

**Characterization and *in vivo* Evaluation of Amniotic Membrane
and Collagen Based Hydrogel on Rat Model**

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Submitted By

MD. AKIB ULLAH

Examination Roll No: MS 170712

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**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY
KHULNA-9208, BANGLADESH**

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Md. Akib Ullah

Examination Roll No: MS 170712

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Approved as to the style and content by


.....
18/09/19

External Supervisor

S. M. Asaduzzaman, PhD

Chief Scientific Officer and Director

Institute of Tissue Banking and Biomaterial Research

Atomic Energy Research Establishment

Savar, Dhaka.


.....

Internal Supervisor

Dr. Md. Shamim Akhter

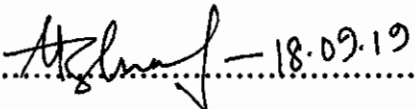
Professor

Biotechnology and Genetic Engineering

Discipline, Life Science School,

Khulna University

Khulna.


.....
18.09.19

Professor Dr. Ayesha Ashraf

Chairman

Examination Committee

Biotechnology and Genetic Engineering Discipline

Khulna University

Khulna-9208

Bangladesh.

Characterization and *in vivo* Evaluation of Amniotic Membrane and Collagen Based Hydrogel on Rat Model

DECLARATION

I hereby declare that this thesis is based on my original work except quotations and citations which have been duly acknowledged. The material here has not ever been submitted in a whole or in a part for a degree from Khulna University or any other institutions. This research work will not be published anywhere without the concern of supervisor.



MD. AKIB ULLAH

Examination Roll No: MS 170712

DEDICATED TO

MY BELOVED PARENTS

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Md. Akib Ullah
BGE Discipline, Khulna University

ABSTRACT

Skin burn wounds and injuries continue to be a major problem affecting communities worldwide. Rapid and effective treatment of burnt skin is crucial to fasten wound closure and healing properly. Amniotic grafts are widely used as wound healing biomaterials. Sophisticated processing, high cost, availability, and the need of medics for transplantation limit the application of amnion grafts. This study was aimed to prepare a novel hydrogel from amniotic membrane and collagen extract combined with external epithelial sheath for burn wound healing which overcome the limitations of graft. 2% human amniotic membrane (AM), Collagen and AM-Collagen gels were formulated using different concentrations of carboxymethyl cellulose sodium (CMC-Na) salt. An external thin protective covering known as epithelial sheath (ES) was also prepared using different concentrations of collagen and chitosan. Various physical properties like swelling ratio, water absorption (%), equilibrium water content (%), gel fraction (%) and spreadability of gel formulations were analysed to find out the best concentration of CMC-Na for final wound healing applications. The formulated gels were found at neutral pH, acceptable swelling ratio, good spreadability and acceptable gel fraction (%). But 6% CMC-Na concentration was found to be best for final application. The same process was adopted for epithelial sheaths as well and epithelial sheath containing 10 mg/ml concentration of both collagen and chitosan was found to be optimum for final animal experiment. *In vitro* cytotoxicity and red blood cell biocompatibility tests of final formulated gels were performed. The gels were observed non-cytotoxic at brine shrimp lethality test and compatible with human red blood cells. Following skin irritation study, second-degree burns were induced on dorsal region of wistar rats; and gels along with ES were applied to observe the wound healing potential at *in-vivo* study. Wound contraction and re-epithelialization of gel treated skin were macroscopically measured. No erythema and edema were observed in skin irritation experiments confirming the applicability of the gels. AM-Collagen-ES treated group had maximum average body weight, food intake and water intake. At the end of the study (day 16), AM-Collagen-ES treated group was superior in wound contraction ($72\pm3.27\%$) over other treatment groups and it was statistically more significant ($P<0.0000009$). While negative control group was healed only $22.67\pm2.31\%$, AM-Collagen gel treated group, AM gel treated group, Collagen gel treated group and positive control group were healed $62.5\pm4.43\%$ ($P<0.00001$), $52\pm3.27\%$ ($P<0.00004$), $36\pm4\%$ ($P<0.001$) and $41\pm3.83\%$ ($P<0.0004$) respectively. For complete epithelialization, AM-Collagen-ES treated group was required only (16.75 ± 0.96) days while AM-Collagen gel treatment, AM gel treatment, Collagen gel treatment, positive control and negative control group were needed (18.75 ± 0.50), (20.5 ± 1.29), (24.75 ± 0.96), (22.25 ± 0.96) and (28 ± 0.82) days respectively. From the above result, we can demonstrate that a gel consisting a combination of collagen and amnion with the presence of epithelial sheath has promising effect on the wound healing process.

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ABBREVIATIONS

Symbol:

%	:	Percentage
$^{\circ}\text{C}$	:	Degree Centigrade
=	:	Equal
d	:	Day
g	:	Gram
h	:	Hour
cm	:	Centimeter
w/v	:	Weight/Volume
rpm	:	Revolution Per Minute
M	:	Molar
N	:	Normal
&	:	And
et.al	:	And Others
etc.	:	Etcetera
ml	:	Milliliter
mg	:	Milligram
μl	:	Microliter
μm	:	Micrometer
mm^2	:	Millimeter Square
kGy	:	Kilo Gamma Ray

General Terms:

TE	:	Tissue Engineering
AM	:	Amniotic Membrane
CMC-Na	:	Carboxymethyl Cellulose Sodium
NaCl	:	Sodium Chloride
ES	:	Epithelial Sheath
EWC	:	Equilibrium Water Content
WHO	:	World Health Organization
SD	:	Standard Deviation
RBC	:	Red Blood Cell

ESC	:	Embryonic Stem Cell
ASC	:	Adult Stem Cell
Ang	:	Angioproten
BMP	:	Bone Morphogenic Protein
EGF	:	Epidermal Growth Factor
EPO	:	Erythropoietin
FGF	:	Fibroblast Growth Factor
G-CSF	:	Granulocyte- colony stimulating Factor
GM-CSF	:	Granulocyte-macrophage colony-stimulating Factor
HGF	:	Hepatocyte Growth factor
IGF	:	Insulin-like growth Factor
GDF	:	Growth and differentiation Factor
NGF	:	Nerve Growth Factor
PDGF	:	Platelet Derived Growth factor
TGF	:	Transforming Growth Factor
VEGF	:	Vascular Endothelial Growth factor
GAG	:	Glycosaminoglycan
ECM	:	Extracellular Matrix

