal growth factor receptor (EGFR) on the cell surface (Fig.8). This stimulates ligand-induced dimerization (Dawson *et al*, 2005), activating the intrinsic protein-tyrosine kinase activity of the receptor. The tyrosine kinase activity, in turn, initiates a signal transduction cascade that results in a variety of biochemical changes within the cell - a rise in intracellular calcium levels, increased glycolysis and protein

synthesis, and increases in the expression of certain genes including the gene for EGFR - that ultimately lead to DNA synthesis and cell migration, adhesion and proliferation (Oda *et al*., 1984).

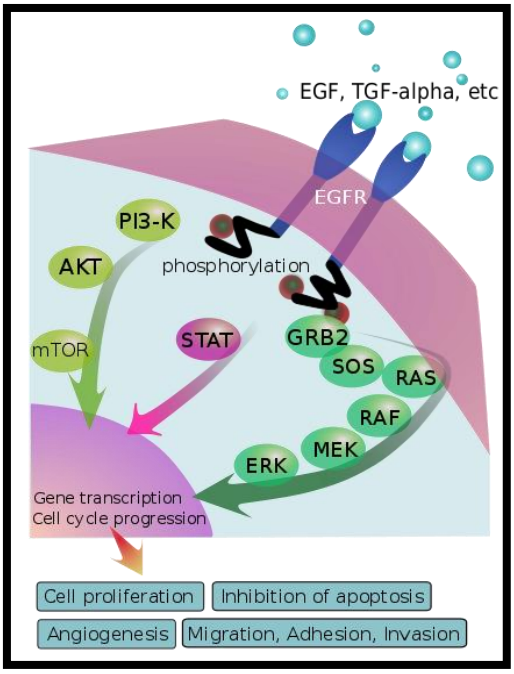

Figure 8: The action pattern of EGF in cell proliferation.

Hydrocortisone

Hydrocortisone (HC) is the pharmaceutical term for cortisol, a steroid hormone (Fig.9), which in vivo increases blood sugar through gluconeogenesis and aids the metabolism of fat, protein and carbohydrate.

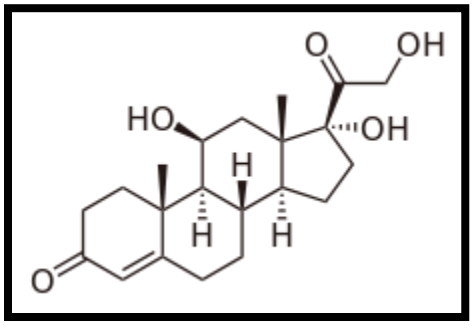

Figure 9: Hydrocortisone (cortisol).

Since four decades earlier, hydrocortisone has been used in cell cultures, for example, to improve growth in 3T3 cultures (Armelin, 1973), although the reason for its effect was obscured then. The experiment of Reinwald and Green (1975) manifested that hydrocortisone makes colony morphology of keratinocytes more orderly and distinctive, and maintains cell proliferation at a slightly greater rate when culture medium is supplemented with low concentration of hydrocortisone (0.4 µg/ml), however, a suppression of keratinocyte growth in culture was observed at concentrations greater than 10 µg/ml (Reinwald and Green, 1975). Peehl and Ham also applied the same concentration (0.4 µg/ml) of hydrocortisone supplementation, while growing human keratinocytes in medium conditioned by 3T3 cells, eliminating the use of feeder layer in culture (Peehl and Ham, 1979). But in contrast to Reinwald and Green (1975), they claimed to get clonal growth of keratinocytes without any conditioning of the medium when increasing the concentration of HC to 10 µg/ml (Peehl and Ham, 1979).

Insulin

Insulin is a peptide hormone (Fig.10) with a molecular weight of 5808 Da and 51 amino acid residues, produced by beta cells in the pancreatic islets of Langerhans, which regulates the metabolism of carbohydrates and fats by promoting the absorption of glucose from the blood to skeletal muscles and fat tissue.

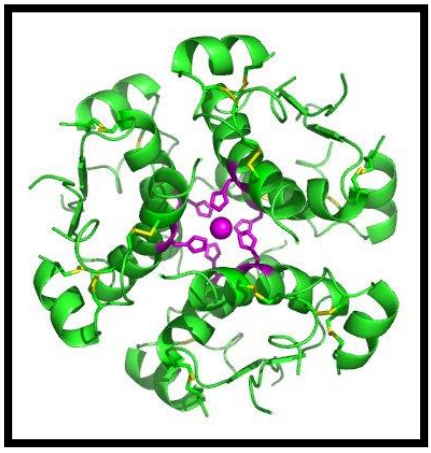

Figure 10: Model of six insulin molecules assembled in a hexamer.

Insulin supplementation carries out an important role in cell culture as it helps cells utilize glucose and amino acids. It was Barnes and Sato (1980) who, for the first time, revealed the stimulatory effect of insulin as a rather 'universal' growth factor for every cell type they examined (Barnes and Sato, 1980). Though the role of insulin is primarily metabolic, it also acts as a growth factor and exerts mitogenic effects on cultured keratinocytes. Insulin also inhibits apoptosis induced by serum withdrawal, and is, therefore, used for clonal growth of keratinocytes in serum-free media (Boyce and Ham, 1983).

Insulin and the related insulin-like growth factors IGF-I and IGF-II act on cells through binding to specific receptors, the insulin receptor (IR) (De Meyts and Whittaker, 2002) and the type 1 IGF receptor (IGF-1R) (Adams, 2000; De Meyts *et al*, 1994). Insulin binding to its receptor stimulates intrinsic tyrosine kinase activity (Fig.11) leading to receptor auto-phosphorylation and the recruitment of intracellular signaling molecules, such as insulin receptor substrates (IRS). IRS and other adaptor proteins initiate a complex cascade of phosphorylation and dephosphorylation reactions, resulting in the widespread metabolic and mitogenic effects of insulin.

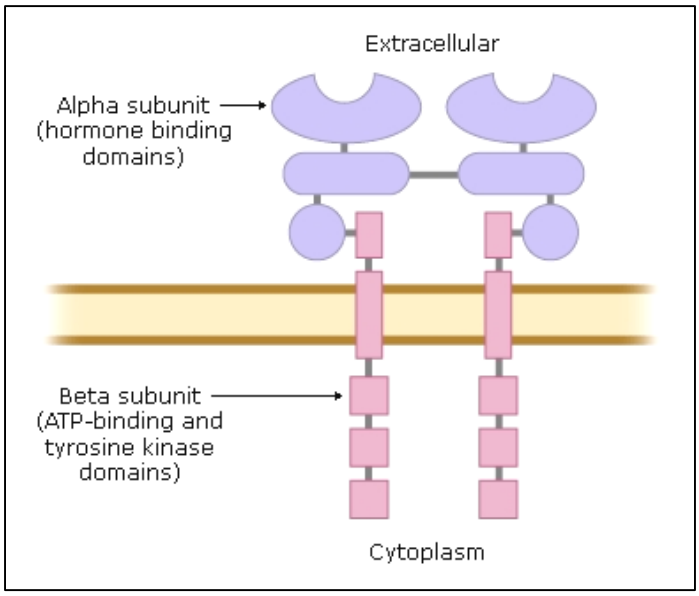

Figure 11: Structure of insulin receptor. The receptor is composed of two extracellular α-subunits that are each linked to a β-subunit and to each other by disulfide bonds.

As an example, activation of the phosphatidylinositol-3'-kinase (PI-3-kinase) pathway stimulates translocation of glucose transporters (e.g., GLUT4) to the cell surface, an event that is crucial for glucose uptake (Fig.12), induce glycogen synthesis, protein synthesis, and lipogenesis. Activation of other insulin receptor signaling pathway (MAP kinase) induces cellular proliferation and growth, and regulation of expression of various genes in insulin-responsive cells.

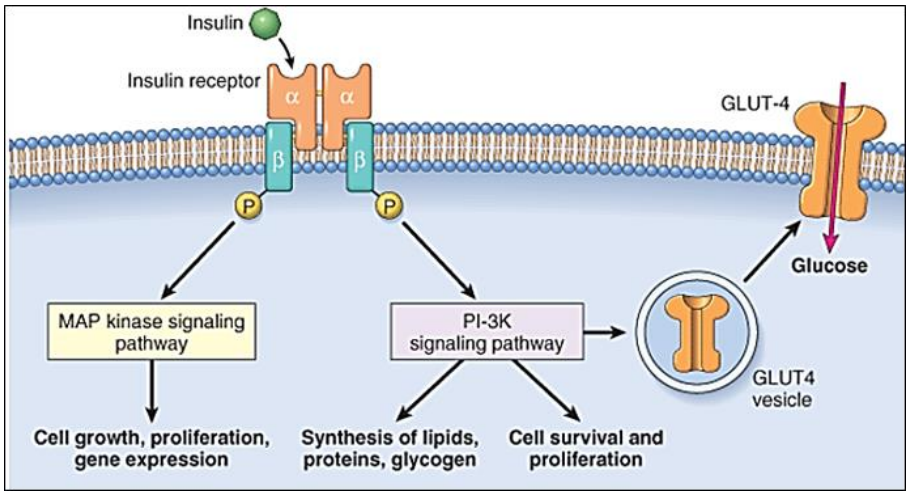

Figure 12: Mechanism of action of insulin.

Transferrin

Transferrins make up an extensive micro-heterogeneous group of single chain glycoprotein isotypes (Fig.13) with approximate molecular weights of 78 to 80 KDa and two specific high-affinity Fe (III) binding sites, which control the level of free iron in biological fluids and are also appropriate for providing iron to cells in culture.

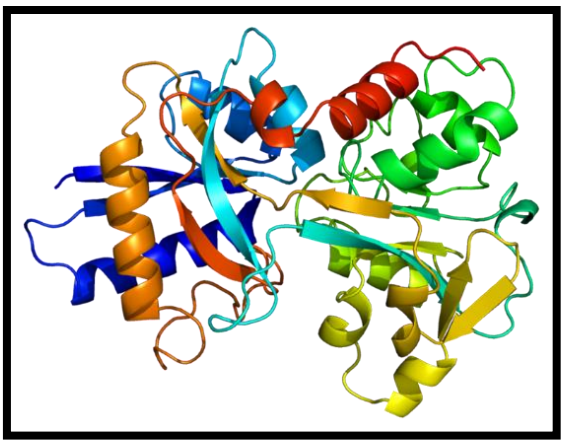

Figure 13: Model of transferrin.

Primary functions of transferrin in cell culture systems are:

- Transferrins facilitate extracellular iron storage, and transport.
- Transferrins are important extracellular antioxidants. They bind iron so tightly under physiological conditions that virtually no free iron exists to catalyze the production of free radicals.
- The delivery of iron to cells by transferrins is a receptor-mediated and controlled process. Cells regulate the amount of iron they receive from the extracellular environment by varying transferrin receptor expression.

Transferrin is a universal iron carrier that provides iron and also helps cells maintain homeostasis by helping cells regulation of iron uptake. When a transferrin protein loaded with iron encounters a transferrin receptor on the surface of a cell, it binds to it and, as a consequence, is transported into the cell in a vesicle by receptor-mediated endocytosis (Fig.14). The pH of the vesicle is reduced by hydrogen ion pumps (H^+ ATPases) to about 5.5, causing transferrin to release its iron ions which are transported out of endosomes via the divalent metal transporters DMT1. The receptor (with its ligand, apo-transferrin, bound) is then transported through the endocytic cycle back to the cell surface, where they dissociate at neutral pH, and both participate in another round of iron uptake. Each transferrin molecule has the ability to carry two iron ions in the ferric form (Fe^{3+}). Intracellular iron is either incorporated into heme or stored in ferritin.

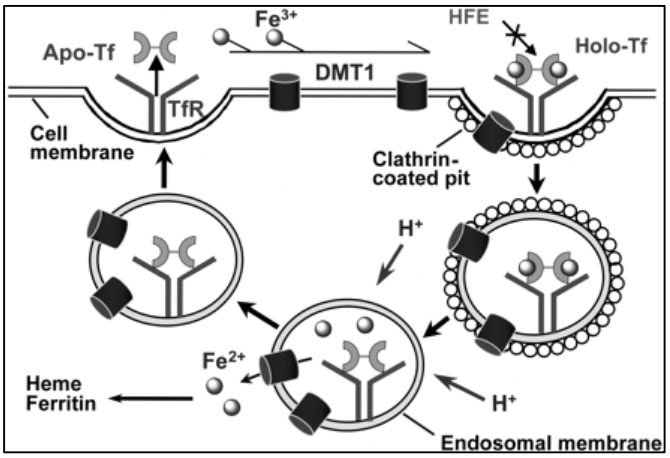

Figure 14: The cycle of transferrin-mediated cellular iron uptake.

Cholera toxin

Cholera toxin, secreted by the bacterium *Vibrio cholera*, is an oligomeric protein complex with molecular weight of 84 KDa, made up of two subunits: subunit A (monomer) and subunit B (pentamer). A subunit contains A1 domain, which includes the enzymatic active site, and A2 domain, which has a α–helix tail. The B subunit contains five chains that form a pentameric ring around the central pore in structure (Fig.15).

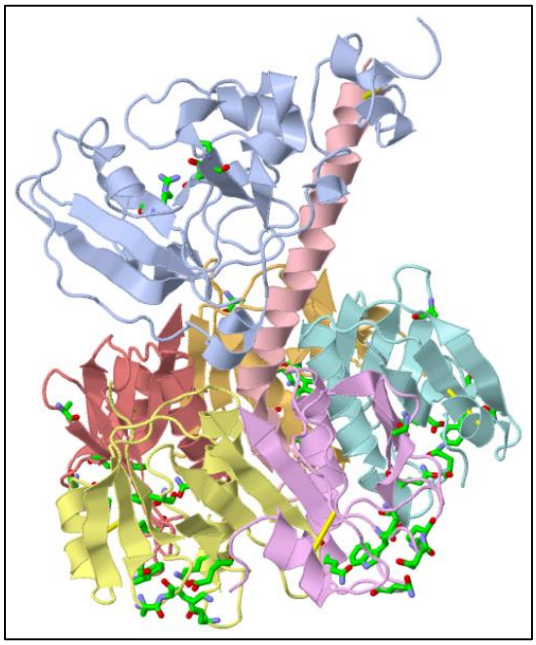

Figure 15: Crystal structure of cholera toxin.