CHAPTER ONE

INTRODUCTION



1.1 Background

Wound healing is a multi-factorial physiological process. The complexity of this phenomenon makes it prone to several abnormalities. Apart from cellular and biochemical components, several enzymatic pathways also become active during repair and help the tissue to heal. Burn injury to the skin or other organic tissue is mainly caused by heat or due to radiation, radioactivity, electricity, friction or contact with chemicals. Skin injuries due to ultraviolet radiation, radioactivity, electricity or chemicals, as well as respiratory damage resulting from smoke inhalation, are also considered to be burns (Sabale and Bhimani, 2012). Burn injury is a common phenomenon in our country. According to World Health Organization (WHO), nearly 173,000 Bangladeshi children are moderately or severely burnt every year (<http://www.who.int/violence injury prevention>). Globally, 265000 deaths every year are caused by burn. The majority of these occur in low- and middle-income countries and almost half occur in the South-East Asia Region (Sabale and Bhimani, 2012).

Burn healing is a complex procedure. Ideal burn healing in humans can be described by the following events: rapid homeostasis; appropriate inflammation; mesenchymal cell differentiation, proliferation, and migration to the wound site; suitable angiogenesis; prompt re-epithelialization and proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue (Singh et al., 2011).

Human amniotic membrane (AM) is a gift of nature that has the potential to be used in burn healing. Application of AM in the treatment of disease has been started in 1910 (Mathieu et al., 2006). It is widely used in skin substitution, treatment of acute Stevens-Johnson syndrome and chronic leg ulcer treatment. Amniotic membrane acts as a scaffold for proliferation and differentiation due to the presence of fibronectin, elastin, nidogen, collagen types I, III, IV, V and VI and hyaluronic acid which finally lead to the promotion of epithelialization. Another important advantage of using AM in allotransplant or xeno-transplant is lack of immune response due to the absence of histocompatibility antigens HLA-A, B and DR.

AM also possesses anti-inflammatory effect. Inhibition of expression of certain pro-inflammatory cytokines from damaged ocular surface such as interleukin (IL) 1 α , IL-2, IL-8, tumor necrosis factor- β , interferon- γ and certain growth factors like, basic fibroblast growth

factor and platelet derived growth factor render AM's anti-inflammatory effect. Anti-angiogenic chemicals identified in both epithelial and mesenchymal cells of AM are thrombospondin-1, endostatin and all four types of tissue inhibitors of metalloproteases (TIMP-1, 2, 3 and 4) thus providing anti-angiogenic effect. Mainly inhibition of TGF signal transduction is attributed to anti-scarring effect of AM (Riau and Beuerman, 2010).

Collagen, which is produced by fibroblasts, is the most abundant protein in the human body. A natural structural protein, collagen is involved in all 3 phases of the wound-healing cascade. It stimulates cellular migration and contributes to new tissue development. Because of their chemotactic properties on wound fibroblasts, collagen dressings encourage the deposition and organization of newly formed collagen, creating an environment that fosters healing. Collagen-based biomaterials stimulate and recruit specific cells, such as macrophages and fibroblasts, along the healing cascade to enhance and influence wound healing. These biomaterials can provide moisture or absorption, depending on the delivery system (Fleck and Chakravarthy, 2007).

Formulating a hydrogel using both AM extract and collagen extract is supposed to work better than other existing burn healing ointment as it would contain biomolecules that are necessary to accelerate burn healing.

1.2 Objectives of the Study

The ultimate goal of our study is to formulate a novel cost efficient wound healing product which is in fact easy to produce and store, is physiologically effective and application does not require medication. To secure our project goals we have the following objectives-

- To optimize and formulate a gel consisting of amniotic membrane and collagen extract.
- To characterize and determine the suitable formulated gels for wound healing.
- To prepare, characterize and determine suitable epithelial sheaths for wound healing.
- Evaluation of biocompatibility of the formulated gel for further implementation in burn patients.
- *In vivo* evaluation of the prepared gels and epithelial sheaths on rat model.

CHAPTER TWO
LITERATURE REVIEW

2.1 Structure and Functions of Skin

Skin is the largest organ of the body and consists of two layers-the epidermis and dermis-that are supported by a number of underlying structures. This organ has multiple functions. It is a barrier between the external environment and the internal organs, protecting against trauma from water, chemicals, micro-organisms, mechanical stress and radiation. It also performs sensory functions related to touch, pressure, temperature, pain and alerts for potential tissue damage. Additionally, it has a role in exchanging fluids, salts, gases & heat (Mareib et al., 2014; Saladin 2007; Sussman and Bates-Jensen, 2007).

Temperature of skin varies in a range of 30 to 40 °C degree depending on the environmental conditions (Noble, 1993). The skin is made up of 2 primary layers that interact anatomically and functionally. The epidermis forms a thin overlying protective coat that is easily regenerated after injury and serves to keep moisture inside the body while resisting external chemical corrosion. The dermis is the thick fibrous portion that gives skin its strength and contains blood vessels, nerves, and adnexal structures, such as hair follicles, sweat glands, and sebaceous glands. The skin rests on a subcutaneous tissue that provides support and padding (**Figure 2.1**).

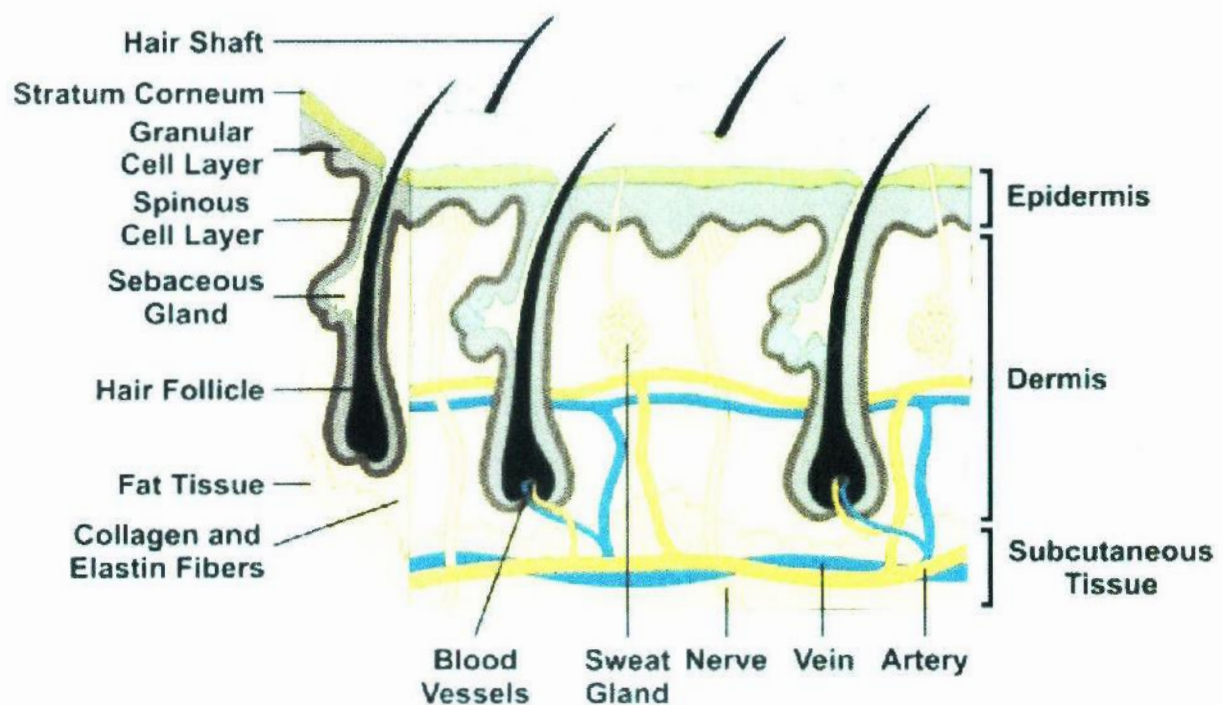


Figure 2.1: Human skin anatomy.

2.1.1 Epidermis

The epidermis is the outermost of the three layers that make up the skin. The epidermis is composed of multiple layers of flattened cells that overlie a base layer (stratum basale) composed of columnar cells arranged perpendicularly (McGrath et al., 2004). The rows of cells develop from stem cells in the basal layer. Cellular mechanisms for regulating water and sodium levels (ENaCs) are found in all layers of the epidermis (Hanukoglu et al., 2017). The human epidermis is a familiar example of epithelium, particularly a stratified squamous epithelium. The epidermis primarily consists of keratinocytes (proliferating basal and differentiated suprabasal), which comprise 90% of its cells, but also contains melanocytes, Langerhans cells, Merkel cells and inflammatory cells (McGrath et al., 2004). Blood capillaries are found beneath the epidermis, and are linked to an arteriole and a venule.

The epidermis is composed of 4 or 5 layers, depending on the region of skin being considered (Figure 2.2). Those layers in descending order are:

- cornified layer (stratum corneum)
- clear/translucent layer (stratum lucidum, only in palms and soles)
- granular layer (stratum granulosum)
- spinous layer (stratum spinosum)
- basal/germinal layer (stratum basale/germinativum)

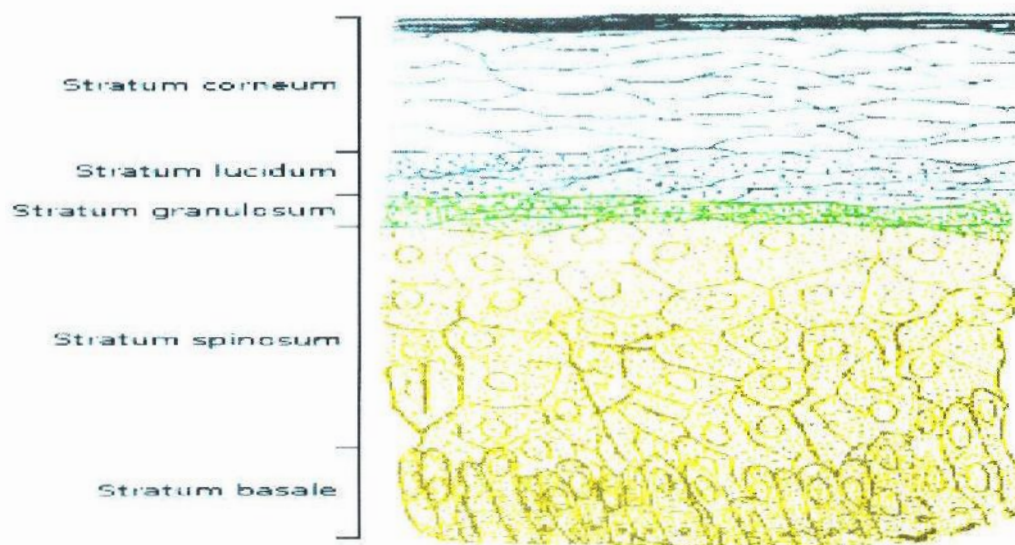


Figure 2.2: Schematic image showing a section of epidermis, with epidermal layers labeled.

2.1.1.1 Epidermis Morphogenesis

Epidermal organogenesis, the formation of the epidermis, begins in the cells covering the embryo after neurulation, the formation of the central nervous system. In most vertebrates, this original one-layered structure quickly transforms into a two-layered tissue; a temporary outer layer, the periderm, which is disposed once the inner basal layer or stratum germinativum has formed (Gilbert, 2000).

This inner layer is a germinal epithelium that gives rise to all epidermal cells. It divides to form the outer spinous layer (stratum spinosum). The cells of these two layers, together called the Malpighian layer(s) after Marcello Malpighi, divide to form the superficial granular layer (Stratum granulosum) of the epidermis (Gilbert, 2000).

The cells in the stratum granulosum do not divide, but instead form skin cells called keratinocytes from the granules of keratin. These skin cells finally become the cornified layer (stratum corneum), the outermost epidermal layer, where the cells become flattened sacks with their nuclei located at one end of the cell. After birth these outermost cells are replaced by new cells from the stratum granulosum and throughout life they are shed at a rate of 0.001 - 0.003 ounces of skin flakes every hour, or 0.024-0.072 ounces per day (Weschler, 2011).

Epidermal development is a product of several growth factors, two of which are (Gilbert, 2000):

- Transforming growth factor Alpha (TGF α) is an autocrine growth factor by which basal cells stimulate their own division.
- Keratinocyte growth factor (KGF or FGF7) is a paracrine growth factor produced by the underlying dermal fibroblasts in which the proliferation of basal cells is regulated.