Cholera toxin exhibits its biological effects mediated by cyclic AMP. The initial event in the activation of adenylate cyclase is the binding of the pentameric B subunit of the toxin with the GM1 ganglioside receptor on the cell surface, triggering endocytosis of the toxin, whereas inside the cell, the A subunit activates adenylate cyclase to produce cAMP (Moss, 1979) (Fig.16). Cholera toxin was shown to be a potent activator of adenylate cyclase in the keratinocyte (Marcelo, 1979), to have an effect uniformly over a wide range of concentration (Green, 1978) and to have very low nonspecific cytotoxicity on cultured cells.

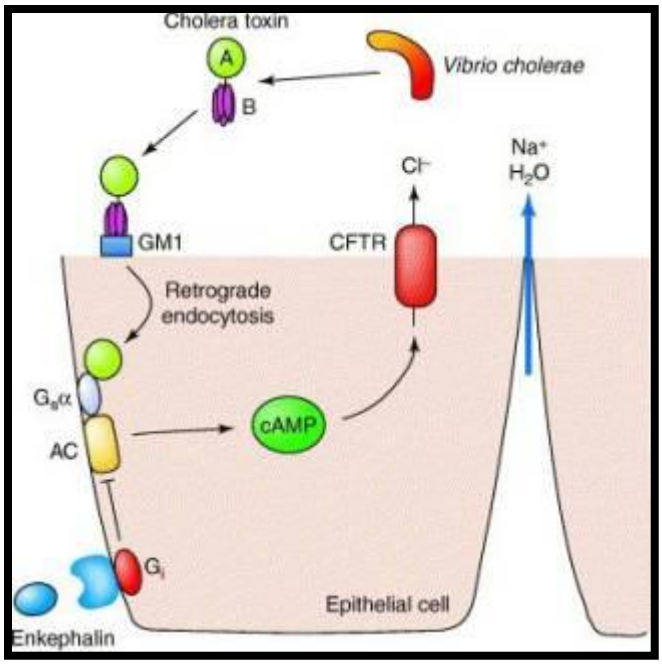

Figure 16: The action pattern of cholera toxin in cell proliferation.

In 1982, Okada and his co-workers extensively studied the effects of cholera toxin on human keratinocytes in relation to cAMP and confirmed about 100-fold increase in the intracellular level of cAMP of cultured keratinocytes when treated with cholera toxin over a range of 10^{-14} to 10^{-8}. Their results indicated that cholera toxin, along with cyclic AMP, exerts its biological effects on keratinocytes biphasically according to the cellular concentration in culture. The toxin enhanced the proliferation of keratinocytes in the early days of cultivation, however, it suppressed the proliferation when added to the confluent culture (Okada *et al*, 1982).

d) Other factors

Culture substrate

Most untransformed mammalian cells require an appropriate surface for survival and growth in vitro; this phenomenon has been termed anchorage dependence, which is also appropriate for human epidermal keratinocytes. Glass, tissue culture plastics, fibrin clots and collagen surfaces have long been recognized as substrates able to support the attachment and growth of cells. Collagen is the most widely used extracellular matrix (ECM) protein for cell culture, facilitating cell attachment, growth, differentiation, migration, and tissue morphogenesis. Karasek and

Charlton (1970) demonstrated collagen as a better substrate than a glass surface. They showed that when epidermal cells were plated on a glass surface, some cells settled and attached, but the efficiency was less than on a glass surface coated with collagen gel (Karasek and Charlton, 1970). So far, 28 types of collagen (type I, II, III, IV, V etc.) have been identified and described, which are found in different animal tissues. For example, type II collagen is largely limited to cartilage (Miller and Matukas, 1969) and type IV to basement membrane (Kefalides, 1973). Kleinman and his co-workers (1978) examined that epidermal cells from guinea pig skin had highest affinity to type IV collagen and this preference for type IV collagen for attachment would be expected from the association of these cells with basement membrane in vivo (Kleinman et al., 1978). To use collagen gel as cell culture substrate, previous workers extracted collagen from various animal tissue, which has become commercially available nowadays. Another form of collagen is gelatin, which is commercially produced by irreversibly hydrolyzing collagen and can be used for coating cell culture plates to improve cell attachment for a variety of cell.

1.3. APPLICATION OF CULTURED KERATINOCYTES

The most widespread early use for normal human keratinocyte cultures has been in the treatment of burns and skin ulcers with sheets of cultured human epidermal cells (Clancy *et al*., 1988; Compton *et al*., 1989; Pittelkow and Scott, 1986).

Patients with full thickness burns require skin grafts in the early stages of treatment to reduce bacterial colonization of wounds, systemic sepsis, fluid loss and pain and to promote healing. Split-thickness skin (STSG) autografts are still the "gold standard" for burn wound closure and remain the mainstay of treatment to provide permanent wound coverage and achieve healing. In some massively burned patients, however, the burns are so extensive that healthy donor site availability is limited. An alternative approach to address this drawback is to graft *in vitro*-expanded epithelial keratinocytes, using a reliable method of culturing human epidermal keratinocytes in stratified and coherent layers (Reinwald and Green, 1975; Green *et al*., 1979; Boyce, 2001; Jones *et al*., 2002; Gerlach *et al*., 2008).

In 1975, James Reinwald, Ph.D. and Howard Green, M.D. first demonstrated that human epidermal keratinocytes could be isolated and serially cultured in vitro with mouse fibroblasts that had been lethally irradiated to halt their proliferation (Reinwald and Green, 1975). A few years

later, this process was used to produce large sheets of cultured epithelium suitable for grafting. Such cultured epidermal autografts (CEAs) were first used in the treatment of major burns in 1981 (O'Connor *et al*., 1981) and then in the subsequent years (Gallico *et al*., 1984; Bettex-Galland *et al*., 1988; Langdon *et al*., 1988; Donati *et al*., 1992; Compton *et al*., 1993; Boyce *et al*., 1999).

However, cultured keratinocyte grafts possessed a number of complications (Pye, 1988): (i) an unexpectedly high number of grafts fail to take in spite of adequate preparation of the graft bed, (ii) an interval of at least 2 to 3 weeks is required from the time of the original biopsy before confluent sheets can be obtained in culture. The low take rate may be due to the differences existing between conventional split skin grafts and cultured keratinocyte grafts. A conventional split skin graft contains blood vessels within the dermal elements and is fully keratinised. The capillaries 'link up' with vessels in the graft bed within a matter of 24 to 48 hours (Birch and Branemark, 1969), serving both to secure the graft and to supply nutrients. The keratin layer protects the graft from drying. Although the cultured keratinocyte sheet is multi-layered and terminally differentiated, it contains none of these elements and is therefore very vulnerable in the first 24 hours following grafting (Richard, 1988). Low grade bacterial infection is common in chronic granulation tissue and therefore lysis of cultured keratinocyte grafts might be expected (Pye, 1988). These complications owed further investigations.

The system of keratinocyte cultures has also been used to address dermatological questions such as contact dermatitis (Sainte-Marie *et al*., 1998) and inflammatory conditions (Cowan *et al*., 1998) including psoriasis.

The method of dissociating cell culture has been used to study the growth and differentiation of epidermal cells, an important problem in the study of psoriasis. During the course of this disease, the epidermis undergoes two major changes: an increase in mitosis and a decrease in keratohyalin synthesis (Scott, 1972). Dissociated epidermal cells in monolayer cultures, as in psoriasis, show an increase in mitotic rates of up to 30% (Prunieras *et al*., 1976) and an absence of keratohyalin synthesis (Karasek, 1975; Prunieras *et al*., 1976). Thus, dissociated epidermal monolayer cell cultures appear to be promising in the study of psoriatic epidermis.

Another example of the application of keratinocyte culture to biologic problems is that of skin cancers. Two directions have been particularly considered. In one, epidermal cells in culture were

used to study the induction of malignant changes by chemical carcinogens. In the other, skin cell cultures were used to detect the viral origin of carcinoma in man. The combination of skin cancer cell cultures and nucleic acid hybridization techniques can be helpful to detect viral DNA in the genome of a cancerous cell (Prunieras *et al*., 1976).

1.4. AIM AND OBJECTIVES

The extensive capacity of the epidermis for cell renewal *in vivo* has been demonstrated by their ability to survive, expand, and generate cultured human epidermal sheets capable of rescuing patients with full-thickness burns covering up to 98% of their body surface (Gallico *et al.*, 1984; Compton *et al*., 1989). Not only for covering wounds of burns and ulcers, human epidermal keratinocyte cultures also have vital contribution in the study of various skin diseases like skin psoriasis (Prunieras *et al*., 1976) as well as in numerous pharmacological investigations. Human keratinocyte cultures also have applicability in the study of skin cancers (Prunieras *et al*., 1976). Recently the induced pluripotent stem cell (iPSC) technology has begun a new era where skin keratinocytes offer high reprogramming and therapeutic potential since the epidermis provides the greatest number of easily obtainable proliferative cells including a subpopulation of epidermal stem cells (Racila *et al*., 2011). In Bangladesh, however, human cell culture is comparatively a new field and has not yet been advanced enough for progressive studies. In this context, the goal of my present study was to develop a suitable method to culture human epidermal keratinocytes for their optimum growth, hoping to keep pace with the ongoing cutting-edge technology of the developed world in near future. To optimize human keratinocyte culture, the study was designed to achieve the following objectives:

- Comparative analysis between the methods of cell dissociation culture and explant culture.
- Determination of appropriate trypsin concentration for epidermal cell isolation.
- Evaluation of different types of substrates in keratinocyte culture.
- Assessment of effects of donor age on keratinocyte culture.
- Assessment of effects of different concentrations of serum on keratinocyte culture.
- Assessment of effects of growth factors on keratinocyte culture.

Chapter Two

MATERIALS AND METHODS

2. MATERIALS AND METHODS

The present study on human keratinocyte culture was carried out in the cell culture laboratory of Tissue Banking and Biomaterial Research Unit (TBBRU), Atomic Energy Research Establishment (AERE), Savar, Bangladesh (Fig. 18). The duration of this research work was 10 months from April 2014 to January 2015. The overall study design has been outlined in Fig.19. All media, reagents, chemicals and equipment used in this study have been mentioned in Appendix I and II.

Figure 18: (A) Atomic Energy Research Establishment (AERE); (B) Tissue Banking and Biomaterial Research Unit (TBBRU).

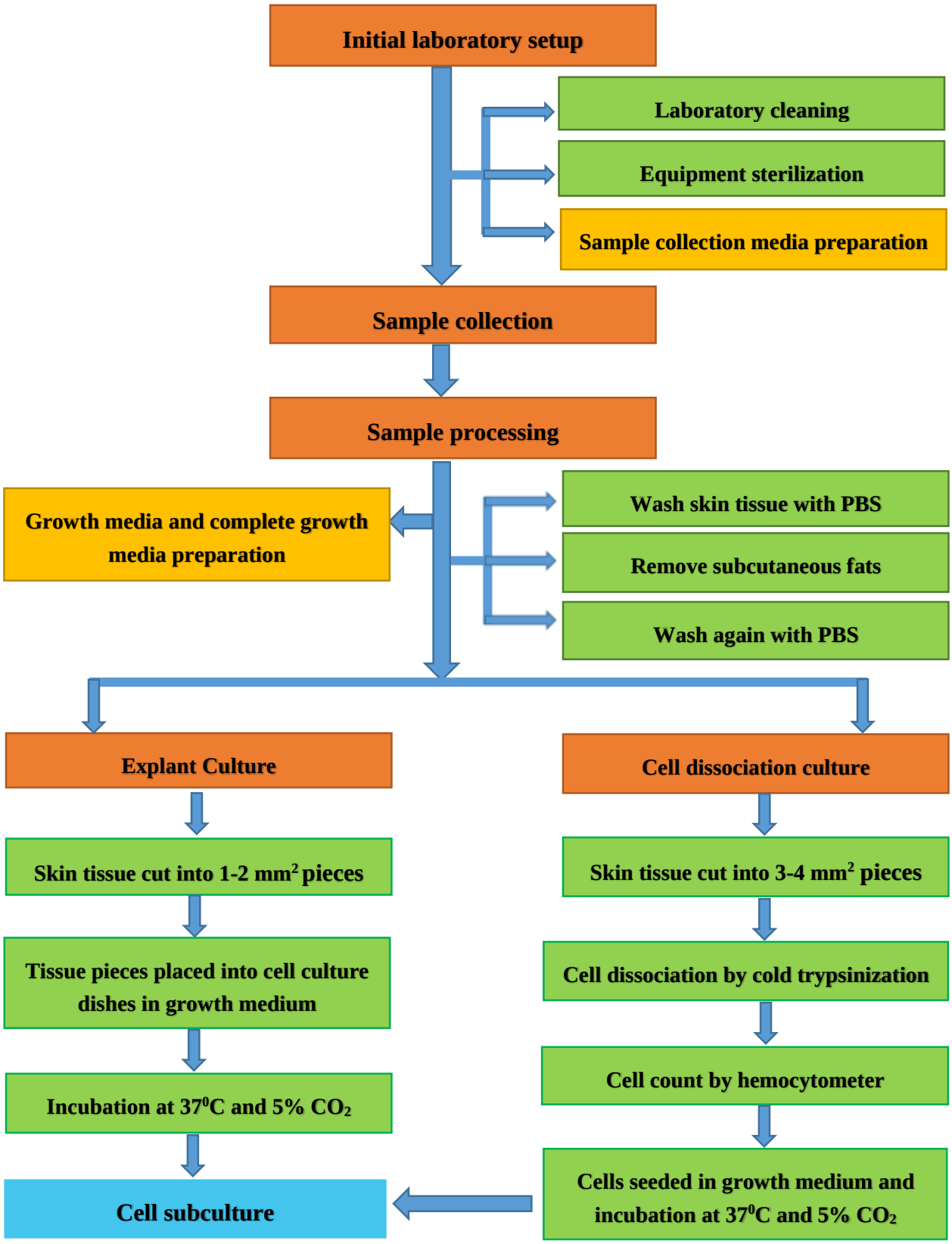

Figure 19: Quick view of the whole study design for human keratinocyte culture.