2.1.1.2 Functions of Epidermis

General functions of epidermis are (Elias, 2007):

- Protection from bacterial entry
- Protection from toxins
- Fluid balance; prevents excess evaporative loss
- Neurosensory social-interactive function

- Provides protection against trauma and harmful environment and organisms
- Abrasion occurs if epidermis is damaged.
- Protection from desiccation

2.1.2 Dermis

The dermis or corium is a layer of skin between the epidermis and subcutaneous tissues that primarily consists of dense irregular connective tissue and cushions the body from stress and strain. It is divided into two layers, the superficial area adjacent to the epidermis called the papillary region and a deep thicker area known as the reticular dermis (James et al., 2005) (**Figure 2.3**). The dermis is tightly connected to the epidermis through a basement membrane. It also contains mechanoreceptors that provide the sense of touch and thermo-receptors that provide the sense of heat. In addition, hair follicles, sweat glands, sebaceous glands (oil glands), apocrine glands, lymphatic vessels, nerves and blood vessels are present in the dermis. The dermis is composed of three major types of cells: fibroblasts, macrophages, and adipocytes. Apart from these cells, the dermis is also composed of matrix components such as collagen (which provides strength), elastin (which provides elasticity), and extrafibrillar matrix, an extracellular gel-like substance primarily composed of glycosaminoglycans (most notably hyaluronan), proteoglycans, and glycoproteins (Marks and Miller, 2006).

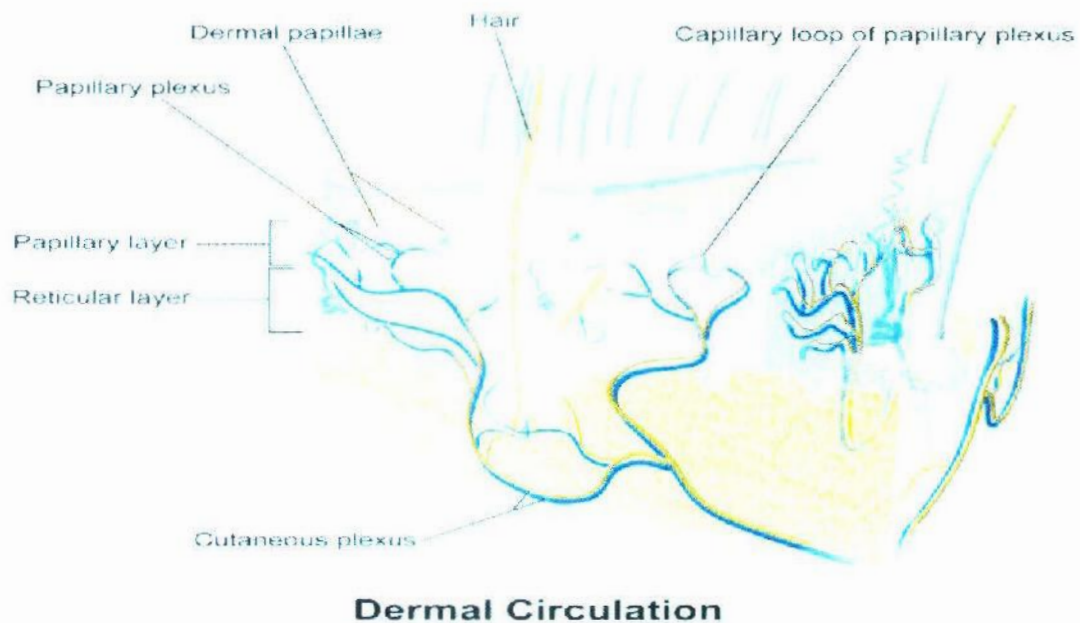


Figure 2.3: Illustration of dermal circulation and layers.

2.1.2.1 Dermis Development

Dermal cells undergo multiple developmental stages to build a functional layer of the skin with constant interactions with the overlying epidermis. Dermal fibroblast cells begin as loosely packed mesenchymal dermal precursor cells underneath the ectoderm. Then, a thin layer of dense dermis forms underneath the ectoderm, which is distinguishable from the underlying mesenchyme (2.60 nuclei/1000 μm^3 vs 1.96 nuclei/1000 μm^3) (Mayerson and Fallon, 1985). The overlying epithelium subsequently forms localized structures called epidermal placodes, presumably by dermal signal (Olivera-Martinez et al., 2001). Placode formation is considered the first sign of differentiation of appendages such as feathers and scales in avians, or hair follicles in mammals (Olivera-Martinez et al., 2001). In turn, epidermis signals back to the dermis to redistribute the dense dermis into dermal condensates of the placodes. Both components then undergo extensive cell proliferation, movement and differentiation to give rise to appropriate appendages.

2.1.2.2 Functions of Dermis

General functions of dermis are (Mast, 1992):

- Fluid balance through regulation of skin blood flow
- Thermoregulation through control of skin blood flow
- Growth factors for epidermal replication and dermal repair
- Provides skin flexibility and strength
- Bleeding occurs and the body's first line of defense is breached. Healing is multifactorial.
- Protection from trauma (due to elasticity and durability properties of the dermis)

2.1.3 Sweat Gland

Sweat glands, also known as sudoriferous or sudoriparous glands, from Latin sudor, meaning 'sweat', are small tubular structures of the skin that produce sweat. Sweat glands are a type of exocrine gland, which are glands that produce and secrete substances onto an epithelial surface by way of a duct (**Figure 2.4**). There are two main types of sweat glands that differ in their structure, function, secretory product, mechanism of excretion, anatomic distribution, and distribution across species (Bullard et al., 1970):

- Eccrine sweat glands are distributed almost all over the human body, in varying densities, with the highest density in palms and soles, then on the head, but much less on the trunk and the extremities. Its water-based secretion represents a primary form of cooling in humans.
- Apocrine sweat glands are mostly limited to the axilla (armpits) and perianal areas in humans. They are not significant for cooling in humans, but are the sole effective sweat glands in hoofed animals, such as the camels, donkeys, horses, and cattle.

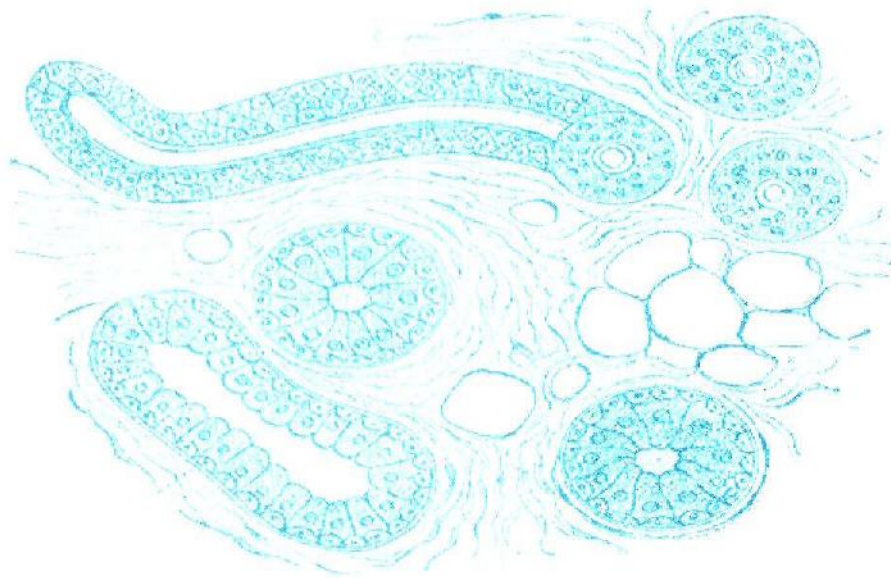


Figure 2.4: Body of a sweat gland cut in various directions.

2.1.3.1 Sweat Gland Development

Approximately 3 million eccrine sweat units are present at birth, and no additional ones are formed thereafter. It is during the fourth week of embryologic development (the embryo measures about 1.25 cm or 0.5 inch at this point), that certain cell types (ectoderm and mesoderm) begin to multiply and differentiate into noticeable and specific structures. The specialized structures formed by the skin, including teeth, hair, hair follicles, fingernails, toenails, sebaceous glands, sweat glands, apocrine glands, and mammary glands begin to appear during this period in development.

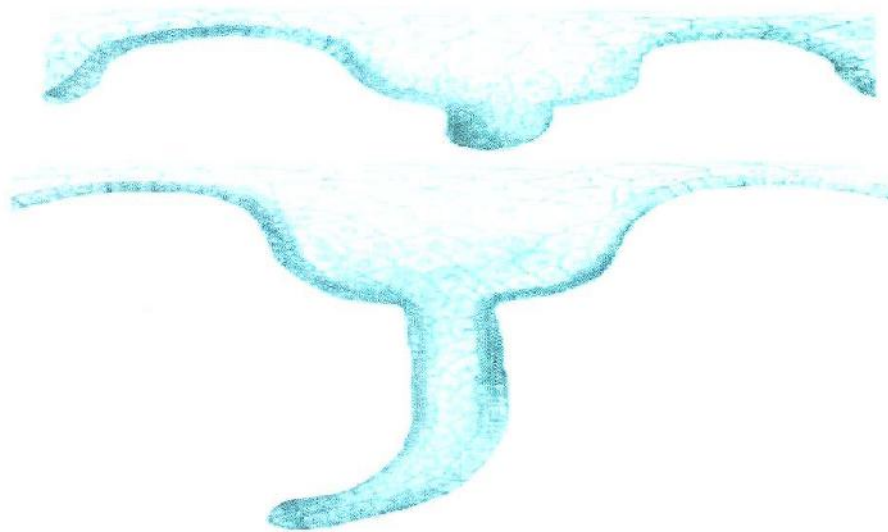


Figure 2.5: Sweat glands develop from outside in.

Eccrine sweat glands first appear on palms and soles during the 12th week of development. Sweat glands actually develop by budding their way as downgrowths from the epidermis (**Figure 2.5**). By the sixth month of intrauterine life, when secretion of sweat begins, the sweat glands look very much like mature glands consisting of the three structural components discussed above. No new sweat glands are formed after birth (www.drypharmacist.com).

2.1.3.2 Functions of Sweat Gland

Each one of these portions or segments of the sweat gland have a specific function (www.drypharmacist.com):

- As its name implies, the secretory segment is responsible for the production of sweat - without the hard work of these little sweat gland components, we would all be in serious trouble: our bodies would heat up (hyperthermia) and damage to our vital organs would result.
- During its passage through ductal portion of the gland, sweat is desalinated by cells that line the duct. In other words, salty elements such as sodium and chloride are reabsorbed to render a significantly less salty or hypotonic solution
- The acrosyringium is the least active part of the sweat gland - it serves as a conduit for perspiration's final journey to the surface of the skin.

2.1.4 Sebaceous Gland

Sebaceous glands are microscopic exocrine glands in the skin that secrete an oily or waxy matter, called sebum, to lubricate and waterproof the skin and hair of mammals. In humans, they occur in the greatest number on the face and scalp, but also on all parts of the skin except the palms of the hands and soles of the feet. The type of secretion of the sebaceous glands is referred to as holocrine. In the eyelids, meibomian glands, also called tarsal glands, are a type of sebaceous glands that secrete a special type of sebum into tears. Areolar glands surrounding the female nipples are another example of sebaceous gland. Fordyce spots are ectopic (misplaced) sebaceous glands found usually on the lips, gums and inner cheeks, and genitals.

2.1.4.1 Sebaceous Gland Development

Sebaceous glands are first visible from the 13th to the 16th week of fetal development, as bulgings off hair follicles (Thiboutot, 2004) (**Figure 2.6**). Sebaceous glands develop from the same tissue that gives rise to the epidermis of the skin. Overexpression of the signaling factors Wnt, Myc and SHH all increase the likelihood of sebaceous gland presence (Smith and Thiboutot, 2007).

The sebaceous glands of a human fetus secrete a substance called vernix caseosa, a waxy, translucent white substance coating the skin of newborns. After birth, activity of the glands decreases until there is almost no activity during ages 2–6 years, and then increases to a peak of activity during puberty, due to heightened levels of androgens (Thiboutot, 2004).