2.1. SAMPLE COLLECTION

The samples of human foreskin tissues were collected after circumcision through informed consent from Dhaka Shishu Hospital (Fig.20A). Donors were male and of different ages (few months-15 years). Immediately after circumcision the samples were transferred under proper aseptic technique in sterile plastic vials containing Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Fig.20B & C). The sample containing vials were packed in double polybag, carried in an ice-box and transported from Shishu hospital to the laboratory of TBBRU on the same day or within one week after collection.

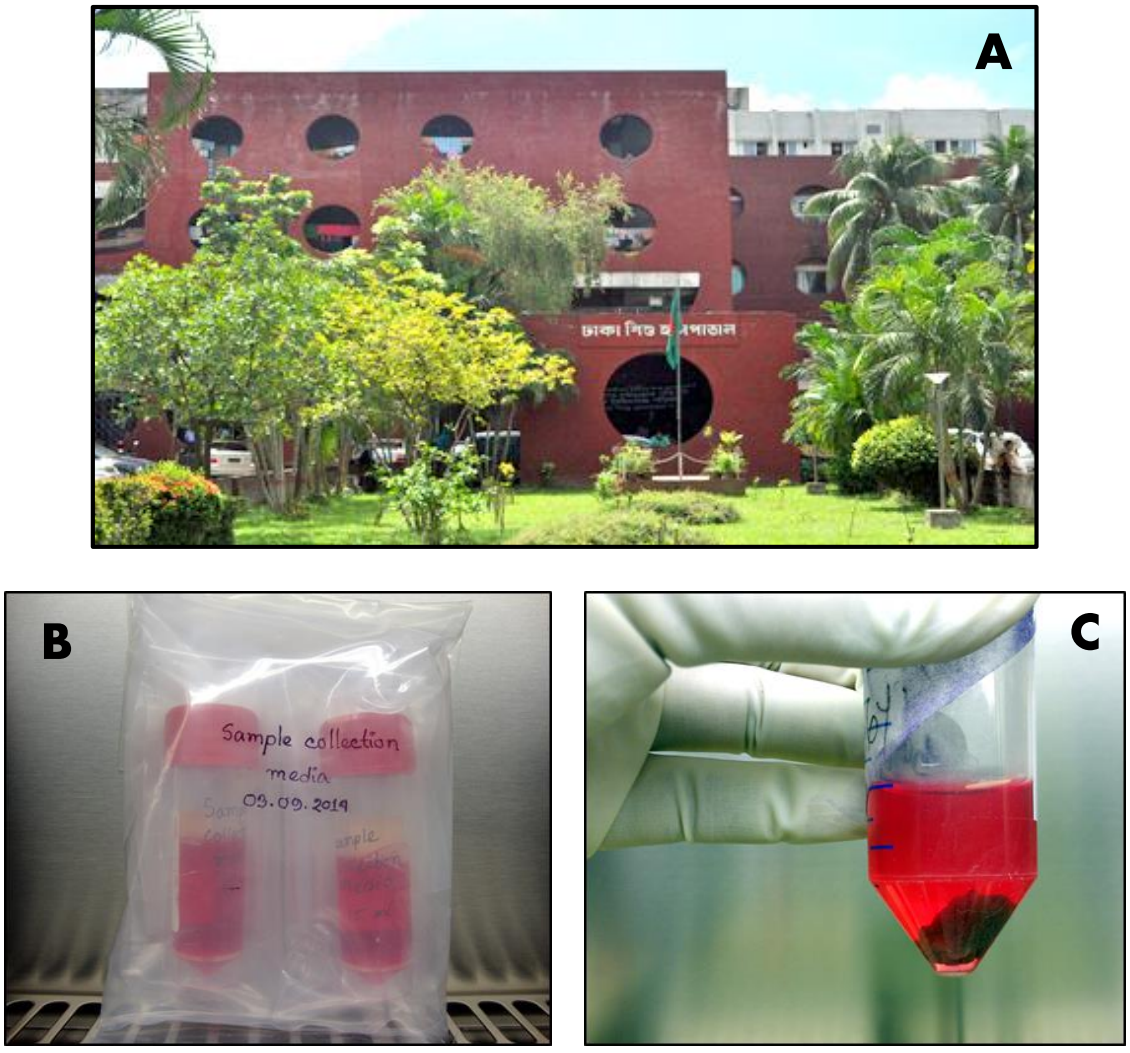

Figure 20: Sample collection. (A) Dhaka Shishu Hospital; (B) & (C) Sample in collection media.

2.2. SAMPLE PROCESSING

The collected samples were stored in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 4^0C until use. Samples were processed as early as possible. The duration before processing was not more than 1 week after collection. However, it is ideal to use the sample the same day of collection or the next morning owing to gradual loss of yield (particularly after 24 h).

The foreskin samples were processed before subjecting to two culture techniques. Each tissue sample was placed in a 90-mm Petri dish and rinsed several times with PBS to remove blood and serum (Fig.21A). Most of the subcutaneous fat and membranous material was removed using fine tweezers and a sharp scissor. Then the skin was flattened on the dish placing the epidermis side down (Fig.21B) and the tissues were scraped away using the edge of the scalpel until only the thin epidermis and the dense dermis remain (Fig.21C). The skin was washed once more with PBS.

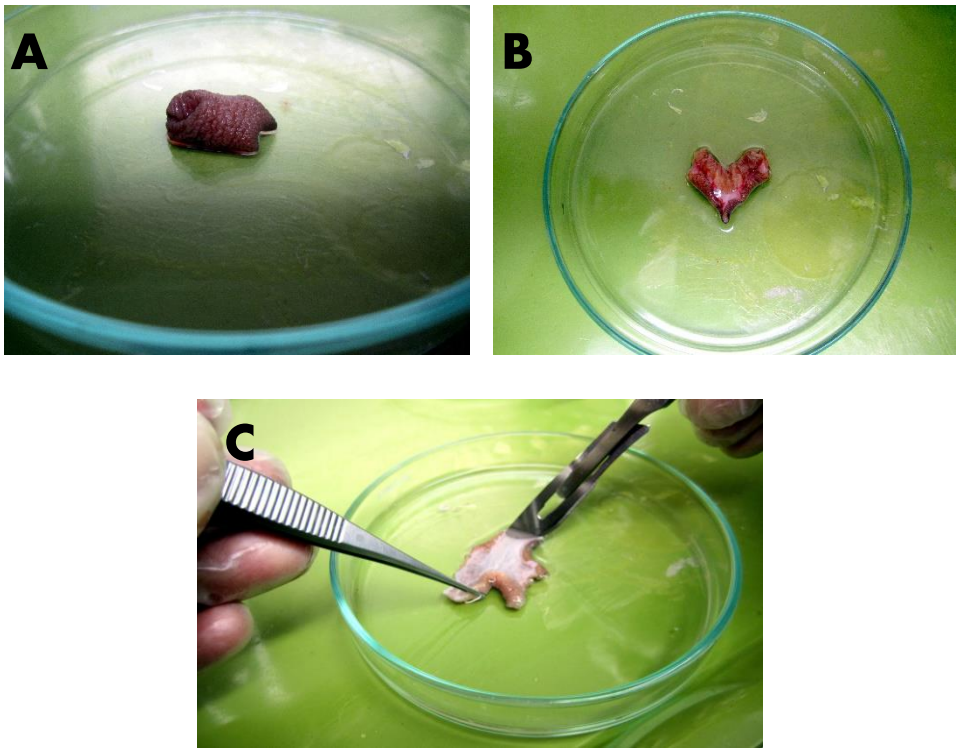

Figure 21: Human foreskin sample processing before culture. (A) Skin sample placed on petri dish; (B) Skin flattened on the dish to remove subcutaneous tissue; (C) Fatty tissues scraping away from the skin using scalpel and tweezer.

After the initial steps of sample processing, the skin cells were cultured by two different methods:

i. Cell dissociation culture by cold trypsinization and
ii. Explant culture.

2.3. CELL DISSOCIATION CULTURE

2.3.1. ISOLATION OF EPIDERMAL CELLS

For cell dissociation culture, the skin was cut into 1-2 cm long and 0.5 cm wide stripes using a scalpel after trimming off the underlying fatty tissues (Fig.22A,B,C&D). This step was performed late in the afternoon since overnight incubation was performed, so that the duration of incubation does not exceed 16 hours. The small tissue pieces were then put into 5 falcon tubes, each containing 5ml of 0.125%, 0.2%, 0.25%, 0.3% and 0.35% trypsin in PBS and incubated overnight (~16h) at 4^0C in refrigerator (Fig.22E).

After incubation, trypsin was removed without disturbing the tissue pieces and 5ml of serum-containing growth medium was added at 37^0C to inactivate remaining trypsin if any (Fig.22F). Then the overnight digested tissue pieces were placed on a petri dish and the edge of the dermal part of the tissue was grabbed with one tweezer and the thin epidermal part with another set of thin tweezers and the epidermis (almost transparent) was slowly peeled off (Fig.22G). The epidermis was minced into the smallest possible fragments with the help of scissors in PBS, transferred to a falcon tube containing growth medium and gently triturated for 20-25 times with a wide-bore pipette to release the epidermal cells. The epidermal cells were pooled and centrifuged at 80g for 5 min (Fig.22H). The supernatant was removed without disturbing the pellet (Fig.22I) and the pellet was resuspended in 5ml of growth medium to produce single cell suspension. Then the number of cells and cell viability were measured with a hemocytometer through trypan blue exclusion method before seeding the isolated epidermal cells in growth medium into T25 culture flask (Fig.22J). The whole procedure of isolating epidermal cells from human foreskin through cold trypsinization has been chronologically displayed in Fig.22.

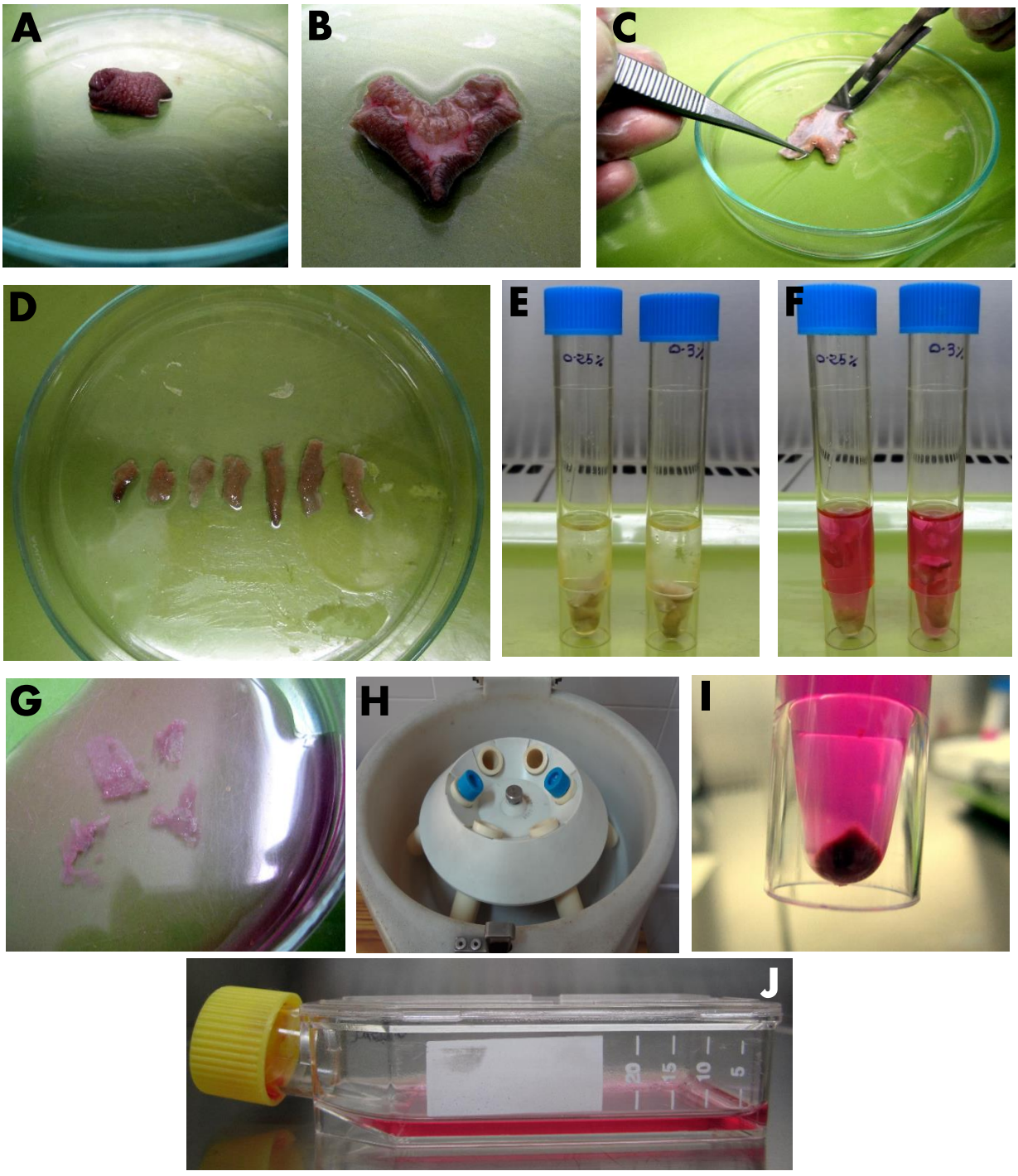

Figure 22: Chronological steps of cell dissociation culture; (A) human foreskin, (B) skin tissue flattened on petri dish, (C) scraping of fatty tissue from skin, (D) 1-2 cm long tissue pieces, (E) tissue in 0.25% trypsin, (F) serum addition to neutralize trypsin after overnight digestion, (G) isolated epidermis, (H) centrifugation of epidermal cell suspension, (I) pellet, (J) seeding of epidermal cells in growth medium in T25 culture flask.

2.3.2. CELL COUNT AND VIABILITY TEST

Total cell count and viability count of human epidermal keratinocytes were made simultaneously by dye exclusion method with trypan blue using hemocytometer. 100 µl of cell suspension was taken into a micro-centrifuge tube. The cell suspension was diluted at 1:1 ratio by adding 100 µl of 0.4% trypan blue. The cover slip was placed on the chambers of the hemocytometer and both chambers were loaded by pipetting the suspension under the cover slip (Fig.23A). The hemocytometer was then observed under the microscope and the cells were counted. Each chamber of hemocytometer is engraved with a laser-etched grid of perpendicular lines, consisting of 9 large squares, where each square contains 10^{-4} ml of cell suspension. The cell count was done in the hemocytometer using four large corner squares and the middle square (Fig.23B). The total cells and the dye stained cells (black) resting on those squares were counted.

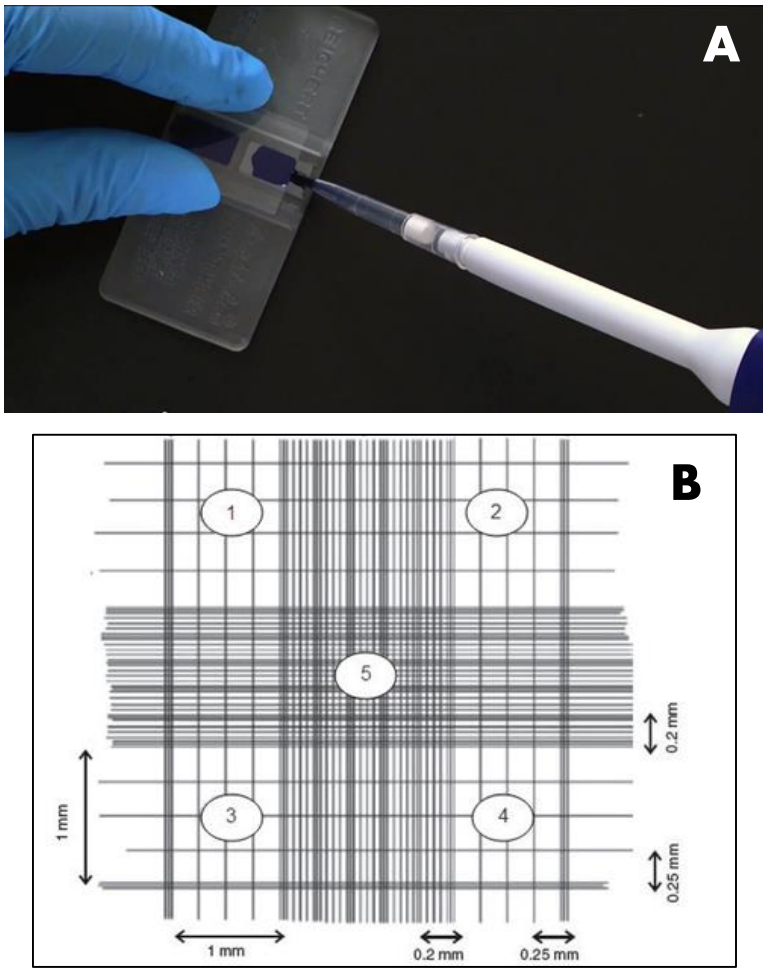

Figure 23: Cell count in hemocytometer. (A) Cell suspension loading in the chamber of hemocytometer; (B) Grid pattern of a hemocytometer.

With the help of trypan blue dye the living and dead cells were distinguished, as the dye passed through the membrane of dead cells, making their appearance blue under microscope. On the other hand, living cells with intact cell membrane excluded the dye and appeared mostly clear during observation through microscope (Fig.24). The cell count was done within 15 minutes since the proportion of stained cells gradually increases with time due to trypan toxicity.

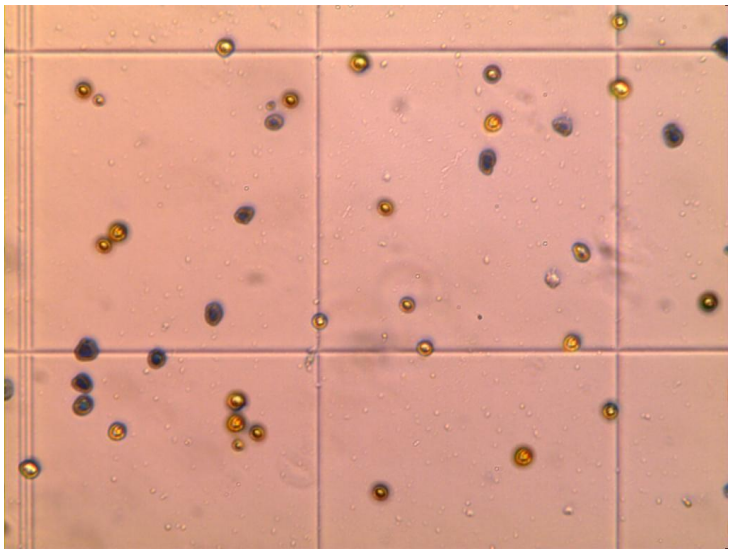

Figure 24: Epidermal cell counting on hemocytometer slide under microscope.

Calculations were made according to the following formulas:

1. Percentage (%) of viable cells:

$$\frac{\text{Total cells counted} - \text{dead (stained) cells}}{\text{Total cells counted}} \times 100$$

2. Concentration of viable cells (viable cells/ml)

   Viable cells/square X dilution factor X 10^4

The cell count on the epidermal cell suspension was adjusted to the desired number by addition of the culture medium to the suspension.

2.3.3. CELL CULTURE

The human epidermal keratinocytes isolated through cold trypsinization were suspended at concentrations of 2×10^4 cells/cm^2 in growth medium, plated in 25 cm^2 tissue culture flask and incubated at 37^0C in an incubator with an atmosphere of 95% humidity and 5% CO_2 to maintain

the pH at 7.4 (Fig.25A). The medium was changed the next day of plating and subsequently for every 3 days, paying close attention to cell density. The cultures of epidermal keratinocytes were subjected to various analysis and observed under an inverted microscope with phase contrast optics and photographed using xlicap software at regular interval (Fig.25B).

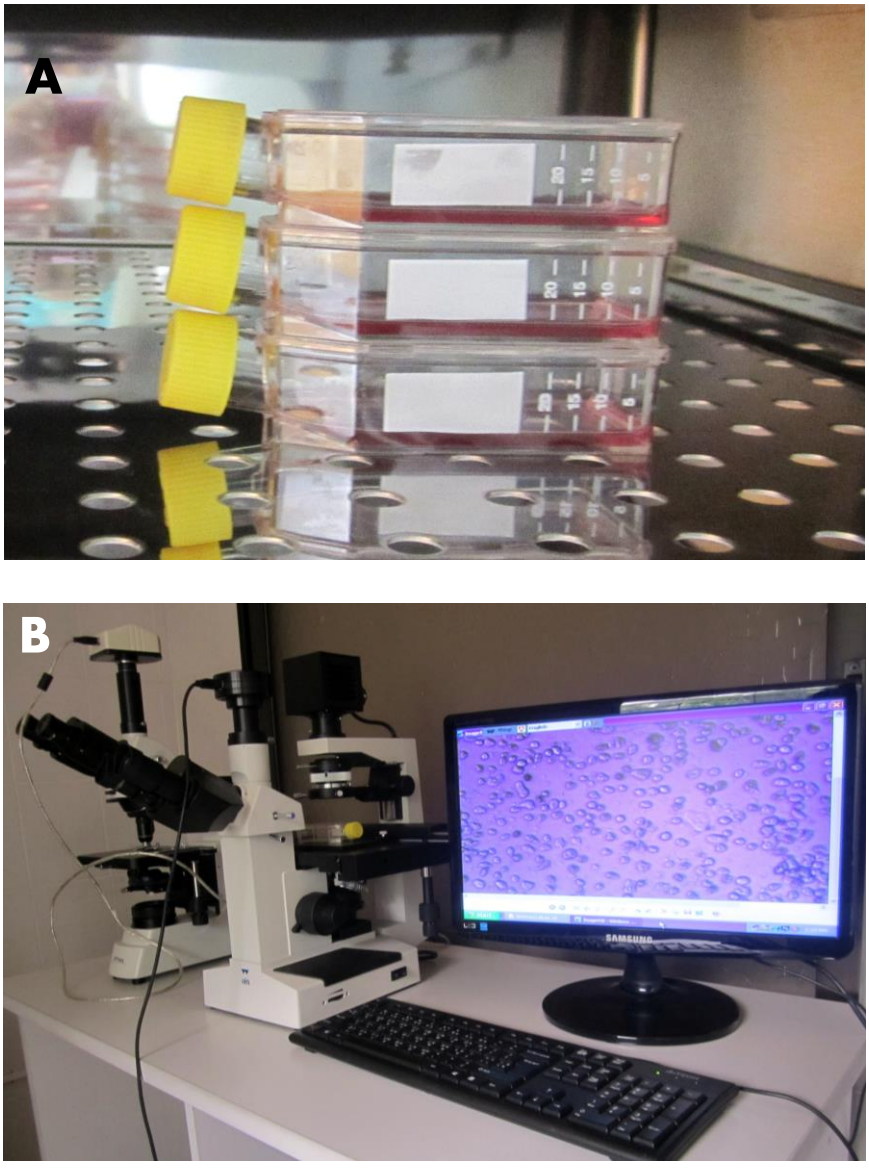

Figure 25: Culture of keratinocytes and observation. (A) Keratinocyte cultures incubating at 370C and 5% CO2; (B) Inverted microscope used for observing keratinocyte culture.

2.3.4. SUBSTRATE ASSAY

For assessing the effect of substrate on keratinocyte culture, the cells were cultured on medium-conditioned flask and gelatin-coated flask, along with plastic culture flask (Fig.24A). To prepare the culture flask conditioned with medium, 2 ml of DMEM containing 10% FBS and antibiotics was added to the flask and the flask was tilted to cover the whole surface with medium. The flask was then incubated overnight at 37 °C before plating epidermal cells onto it. Gelatin-coated substrate was prepared by adding 2 ml of 0.1% gelatin solution to cover the surface of the culture flask. The flask was incubated for at least 1 hour at 37^0C. The gelatin solution was then aspirated and the flask was allowed to dry for at least 10 min in the tissue culture hood before inoculation of epidermal cells. All these 3 types of substrates were analyzed for their effects exerted upon cell attachment after plating and subsequent cell growth.

2.3.5. ANALYSIS OF EFFECT OF SERUM AND GROWTH FACTORS

Human epidermal keratinocytes were cultured in DMEM containing various concentrations of fetal bovine serum, 5%, 10%, 15%, 20% and 25%, to determine the suitable concentration for optimal keratinocyte growth. To evaluate the effect of growth factors, keratinocytes were cultured in serum containing media supplemented with hydrocortisone (0.4 µg/ml), or insulin (5 µg/ml), or both and the cell proliferation rate, plating efficiency and duration to develop confluent culture of keratinocytes were analyzed in each condition.

2.3.6. CELL SUBCULTURE

When the keratinocyte cultures became 70-75% confluent under optimum culture condition, they were subcultured. For this, the spent medium from the culture flask was discarded using a pipette. The culture flask was washed once with calcium- and magnesium-free phosphate buffered saline (PBS). The flask was then incubated with 2 ml of 0.25% trypsin at 37^0C for 4-5 minutes. The flask was gently tapped to release the cells. The trypsin was inactivated by the addition of serum-containing growth medium and the cell suspension was centrifuged at 80g for 5 min. The

supernatant was removed without disturbing the pellet and the pellet was resuspended in 5 ml of growth medium in a 25 cm^2 tissue culture flask and incubated at 37^0C and 5% CO_2.

2.4. EXPLANT CULTURE

For explant culture, the skin tissue was processed to remove the fatty tissues and then cut into 1-2 mm^2 pieces. Using tweezers, the tissue pieces were placed in 3 types of culture dishes: gelatin-coated culture dish, DMEM-conditioned culture dish and plain culture dish, at a density of about 15-20 pieces/dish (Fig.26A,B,C). The skin explants were oriented in the culture dish with the epidermis facing up. The culture dish with explants were incubated at 37^0C and 5% CO_2 for 30 minutes to 1 h (according to the amount of liquid associated with the specimens) to secure attachment of the explants to the culture dish. It was carefully observed that the explants do not dry out completely. Then about 1.5–2 ml of the growth medium was added very gently to cover the explants and the dishes were returned to the incubator (Fig.26D). It is essential that the pieces of tissue remain attached to the plate. Floating pieces were removed from the dish. Cultures were checked for cell growth the next day. On day 3 or 4 of culture the cells were subcultured by the method mentioned in section 2.3.6.

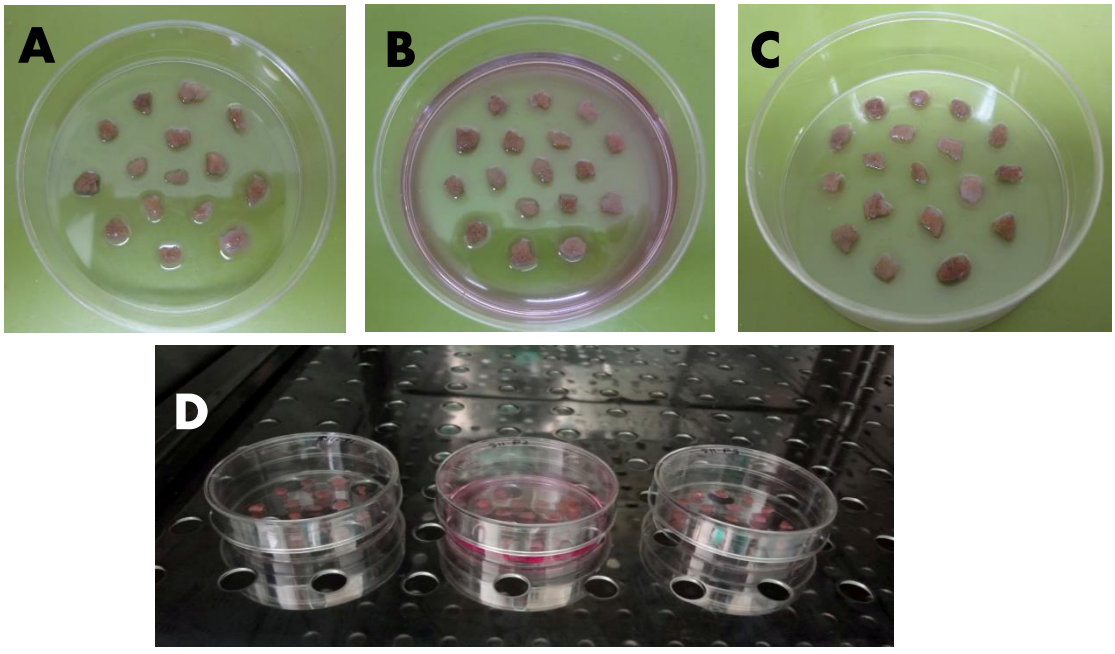

Figure 26: Skin explants in (a) gelatin-coated culture dish, (b) DMEM-conditioned culture dish and (c) plain culture dish; (d) Incubation of skin explant culture.