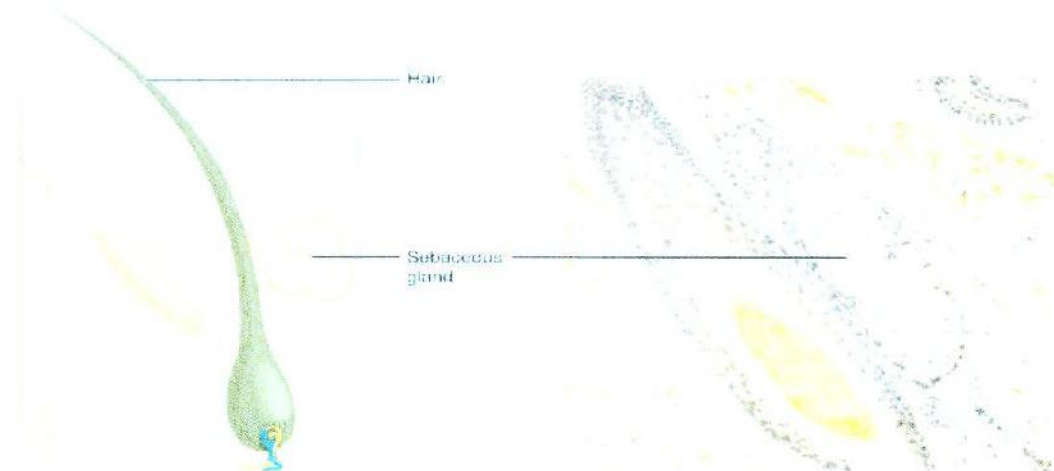


Figure 2.6: Illustration of sebaceous gland.

2.1.4.2 Functions of Sebaceous Gland

Relative to keratinocytes that make up the hair follicle, sebaceous glands are composed of huge cells with many large vesicles that contain the sebum. These cells express Na^+ and Cl^- ion channels, ENaC and CFTR (Hanukoglu et al., 2017).

Sebaceous secretions in conjunction with apocrine glands also play an important thermoregulatory role. In hot conditions, the secretions emulsify the sweat produced by the eccrine glands and this produces a sheet of sweat that is not readily lost in drops of sweat. This is of importance in delaying dehydration. In colder conditions, the nature of sebum becomes more lipid and in coating the hair and skin, rain is effectively repelled (Zouboulis, 2004).

Sebum is produced in a holocrine process, in which cells within the sebaceous gland rupture and disintegrate as they release the sebum and the cell remnants are secreted together with the sebum. The cells are constantly replaced by mitosis at the base of the duct (Young et al., 2006).

Sebaceous glands are part of the body's integumentary system and serve to protect the body against microorganisms. Sebaceous glands secrete acids that form the acid mantle. This is a thin, slightly acidic film on the surface of the skin that acts as a barrier to microbes that might penetrate the skin. The pH of the skin is between 4.5 and 6.2, an acidity that helps to neutralize the alkaline nature of contaminants. Sebaceous lipids help maintain the integrity of the skin barrier, and supply vitamin E to the skin (Zouboulis, 2004; Youn, 2010).

2.1.5 Hair Follicle

The hair follicle is a dynamic organ found in mammalian skin. It resides in the dermal layer of the skin and is made up of 20 different cell types, each with distinct functions. The hair follicle regulates hair growth via a complex interaction between hormones, neuropeptides and immune cells. This complex interaction induces the hair follicle to produce different types of hair as seen on different parts of the body. For example, terminal hairs grow on the scalp and lanugo hairs are seen covering the bodies of fetuses in the uterus and in some new born babies (Gilhar et al., 2012).

2.1.5.1 Hair Follicle Development

Hair growth occurs in a cyclical manner, but each follicle functions as an independent unit. The hair growth cell cycle is composed of three stages: anagen, catagen, and telogen (St-Jacques et al., 1998). Anagen, the active growth stage, typically lasts approximately three to five years on the scalp, during which hairs grow at a rate of about 0.33 mm per day. The length of the anagen phase decreases with age and decreases dramatically in individuals with alopecia (James et al., 2005).

Catagen usually lasts about two weeks and is a period of involution resulting in club hair formation after many cells in the outer root sheath undergo apoptosis. The resting phase, telogen, lasts about three to five months on the scalp, and hairs in this stage are eventually pushed out by the growing anagen hair shaft. Other sites on the body tend to have shorter anagen and longer telogen phases, causing most body hair to be shorter and remain in place for longer periods of time (James et al., 2005) (**Figure 2.7**).

Two secreted molecules that may have important roles in hair follicle development and cycling are the insulin-like growth factor 1 and fibroblast growth factor 7. In mice, both are produced by the dermal papilla and have receptors predominantly in overlying matrix cells. Hormonal factors controlling hair growth include estrogens, thyroid hormones, glucocorticoids, retinoids, prolactin, and growth hormone.

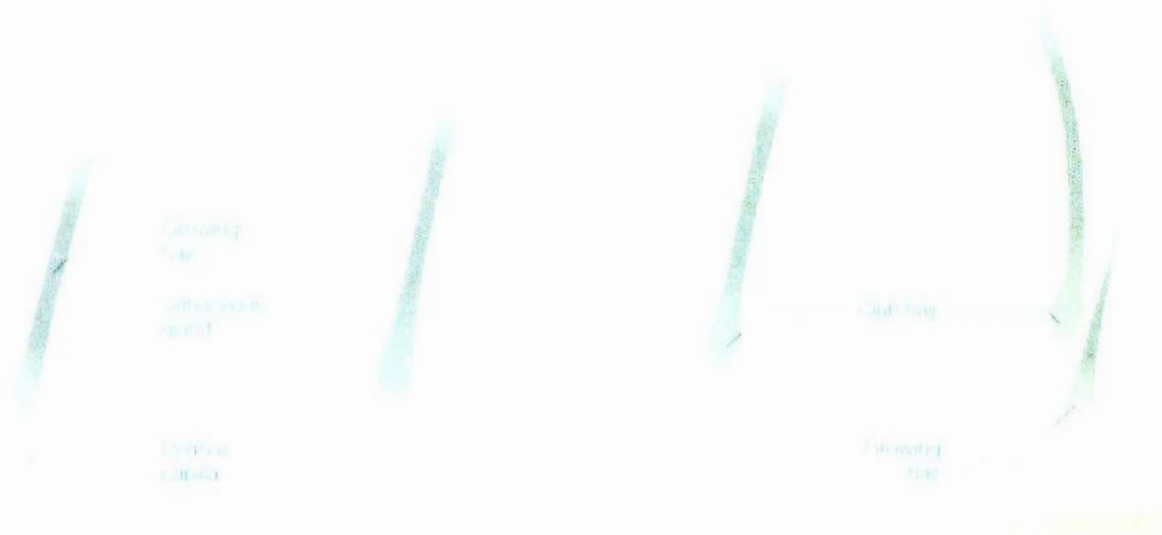


Figure 2.7: Phases of hair growth (James et al., 2005).