Chapter Three

RESULTS

3. RESULTS

3.1. DISSOCIATED CELL CULTURE

3.1.1. EPIDERMAL CELL ISOLATION AND CELL VIABILITY

Human foreskins floated overnight in cold trypsin were successfully separated at the dermal-epidermal junction, yielding transparent sheets of epidermis free of dermis (Fig. 27A). No dermal cells or tissue remnants were found on the dermal side of the epidermal sheets when observed under electronic microscope (Fig. 27B).

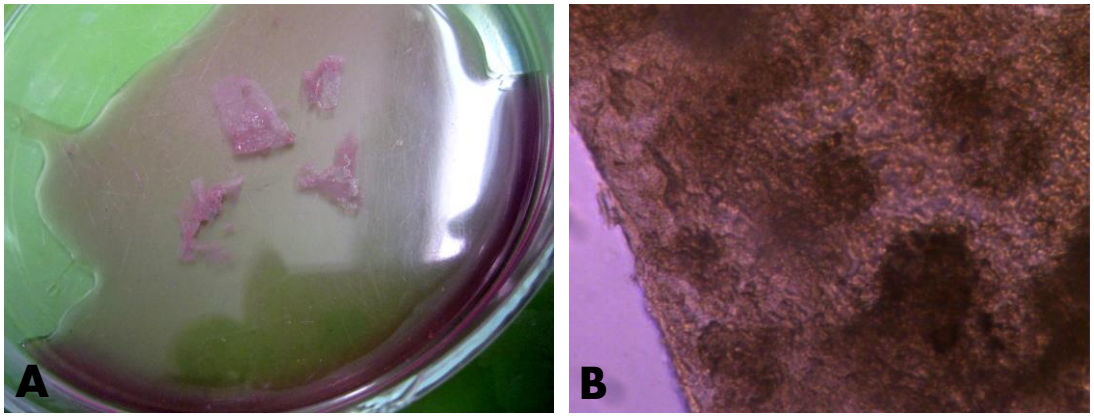

Figure 27: (A) Isolated epidermis from human foreskin by cold trypsinization method; (B) Microscopic view of epidermis revealed no remnants of dermal tissue with the isolated epidermis.

For the separation of epidermal layer, the appropriate concentration of trypsin-EDTA solution was found to be 0.25%, which yielded viable cells ranging from 70% to 90% depending on different aged donors and sample storage time. The cell viability was higher, along with highest yield of cells, when skin samples from children of few months old, stored not more than 2 days, were treated with 0.25% trypsin-EDTA (Fig. 28A). At low trypsin concentration, with 0.2%, highest cell viability was obtained (Fig.29), but the separation of epidermis from underlying connective tissue was difficult and the total cell count was significantly low and at 0.125%, the separation was not possible at all. On the other hand, when higher trypsin concentration (0.3% and 0.35%) was used, the epidermis was damaged, which caused marked reduction of cell viability as well as low

cell count (Fig. 28B, 29). The overnight tissue digestion by trypsin for 16-18 hours was optimal for the cell isolation and seemed to be critical for cell viability. In one instance, the tissue sample was digested in trypsin solution for more than 20 hours, after which, the epidermal layer was found to be highly damaged and the cell viability was sharply decreased. In another case, skin tissue was treated with optimal concentration of trypsin-EDTA (0.25%) after storing the sample for 7 days and the cell viability was still reduced. Therefore, in other cases, the samples were processed mostly within 2-4 days and the storage time did not exceed 7 days after collection.

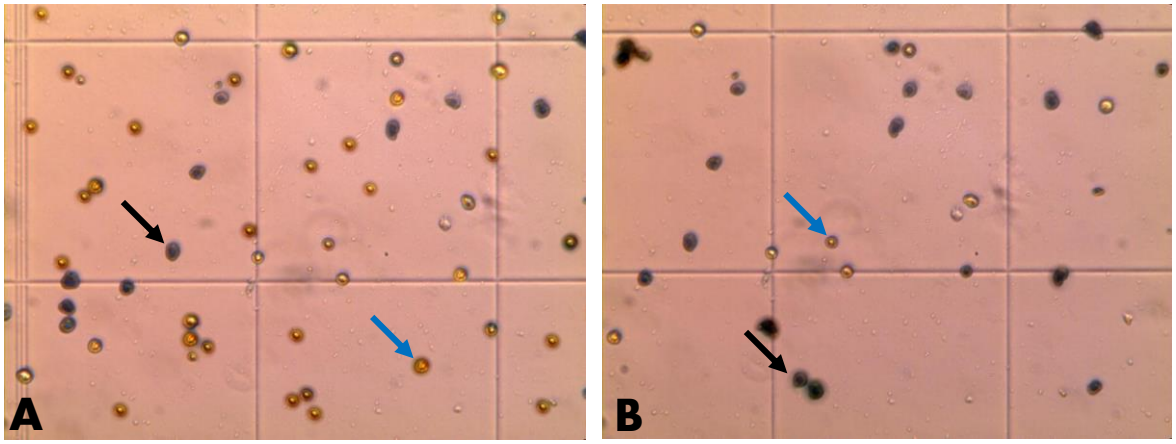

Figure 28: Count of cell viability on hemocytometer through trypan blue exclusion method after disaggregating the skin sample with (A) 0.25% trypsin and (B) 0.35% trypsin; (blue arrows showing live cells and black arrows showing dead cells).

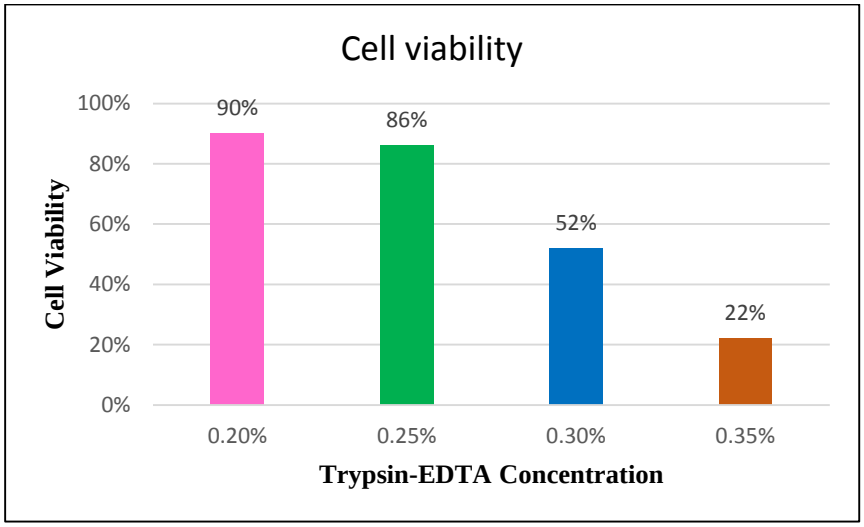

Figure 29: Relation between different concentrations of trypsin-EDTA solution and cell viability of human epidermal cells isolated from a child's foreskin (3 years 6 months old).

The cell suspension obtained from isolated epidermis was composed predominantly of single cells. A few small clumps containing two to ten cells were also present; clusters of more than ten cells were rare. Approximately 2.5×10^6 mean epidermal cells were obtained from each foreskin sample measuring about 3 cm^2 area, which were seeded in serum containing growth medium at a concentration of 2.5×10^5 cells/ml medium in 25 cm^2 culture flask. After plating, the cells were observed under inverted microscope. Morphologically, most of the keratinocytes were small in size and somewhat rounded or of polygonal shape (Fig.30). The rest of the cells were larger, flat keratinocytes at different stages of differentiation (green arrow). A very few dendritic cells were exhibiting long cytoplasmic process extending from those cells (yellow arrow). These cells were presumed to be Langerhans cells according to previous workers (Eisinger *et al*., 1979). The epidermal cells settled after plating and many of them attached to the substrate within 24-36 hours. The unattached cells got washed away when the medium was changed on the next day of plating.

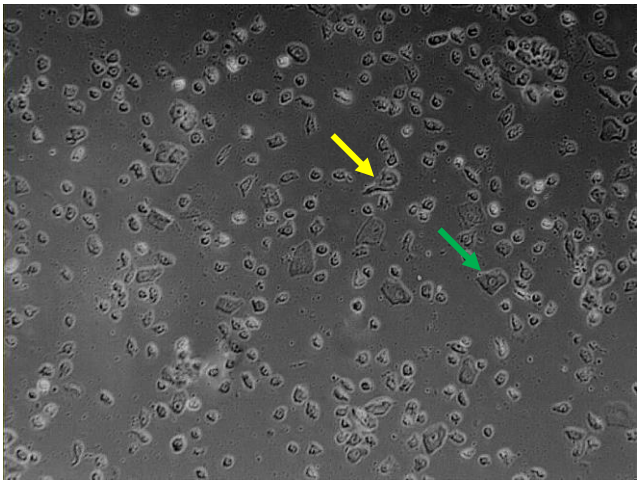

Figure 10: Plating of epidermal cells after isolation by cold trypsinization; the keratinocytes were mostly small and round shaped and some were larger and flat (green arrow); a very few dendritic cells with cytoplasmic process were present (yellow arrow).

3.1.2. EFFECT OF SUBSTRATE ON KERATINOCYTE CULTURE

The effect of substrate on keratinocyte culture was evaluated by plating them in growth media supplemented with 10% FBS on 3 types of culture flasks: plastic tissue culture flask, culture flask conditioned with medium and culture flask coated with 0.1% gelatin (Fig.31).

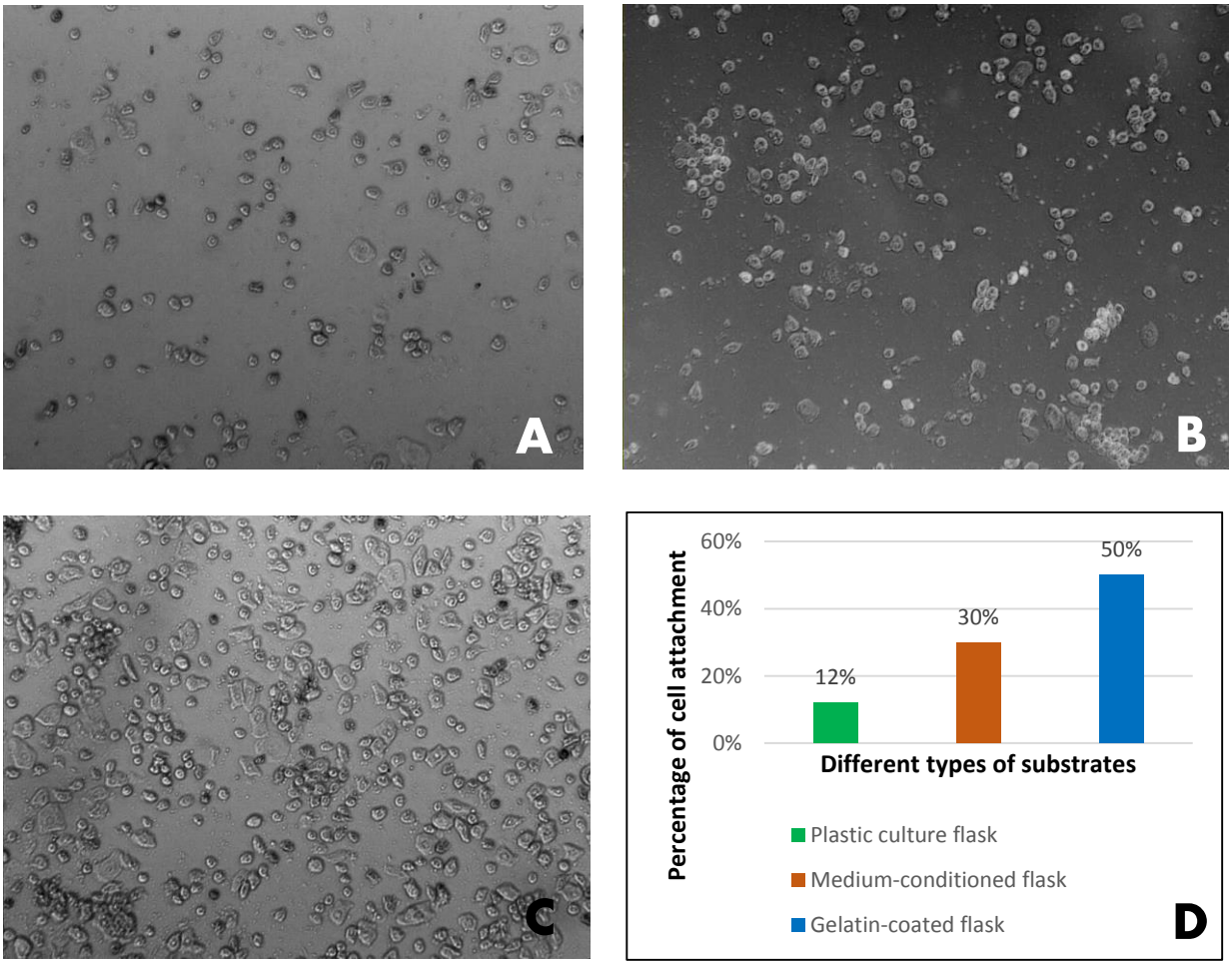

Figure 11: Human epidermal cells from young donor at 8th day of culture in 10% serum-containing medium on (A) plastic culture flask, (B) media-conditioned flask, and (C) gelatin-coated flask; (D) Graphical presentation of the relation between cell attachment and the nature of substrate.

When the epidermal cells were plated on plastic culture flask, very few cells (<15%) attached to the surface within 36 hours and the majority remained unattached (Fig.31D), which were removed with the first media change. Some of the remaining cells were also loosely settled, which could be easily detached from the surface by gentle tapping of the culture flask. Consequently, the epidermal cells on plastic surface showed very poor cell growth and failed to grow to confluency (Fig.31A). The attachment and growth of keratinocytes could be improved by incubating the culture flask overnight with growth medium prior to plating the epidermal cells, which increased cell attachment from less than 15% to 25-30% (Fig.31D). The subsequent growth of the epidermal

cells on medium-conditioned surface was also higher than those on plastic surface (Fig.31B). However, the most significant cell growth was obtained when the epidermal cells were plated on gelatin-coated flask (Fig.31C). Of the viable cells plated at cell density of 2.5×10^5 cells/ml of serum-containing medium, 35-50% cells attached to the gelatin-coated surface during the first 24 hour and they developed to confluent culture in 14-21 days. So, further experiments were carried out on gelatin-coated culture flasks.

3.1.3. EFFECT OF DONOR AGE

In this experiment, the *in vitro* proliferative behavior of four keratinocyte cultures of young donors (from few months to 2 years old) was compared to that of six cultures derived from donors aged 3-15 years. All the human keratinocyte cultures established from donors ranging in age from few months to 15 years showed a finite culture lifespan and exhibited an inverse relationship between the age of donors and the proliferative capacity of the cells in culture. Skin samples from young aged (< 3 years) donors, specially from newborns, exhibited higher cell viability than those from adult donors (Fig.32A). When these cells were plated, the attachment rate for keratinocytes derived from newborns and adult donors varied from 35% to 50%. The newborn keratinocytes, in all instances, grew better than adult keratinocytes plated at equal density and maintained under identical conditions. The current study also demonstrated that the keratinocytes obtained from foreskin of young donors had a faster growth rate compared to those from adult donors and reached to confluency in 14-17 days (Fig.32C), on the other hand cultures from adults required an additional 4-5 days to become confluent (Fig.32F).

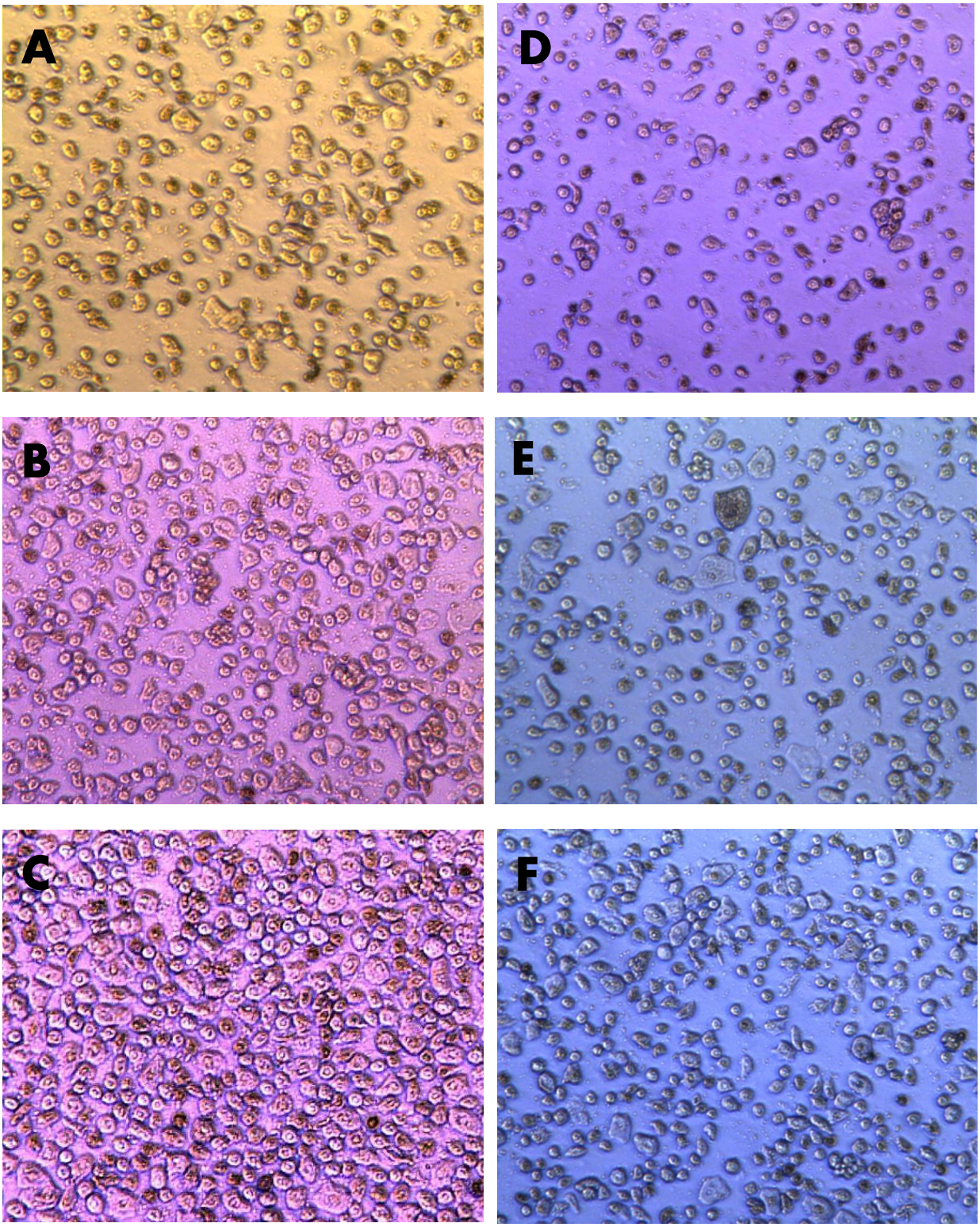

Figure 12: Effect of donor age on human keratinocyte culture. Keratinocytes cultures from newborn donor at (A) 7th day, (B) 10th day, and (C) 14th day; keratinocyte cultures from adult donor at (D) 7th day, (E) 10th day, and (F) 14th day.

3.1.4. EFFECT OF SERUM CONCENTRATION

The effect of different concentrations of fetal bovine serum (FBS), ranging from 5% to 25%, on the proliferation and replicative lifespan of human epidermal keratinocytes was studied in this experiment. The rate of cell proliferation was very poor when serum concentration in the growth medium was as low as 5% (Fig.33A). The increase in cell proliferation was obtained with the increase in serum concentration from 5% to 20%. The proliferation rate was highest and most uniform in the presence of 10% and 15% serum in the growth medium (Fig.33B), with no significant difference between them. Cells grown in 5% serum supplemented medium failed to reach to confluency even in three weeks, whereas those cultured in 10% and 15% serum attained confluency in 2-3 weeks. When serum concentration was 20% or higher, the cell proliferation rate decreased (Fig.33C) and the cells formed clumps instead of growing in monolayer (Fig.33D).

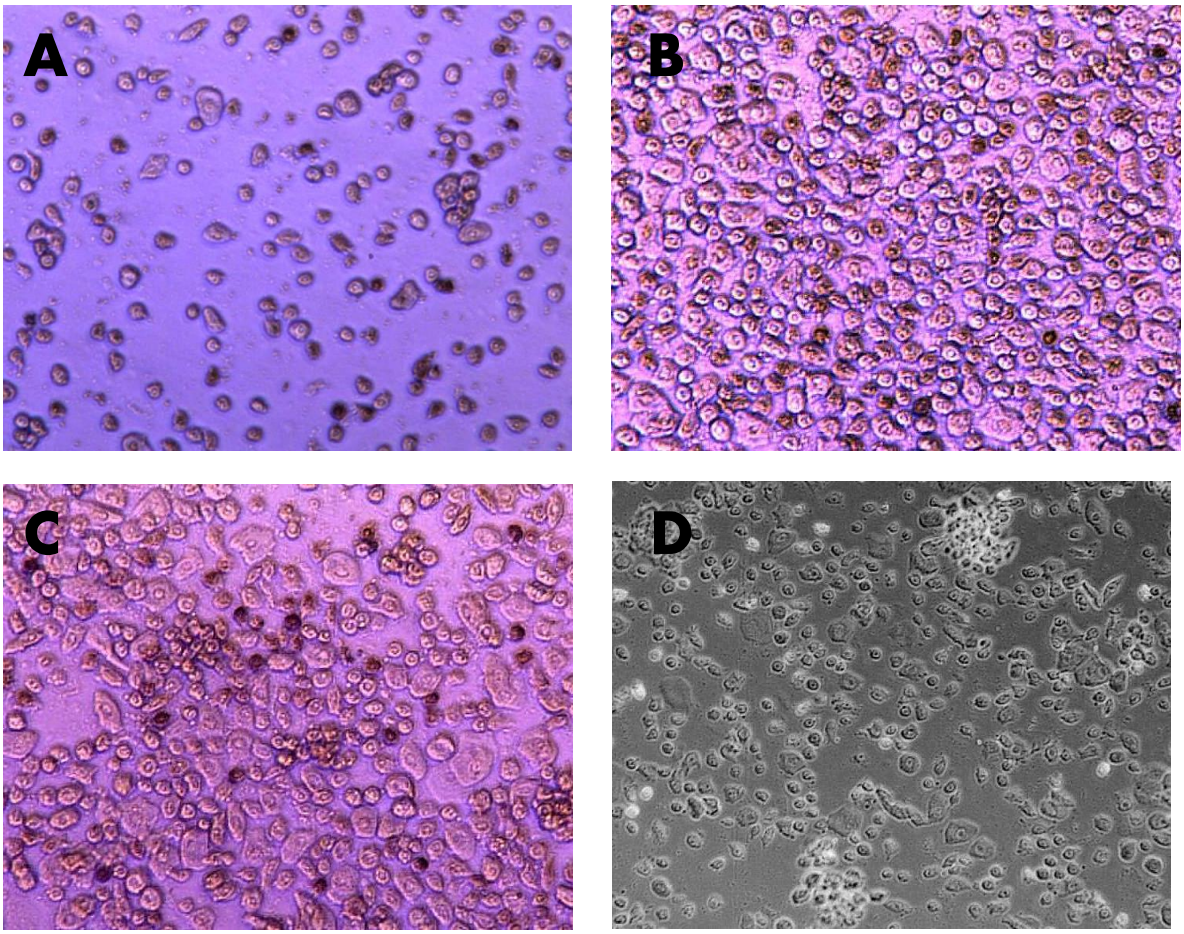

Figure 13: Human epidermal keratinocytes at 14th day of culture in growth medium containing (A) 5%, (B) 10%, (C) 20%, and (D) 25% serum.

3.1.5. EFFECT OF GROWTH FACTORS

Human epidermal keratinocytes obtained from young donors' foreskin and plated at 2.5×10^5cells/ml grew to confluency in two weeks on gelatin-coated culture flask in DMEM containing 10% FBS and antibiotics (100 U⁄ml penicillin and 100 µg⁄ml streptomycin). The addition of hydrocortisone (0.4 µg/ml) to the growth medium increased the rate of cell proliferation about 2-3 fold (Fig.34A). Supplementing medium with hydrocortisone (0.4 µg/ml) and insulin (5 µg/ml) gave better cell growth than hydrocortisone supplement alone and brought the cultures to confluency within one week (Fig.34C). When DMEM was supplemented with only insulin (5 µg/ml), it gave less cell growth than the mixed insulin-hydrocortisone supplement, but better growth than the hydrocortisone supplement alone (Fig.34B).

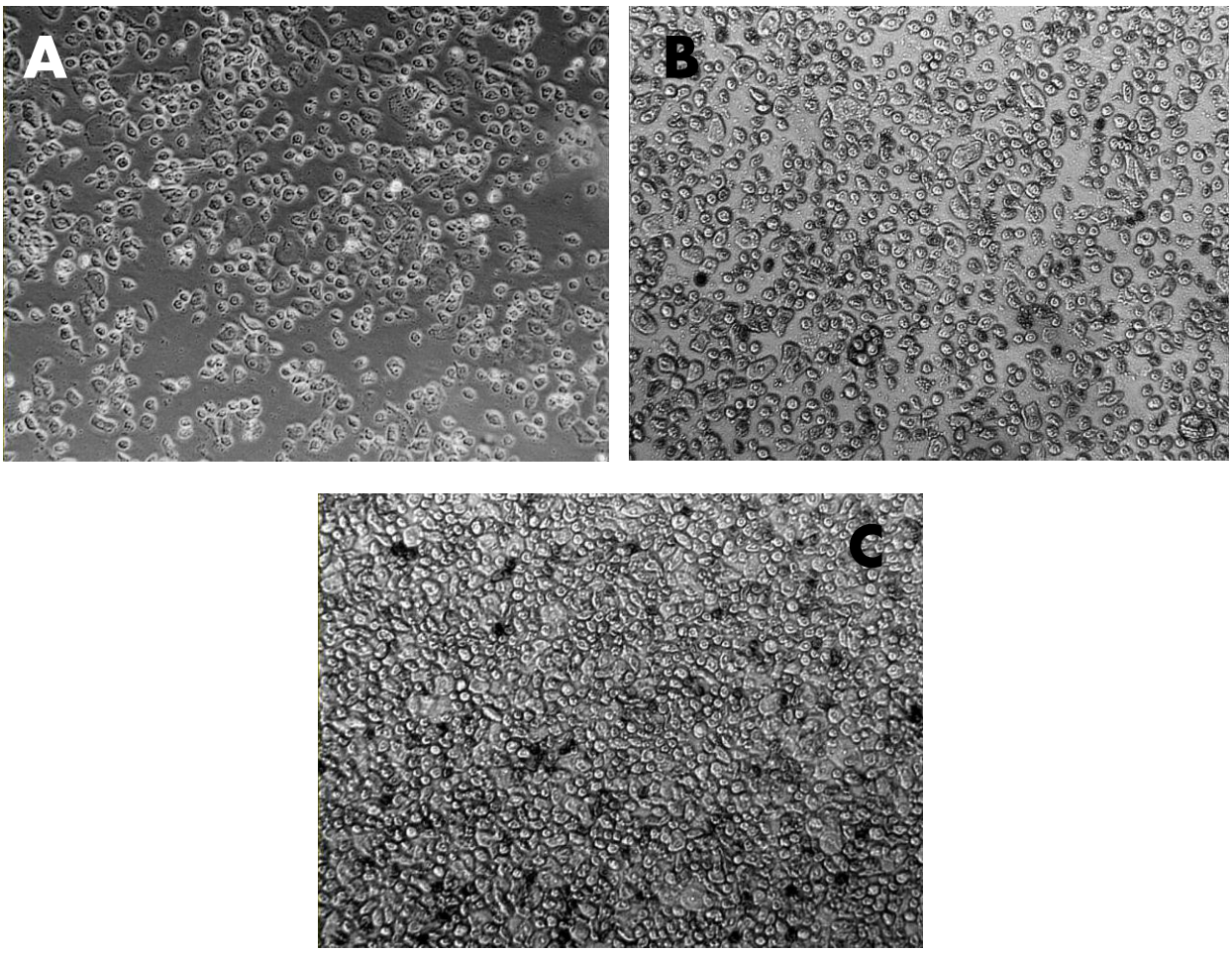

Figure 14: Human epidermal keratinocytes at 6th day of culture in complete growth medium supplemented with (A) hydrocortisone (0.4 µg/ml); (B) insulin (5 µg/ml); (C) both hydrocortisone (0.4 µg/ml) and insulin (5 µg/ml).