2.1.5.2 Functions of Hair Follicle

The main function of the hair follicle is to produce a hair shaft or fiber. The shaft is the physically visible portion of the hair and plays a significant role both biologically and psychologically. The hair shaft is composed of three parts – the cuticle, the cortex and the medulla. The cortex forms the bulk of the hair. This contains melanin, which is responsible for hair pigmentation.

The hair in different parts of the body has an ornamental function-specially in humans. The appearance of a person can be changed by changing the hair pattern and so hair plays an important role in social communication. The hair fibers also form a protective layer on the surface of the epidermis, protecting it from injury, insect bites and electromagnetic rays.

Hair responds to external stimuli and any movement of the fiber is picked up by the follicle to transmit a message to the nervous system. The hair fibers can therefore be said to act like 'antennas' to receive sensory signals.

Hair also plays a part in controlling the body heat by providing insulation against sudden heat loss or gain.

The outer root sheath of the hair follicle is a reservoir of sebaceous glands. The cells of the outer root sheath also secrete keratins, adhesion molecule, cytokines and other growth factor receptors that help to regenerate and repair the epidermal layer after injury. The epithelial cells in the upper outer root sheath can restore ulcerated wounds while the Langerhans cells can restore the cells lost due to ultraviolet radiation.

The hair follicle often acts as a sensory organ for the skin. Mechanical stimuli above the skin are detected by the skin and when the hair moves even slightly, neuro-receptors in the follicles transmit a message to the nervous system. The Langerhan's cells and Merkel cells are the main neuro-secretory cells responsible for this. The Langerhan's cells also activate the immune system when required.

Finally, the hair follicle cycle influences the surrounding skin environment and a study of hair biology is therefore central to the study of the biology of skin itself (<http://www.keratin.com>).

2.2 Burn

Burn wounds and injuries are often devastating. They can have severe long-term consequences for the victims and they continue to be a major problem affecting communities worldwide (Mock et al., 2009). Statistics from the WHO demonstrate that there are over 300,000 deaths per year from fires alone with many more from scalds, electrical burns, and other sources but there is still no accurate global data to confirm these numbers. Over 95% of fatal fire-related burns occur in low and middle-income countries (http://www.who.int/violence_injury_prevention/other_injury/burns/en/index.html). Children and the elderly remain the most vulnerable groups with the highest-mortality.

2.2.1 Severity of Burns

The overall severity of a burn is related to the casualty's age, TBSA affected, burn depth, associated injuries, and, to a lesser extent, associated illnesses (Zawacki et al., 1979) and infection. Burns can leave skin vulnerable to bacterial infection and increase your risk of sepsis

- Scarring
- Bone and joint problems
- Low blood volume
- Dangerously low body temperature
- Breathing problems



2.2.2 Burn and Wound Types

Different types of wounds can result from different types of accidents or casualties. Among them, there are different types and degrees of burn as well.

2.2.2.1 Types of Wounds

A wound is the disruption of the integrity of anatomical tissues caused by exposure to any factor. Wounds are generally examined under two groups:

- **Open Wounds** - This group includes lacerations, cutting-pricking tool wounds, gunshot wounds, surgical wounds, insect bites and stings, radio-necrosis, vascular neurological and metabolic wounds. Wounds except for lacerations cause serious damage to tissues

beneath the skin. In laceration type wounds, skin and subcutaneous tissue have been destroyed, but deep tissues remain healthy. The anatomical integrity of tissues is damaged in cutting pricking tool wounds without any tissue damage at the edges of the wound (Kapoor & Appleton 2005).

- **Closed Wounds** - This group includes contusion, hematoma and abrasion. Contusion-type injuries involve damage to soft tissues, small blood vessels and deep tissue layers, resulting in their separation, but the anatomy of the skin remains intact. Oedema, and in later periods, atrophy and defective pigmentation are observed in wound and the healing is delayed. Vessel rupture or hyperaemia due to vessel damage is called hematoma and wounds such as scrapes are termed abrasions. The healing process is very painful because this type of wound involves damage to sensory nerves and the wound can easily become infected (Mutsaers et al., 1997).

Wounds are also classified according to tissue loss:

- **Wounds without Tissue Loss** - These kinds of wounds occur as a result of tissue crushing. The severity of bleeding occurring in tissue varies according to the condition of the wound. Tissues exposed to this kind of wound heal after granulation tissue formation in minimal level in first phase of healing process (Ruszczak 2003).
- **Wounds with Tissue Loss** - These types of wounds involve damage or loss in some or all of the skin layers. Healing occurs *via* filling of the wound area by granulation tissue typically growing from the base of a wound. Wounds that involve tissue loss are collected in two groups in proportion to the loss. In superficial wounds, the entire epidermis and the papillary layer of the dermis are damaged. The epidermis, all the layers of the dermis and even subcutaneous tissue are damaged in full-thickness wounds covering second group (Mutsaers et al., 1997; Chanson et al., 2005).

2.2.2.2 Types of Burns

Burn is a kind of wound that occurs when skin or organs are damaged by an electrical current, heat, chemical or flammable agent effect. It is known that burn-initiated pathophysiological events differ from other traumas, may cause death risks and lead to increasing capillary permeability, resulting in hypovolemia. Burn causes changes of vascular permeability,

extravasation of plasma proteins, aggregation of platelets and increased fibrinolysis (Atiyeh et al., 2005).

Burns are divided into 4 groups according to the depth and the affected skin layers:

2.2.2.2.1 First Degree Burns

Only the outer layer of the epidermis and Stratum corneum are damaged in this type of burn, and there is no damage in the dermis. First-degree burns generally occur as a result of short term heat or flame contact or long-term exposing to intense sunlight (**Figure 2.8**). First-degree burn areas are characterized by slight edema, which diminishes after 24 hours. At this stage the skin begins to dry, there is no vesicle and infection is not seen. The wound heals within a week (Whitney and Wickline, 2003).