3.2. EXPLANT CULTURE

The attempts of explant culture were not completely successful in the present study due to some technical limitations, for which the epidermal outgrowth was obtained only from two skin samples out of ten. In cases of two samples obtained from newborn donors, epidermal keratinocytes were observed growing out of the skin explant between 24 and 36 hour (Fig.35A). The cell outgrowth, over day 3 and 4, continued to expand (Fig.35B).

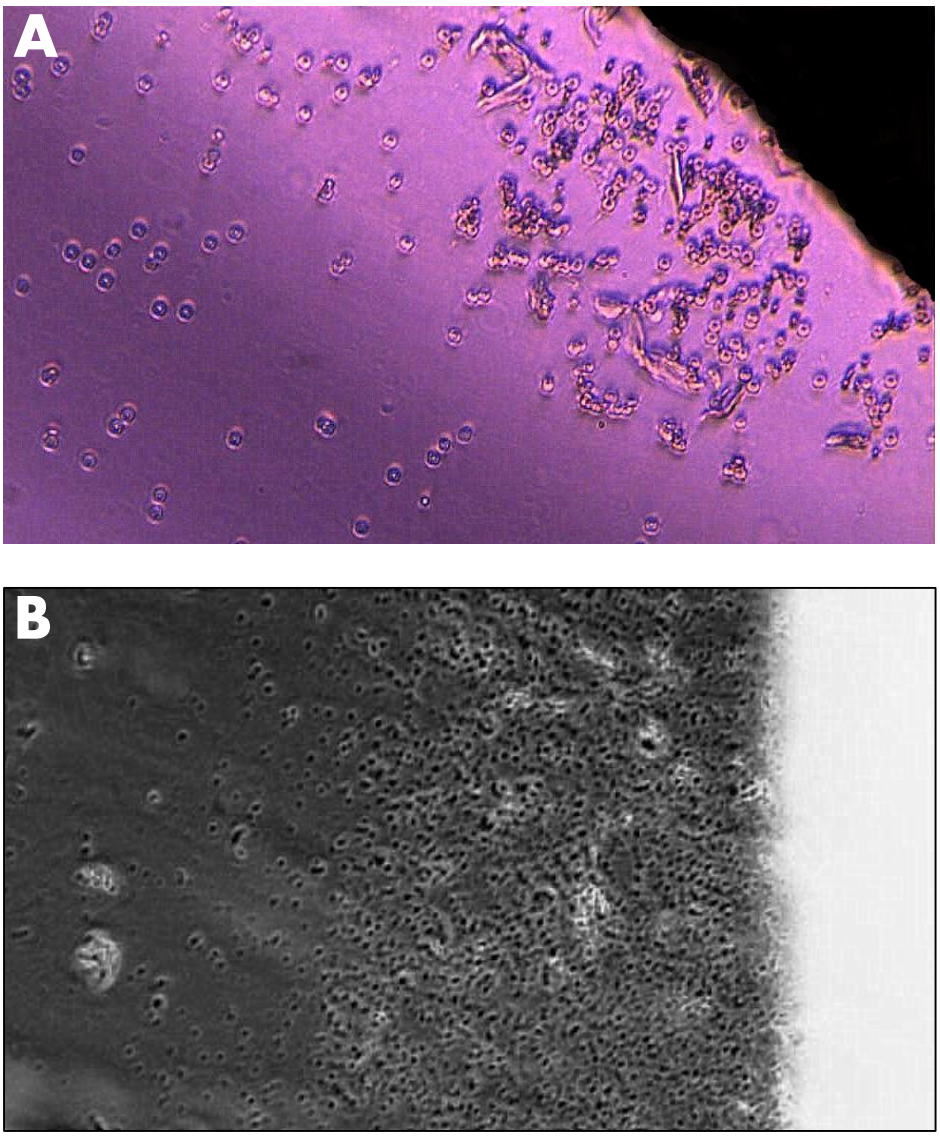

Figure 15: (A) Epidermal cells migrating out of the skin explant in 24 hour; (B) Expanding epidermal cell outgrowth from skin explant at 4th day of culture.

The outgrown epidermal cells, at 6th day of culture, were harvested according to the method reported by Guo and Jahoda (2009) before the migration of fibroblasts from the explant to avoid dermal contamination of culture (Guo and Jahoda, 2009). After seeding these epidermal keratinocytes into new culture dish, the cell proliferation rate was found to be low. Some isolated colonies of epidermal cells developed on the culture dish in 7 days after plating (Fig.36A), which grew to multilayered colonies in 10 days (Fig.36B). But the overall confluent culture was not obtained even in 3-4 weeks.

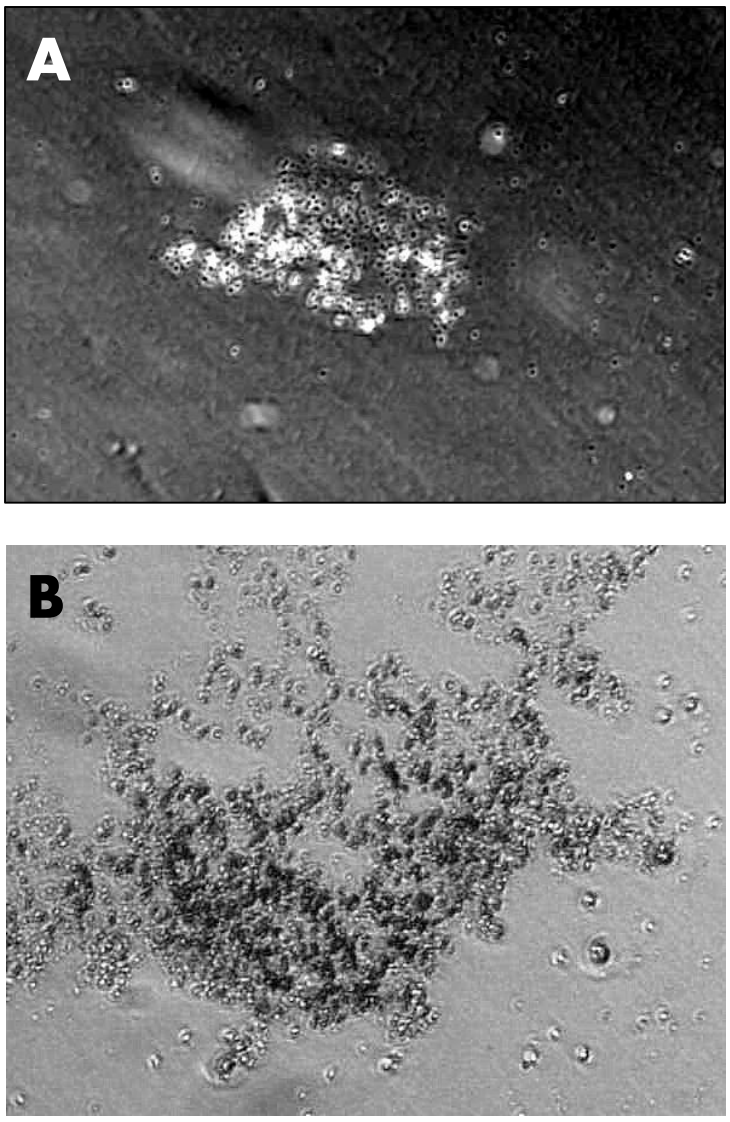

Figure 16: (A) Epidermal cell colony at 7th day of culture after harvesting from skin explant; (B) Epidermal cells grown to multilayered colony at 10th day of culture.

Chapter Two

DISCUSSION

4. DISCUSSION

The present study was designed to optimize culture conditions for the multiplication of human epidermal cells. For this purpose, human foreskin after circumcision was chosen as the source of epidermal cells, because this tissue is readily available than those used by different researchers, such as skin obtained at autopsy (Einbinder *et al*., 1966; Reaven and Cox, 1968; Karasek and Charlton, 1971; Liu and Karasek, 1978), breast skin removed at mastectomy (Reaven and Cox, 1968; Karasek and Charlton, 1971), facial skin after meloplasty (Liu and Karasek, 1978), skin tissue from surgical specimens (Einbinder *et al*., 1966; Briggaman *et al*., 1967), or split-thickness skin (Eisinger *et al*., 1979; Staiano-Coico, 1986). In addition, the use of circumcision skin also sidesteps the matter of ethical concern prevailing in case of human fetal tissue used by some recent workers (Johnen *et al*., 2012; Tan *et al*., 2014).

Human foreskin tissues were subjected to two culture techniques, cell dissociation culture and explant culture, for the cultivation of human epidermal keratinocytes to compare both techniques with each other for the determination of more convenient one.

Explant culture is the oldest method of human skin culture and after various modification in the culture system, attempts to acquire purified adult stem-cell like/progenitor keratinocytes from whole human skin are ongoing in many laboratories (Guo and Jahoda, 2009). In spite of this, the dissociation method of keratinocyte primary culture has become well established, because in many cases, the fibroblasts grow out from the skin explants during culture and it becomes difficult to separate the fibroblasts from the keratinocytes once the two cell types are mixed. This fact was confirmed by Jensen *et al*. in 1981, who reported the appearance of spindle-shaped fibroblastic cells outside the border of the epithelial growth sheet between 7 and 14 days after initiation of explant cultures incubating at 37^0C and the acceleration of their growth afterwards (Jensen *et al*., 1981). In the present study, therefore, the dissociation culture was more extensively studied than explant culture.

For cell dissociation culture, the tissue has to be disaggregated either mechanically or using enzymes or chemicals to obtain cell suspension. Although mechanical disaggregation is faster than enzymatic method and is comparatively cheaper as it does not require large quantities of expensive

enzyme, it results in extensive damage to live cells and consequently lower recovery rate (Shenoy, 2007). On the other hand, using enzymes for dislodging epidermal cells of skin tissue yields high number of viable cells. There are two important enzymes used in tissue disaggregation, collagenase and trypsin. Since the intracellular matrix contains collagen, the epithelial cells can be damaged by collagen but the fibrous tissues remain unaffected (Shenoy, 2007). The crude collagen also contains non-specific proteases. Therefore, trypsin is the most suitable enzyme, which was used for tissue dispersion in this study.

For enzymatic dissociation of epidermis, human foreskin can be subjected to either cold trypsinization, where skin tissue is overnight digested at 4^0C (Eisinger *et al*., 1979; Nelson *et al*., 1980; Ponec *et al*., 1981), or warm trypsinization, where skin tissue is exposed to warm trypsin at 37^0C (Vaughn *et al*., 1971; Liu and Karasek, 1978; Marcelo *et al*., 1978; Stanley *et al*., 1980). At higher temperature, warm trypsinization makes the dissociation of cells faster, but the cell surface proteins may also be cleaved sometimes due to the proteolytic activity of trypsin, which leads to dysregulation of cell functions. On the other hand, cell damage is likely to be less at low temperature, resulting in higher yields. Although lowering the temperature slows protease activity, it permits greater diffusion of aqueous solutions at the undulating epithelial-connective tissue interface (Szabo, 1955). Moreover, some investigators have hypothesized that cold trypsin produces separation in the lamina lucida between basal cell plasma membrane and the basal lamina (Jensen and Moffet, 1970; Briggaman *et al*., 1971) leading to more reliable dermo-epidermal separation.

The method of cold trypsinization, hence, was adopted in this study for the successful disaggregation of epidermis. The effect of different concentrations of trypsin-EDTA solution upon skin tissue was comparatively analyzed in this study. When the trypsin-EDTA concentration was as low as 0.125%, the enzyme could not successfully perform the proteolytic cleavage, for which the epidermal layer could not be separated from the underlying dermis. At a concentration of 0.2%, the cell viability was found to be highest, but the total yield of cells was very poor. The appropriate concentration of trypsin-EDTA in this study was determined to be 0.25%, which resulted in highest yield of cells along with higher cell viability and was similar to other reporters (Eisinger *et al*., 1979; Nelson *et al*., 1980). But unlike Nelson *et al*. (1980), who used an incubation period of 18-24 hours, the incubation period in the present study had not been exceeded over 18 hours. In one instance, the incubation period of tissue in trypsin solution exceeded 20 hours, which led to

damage of epidermis and sharp decrease in cell viability. It supported the work of Sutradhar *et al*. (2010), who studied the time-dependent cytotoxic effect of trypsinization on equine chondrocytes and reported rapid decrease of cell viability, morphological change of cells to blebbing and loss of their membrane integrity by longer trypsinization (Sutradhar *et al*., 2010). For the isolation of epidermis, the skin tissues were processed and trypsinized as soon as possible after sample collection, usually within 2-4 days. Aesen and Belmonte (2010) suggested that samples should ideally be processed on the same day or the next morning of collection owing to gradual loss of yield, which takes part particularly after 24 hour (Aesen and Belmonte, 2010). Due to some technical limitations, however, sample processing could not be done as early in the present study as reported by Aesen and Belmonte (2010). If this limitation could be overcome, that could result in greater yield with higher cell viability.

The epidermal cell population obtained through cold trypsinization was predominantly composed of two types of cells as shown in Fig. 26. One of them were small in size and to some degree rounded or polygonal in shape. The other ones were large, flat cells. A large volume of cells had a morphologic appearance, which was transitional between these two distinct cell types. These results were comparable to that of Briggaman *et al*. (1967), who reported the small cells to be the basal cells and the large, flat cells to be upper malpighian cells (Briggaman et al., 1967) at varying stages of differentiation. Again a very few cells were also observed after plating, which possessed cytoplasmic process and assumed to be Langerhans cells as reported by Eisinger *et al*. (Eisinger *et al.*, 1979). After plating, cell attachment to the substrate occurred within 24-36 hours and the unattached cells were washed away during the first media change. Such attached cells were named as ‘germinative cells’ by Moore and Karasek (1971) and the unattached cells, ‘non-germinative cells’ (Moore and Karasek, 1971).

Human epidermal keratinocytes are anchorage-dependent cells and require appropriate substrate for their survival, growth, migration and differentiation. Stanley *et al*. proposed epidermal cell attachment to substrate to be important in terms of differentiation (Stanley *et al*., 1980). There is evidence to suggest that epidermal cells tend to differentiate terminally if separated from their mesenchymal substrate, and that contact with this substrate is important in preventing terminal differentiation and cell death. McLoughlin (1961) showed morphologically that cells in the epidermis of chick embryos stopped proliferating and differentiated terminally in the absence of contact with mesenchyme, but in its presence proliferation continued and the basal layer was

maintained (McLoughlin, 1961). Briggaman and Wheeler (1968) showed that adult human epidermis required contact with the dermis (even if the dermis was made nonviable by freezing and thawing) in order to maintain a proliferating basal layer (Briggaman and Wheeler, 1968). In the absence of direct contact with the dermis, the basal layer no longer incorporated tritiated thymidine and the epidermis degenerated. Green has shown that human epidermal cells will cease to multiply and terminally differentiate if placed in suspension culture (Green, 1977). Morrissey and Green (1978) have shown that cells from a keratinocyte line from a mouse teratoma will rapidly loose colony-forming ability in suspension culture (Morrissey and Green, 1978). It seems, therefore, that the nature of the substrate and the contact of epidermal keratinocyte with that substrate are important in maintaining cell proliferative capacity and, perhaps, in preventing terminal differentiation.

Influence of substrate was studied by evaluating three types of substrates in this study and the results presented that the attachment to the substrate and the subsequent growth of keratinocytes were most significant in gelatin-coated flask, intermediate in medium-conditioned flask, and poor in plastic culture flask. Gelatin is a heterogeneous mixture of water-soluble proteins extracted from animal collagen and its use to coat culture flask in this study was equivalent to those workers who used collagen-coated dish as culture substrate (Karasek and Charlton, 1971; Liu and Karasek, 1978). Here the gelatin substrate was not a requirement for epidermal growth and maintenance but may select for attachment of basal cells and discourage attachment of cells committed to differentiate (Moore and Karasek, 1971; Liu and Karasek, 1978; Stanley *et al*., 1980). This may provide a proliferative stimulus since differentiated cells inhibit proliferation of basal cells in vitro (Liu and Karasek, 1978) and removal of differentiated cells prior to culture stimulates basal cell proliferation (Reaven and Cox, 1968). The better growth of cells on collagen is not unique for epidermal keratinocytes. In 1956, Ehrmann and Gey noted an improved growth of several cell strains when grown on crude reconstituted rat tail collagen, compared with a glass culture surface (Ehrmann and Gey, 1956). Subsequently, other investigators described the growth of human fetal liver cells and neonatal rat liver explants on collagen (Cleaton and Beswick, 1972; Hillis and Bang, 1962)

In addition to appropriate substrate, donor age was also a critical factor for the growth of human keratinocytes. Rheinwald and Green, in 1975, compared the *in vitro* proliferative behavior of seven newborn-derived keratinocyte cultures to that of three cultures derived from donors aged 3, 12,

and 34 years. Newborn cultures underwent a calculated 25 to 51 cell generations and could be maintained through 3 to 6 passages, while the older cultured underwent 20 to 27 generations and could be maintained through only 2 to 3 passages under identical conditions. Plating efficiency ranged up to 15.7% for the newborn keratinocytes and usually exceeded 2%, while the highest plating efficiency observed for the postnewborn keratinocytes was 0.7% for the 12-year donor. Total keratinocyte population expansion in culture averaged 5737-fold for the newborn keratinocytes, and 333-fold for the older donors. These data strongly suggested that donor age influenced keratinocyte lifespan and proliferative capacity in vitro, which were supported by the reports of other workers (Rheinwald and Green, 1975). A similar result was also observed by Gilchrest in 1983, who indicated that keratinocytes derived from newborn foreskin had a faster growth rate than do keratinocytes derived from skin of healthy adults. In this concern, the results supported the hypothesis that the age-associated decrease for keratinocyte proliferation *in vitro* might be due to progressive loss of mitogenic responsiveness by showing greater response of newborn keratinocytes to specific mitogens, i.e., EGF and KGF, than that of older keratinocytes (Gilchrest, 1983).

The present experiments showed similar results to the previous works concerning the relation between donor age and keratinocyte culture. When epidermal cells obtained from young donors (less than 3 years old) were cultured, it attained confluency approximately in two weeks exhibiting faster cell proliferation rate. On the contrary, the plating efficiency of cultured keratinocytes from adult donors of 3-15 years old reached to confluent culture in 18-21 days. The total cell expansion was higher in the cell cultures of young donors than that of adult donors. These data suggested that donor age influences keratinocyte lifespan and proliferative capacity *in vitro* and that quantitative effects are also comparable with those observed for dermal fibroblasts (Hayflick and Moorehead, 1961; Martin et al., 1970; Schneider and Mitsui, 1976).

In the present study, when epidermal cells from newborn donors were cultured on gelatin substrate in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at a temperature of 37^0C and pH of 7.4, the confluent culture was attained in 2-3 weeks. DMEM is a nutrient-rich formulation containing higher concentration of amino acids, vitamins and glucose to support growth of mammalian cells in culture. Serum contains a number of growth factors, lipids and trace elements and its optimal concentration of 10% as determined in this study is similar to those reported by Karasek (1966) for explant cultures (Karasek, 1966). When the growth medium

was supplemented with insulin (5 µg/ml) it resulted in 3-4 fold increase in keratinocyte culture, which was higher than hydrocortisone (0.4 µg/ml) supplementation alone, although most significant growth was obtained by supplementing serum containing medium with both hydrocortisone (0.4 µg/ml) and insulin (5 µg/ml), which supported the culture to become confluent within one week. However, after performing subculture the growth rate of keratinocytes dramatically decreased and cells could not survive for long term culture. It is assumed that the trypsinization procedure during subculturing might damage the cell surface protein, due to which they could not adhere to the substrate after passaging and led to cellular death. It is comparable to the fact reported by Frisch and Francis (1994) that apoptosis was induced in an immortalized human keratinocyte cell line when it was prevented from attaching to the substratum (Frisch and Francis, 1994). Another reason behind the failure of cell survival might be the occurrence of terminal differentiation. Application of trypsin for least amount of time and addition of epidermal growth factor (EGF) to the culture medium since after passaging might be helpful to improve this condition. According to Rheinwald and Green (1977), EGF delays senescence of keratinocytes by increasing the distance of the multiplying cells from obligatory differentiation. This effect was revealed by a greater ability of the cells to survive subculture and initiate new colonies. (Rheinwald and Green, 1977).

In the present study, the attempts of explant culture were not fully successful. In some instances, no outgrowth of cells was obtained, probably because the edge of the explants could not be sharply cut always and the epidermal cells did not come out through the blunt edges. Other possible reasons might be the size of explants and the inappropriate trimming of underlying subcutaneous tissues for which the explants failed to attach to the substrate and to give cell outgrowth. Although some explants exhibited cell migration out of the explants within 36-48 hours, they did not finally develop to confluent culture, most likely due to the heterogeneity nature of the culture system.

5. CONCLUSION

This study was conducted to investigate the optimum conditions for the cultivation of human epidermal keratinocytes. Cell dissociation culture was successfully adopted for human keratinocyte culture, while explant culture showed slower cell growth, lower cell yield and failure to develop confluent culture. In this study, epidermal cells from newborn and infant exhibited greater proliferation rate than that of middle childhood and teenaged donors. After plating the disaggregated cells, a gelatin-coated plate was found better for both attachment and growth of epidermal cells. Finally, optimal growth of human keratinocytes was obtained in DMEM supplemented with 10% FBS, 0.4 µg/ml hydrocortisone and 5 µg/ml insulin. For cell dissociation culture, further study is required regarding the effects of other growth factors like epidermal growth factor (EGF), cholera toxin etc. Explant culture needs to aim more extensive studies as it provides an easy method of keratinocyte culture and possesses potential to obtain cells of basal layer in particular with high proliferative capacity. In addition, a robust cost-effective culture method is needed to establish cultured stratified epidermal sheets to use them in the treatment of burn patients as allografts or autografts.

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APPENDICES

APPENDIX - I

Equipment

The important equipment used in the study are listed below:

- Glass Filtration unit
- Filter paper
- Glass bottles: 1000 ml
- Duran bottles: 500 ml
- Falcon tubes: 15 ml, 50 ml
- Measuring cylinders: 10 ml, 25 ml, 50 ml, 500 ml
- Petri dishes: 90 mm
- Plastic culture dishes: 60 mm, 90 mm
- Plastic culture flasks: 25 cm^2
- Beakers: 100 ml
- Pipettes: 5-40 µl, 40-200 µl, 100-1000 µl
- Tweezers
- Scissors
- Scalpel
- Sterile gloves
- Waste container
- Biosafety cabinet: Class II, Type A
- Incubator: 37^0C, 90% humidity, 5% CO_2
- Hemoccytometer
- Centrifuge
- Inverted Microscope
- Electric balance
- Autoclave
- Refrigerator

Reagents

All the necessary reagents used in the study are listed below:

• Dalbecco's Modified Eagle's Medium (DMEM)	Sigma Aldrich, USA
• Sodium bicarbonate (Na2CO3)	Sigma Aldrich, Germany
• Fetal bovine serum (FBS)	GIBCO, Newzealand
• Newborn calf serum	GIBCO, Newzealand
• Penicillin	Invitrogen
• Streptomycin	Invitrogen
• Trypsin/EDTA (0.25%)	GIBCO, Canada
• Trypan blue dye	GIBCO, Invitrogen
• Gelatin	Sigma Aldrich, Germany
• Hydrocortisone	Sigma Aldrich, USA
• Insulin	Sigma, USA
• Sodium Chloride (NaCl)	Sigma Aldrich, Germany
• Sodium hydrogen phosphate ($Na_2HPO_4.7H_2O$)	Sigma Aldrich, Germany
• Potassium chloride (KCl)	Sigma Aldrich, Germany
• Potassium di hydrogen orthophosphate (KH_2PO_4)	Sigma Aldrich, Germany

APPENDIX - II

Reagent setup

PBS

Phosphate buffered saline (PBS) was prepared by adding following amounts of KCl, KH_2PO_4, NaCl and $Na_2HPO_4.2H_2O$ in de-ionized water. The container was shaken to dissolve the salts properly into the water. Then the PBS was autoclaved and stored in a refrigerator at 4^0C.

Name of salts	Amount of salts (g/l)
KCl	0.20 g
KH_2PO_4	0.20 g
NaCl	8.00 g
$Na_2HPO_4.7H_2O$	3.60 g

Trypsin solution

Various concentration of trypsin-EDTA solution was prepared by diluting 2.5% trypsin-EDTA in PBS. The amount of trypsin and PBS are as follows:

Concentration of Trypsin	Amount of Trypsin-EDTA (µl/ml)	Amount of PBS (µl/ml)
0.125 % Trypsin	50 µl	950 µl
0.25 % Trypsin	100 µl	900 µl
0.3 % Trypsin	120 µl	880 µl
0.35 % Trypsin	140 µl	860 µl

Gelatin 0.1% solution

0.1 g of gelatin powder was weighed and added to 100 ml of de-ionized water. The container was swirled and then heated to dissolve the gelatin powder. It was then sterilized by autoclaving and stored at 4^0C for up to 2 months.

Basal medium (DMEM)

13.4 g DMEM powder was added to a container containing 950 ml of sterile de-ionized water. The container was shaken to dissolve the powder. Then 3.7 g of $NaHCO_3$ was added to the solution and dissolved properly into it. This basal medium was then filter sterilized and stored at 4^0C up to 6 months.

Growth medium

DMEM was supplemented with 10% fetal bovine serum (FBS) or 10% newborn calf serum and antibiotics (penicillin/streptomycin) to prepare the growth medium. In 22.25 ml of DMEM solution, 2.5 ml of fetal bovine serum (FBS) or newborn calf serum and 250 µl of Penicillin/Streptomycin was added giving a final concentration of 10% serum, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin and the final volume of the media was 25 ml. the media was stored at 4^0C and used within 2 weeks. Based on this measurements different volume (12 ml, 25ml, 50 ml etc.) of growth medium was prepared as per necessity.

Complete growth medium

To prepare complete growth medium, following supplements were added to the growth medium:

Supplements	Final concentration	
Hydrocortisone	0.4 µg/ml	8.3×10^{-7} M
Insulin	5 µg/ml	8.7×10^{-7} M

Medium supplements

Hydrocortisone

Stock solution:

- 0.01 mg Hydrocortisone was dissolved into 2.5 µl of 95% ethanol.
- The solution was diluted at 1:10 (v/v) by adding 25 µl of growth medium to the solution to make 8.3×10^{-4} M, a 1000X stock solution.
- The stock was stored at -20^0C.

Working solution:

- The stock solution was diluted at 1:1000 (v/v) by adding 25 ml of complete growth medium to the solution giving the final concentration of 8.3×10^{-7} M.
- It was stored at -20^0C.

Insulin

Stock solution:

- 0.1 mg insulin was dissolved into 0.02 ml of 0.05M HCl in UPW.
- The solution was diluted at 1:10 by adding 0.2 ml PBS to the solution to make the stock solution.
- The stock was filter sterilized by 0.22 µm filter.
- It was then stored at -20^0C.

Working solution:

- The stock solution was diluted at 1:100 by adding 20 ml of complete growth medium to the solution giving the final concentration of 5 µg/ml, 8.7×10^{-7} M.
- It was stored at 4^0C.