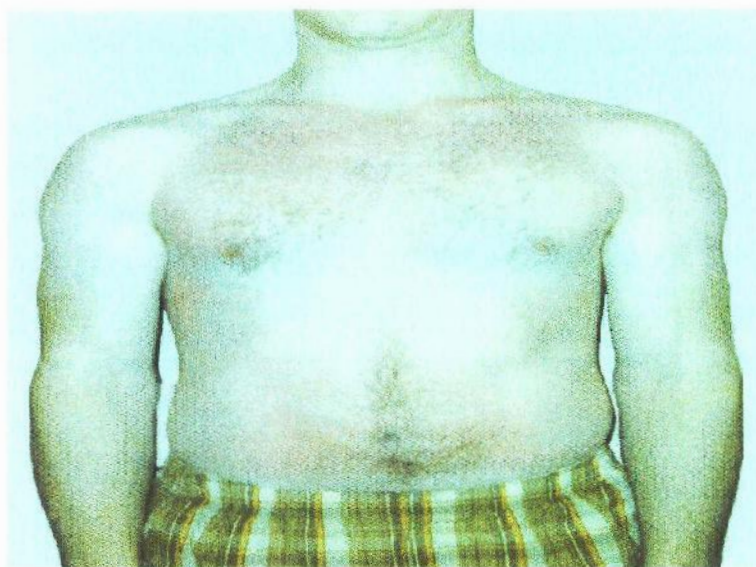


Figure 2.8: Typical first degree sunburn.

2.2.2.2.2 Second Degree Burns

These types of burns are deeper than first-degree burns and necrosis spread into the dermis. Damage covers the entire epidermis and some part of dermis. The wound is clinically characterized by pain, erythema and bullae. The recovery rate depends on the depth of skin injury and formation of infection. Generally, second-degree burns heal spontaneously in a short period if infection does not occur. If infection occurs in the wound, it can easily convert to third degree burn.

The burns in this group may be divided into two categories, termed superficial and deep dermal second-degree burns (Whitney & Wickline 2003):

- **Superficial Second Degree Burns** - These occur due to short period contact with flame or hot liquids (**Figure 2.9**). Generally, the upper portion of the Stratum germinativum is damaged in superficial second-degree burns. The surface is generally humid because of the leakage of liquid plasma from the burned area. Generally, less scarring than the deep dermal burns occurs. Recovery usually occurs within 3-4 weeks with zero or very mild scarring (Madri 1990).

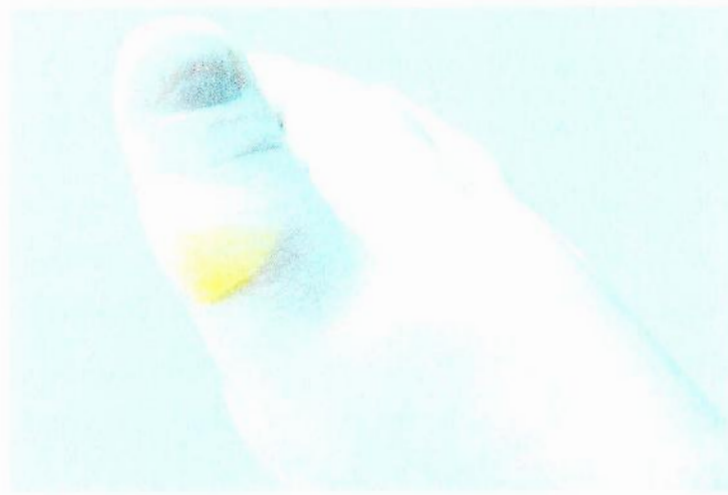


Figure 2.9: Superficial second degree burn of the thumb.

- **Deep Dermal Burns** - These occur due to contact with chemicals such as flame, hot liquids or acids, or exposure to high electrical current (**Figure 2.10**). In these types of burns, whereas the epidermis is completely burned, damage extends to the Stratum germinativum and the bottom section of the dermis. In deep dermal burns, fluid loss and metabolic effects are the same as in third-degree burns. Wound pain is very severe during burning and hyper anesthesia may occur in some areas. The wound may develop into a third-degree burn if infection occurs in the burn area. The time required for re-epithelialization depends on degradation in the dermis, the amount of burnt hair follicles and sweat glands, and the width of infected areas. If the wound is properly preserved, it usually closes within 2 months, leaving some scarring on the skin surface. Scarring and contracture occur in healing areas where closing lasts longer than 2 months. In this case,

it is very difficult to distinguish the wound from third-degree burns and treatment of this kind of wound will last longer (Shakespeare 2001).



Figure 2.10: Second degree deep dermal burn caused by contact with boiling water.

2.2.2.2.3 Third Degree Burn

These kinds of burns result from hot water, fire and prolonged contact with electrical current (**Figure 2.11**). The wound area exhibits tautness and brightness as the elasticity of the skin is lost, causing abnormal shrinkage. In such cases, all structures within the skin sustain damage. The dermis and subcutaneous fat are destroyed as a result of coagulation necrosis. Thrombosis occurs in vessels under the skin. Increased capillary permeability and edema is much higher in third degree burns than second-degree burns. Skin is damaged in all layers and is characterized by autolysis and leuko-cytoclastic infiltration for 2 or 3 weeks. This event is usually associated with suppuration. Capillary bundles and fibroblasts are organized in granulation tissue under scar. If the burn affects subcutaneous fat, healing can take much longer. Burn affecting the muscle causes increasing in degradation of red blood cells. The care of third-degree burns requires removing scar tissue and covering the wound with a graft. If grafting is not carried out, a thick layer of granulation is shaped and the contraction of the area follows it. At this stage, re-epithelialization, slightly, occurs on the edge of wound granulation is soft, can be infected and healing continues over several months. Permanent deep scars in the skin occur following healing in these kinds of wounds and surgical intervention is usually required to restore normal appearance (Moulin et al., 2000; Shakespeare 2001).



Figure 2.11: Eight days old third degree full thickness burn caused by motorcycle muffler.

2.2.2.2.4 Fourth Degree Burn

Fourth degree burn extends through entire skin, and into underlying fat, muscle and bone (**Figure 2.12**). Possible prognosis are amputation, significant functional impairment and in some cases, death (Tintinalli, 2010).



Figure 2.12: Fourth degree burn.

2.2.3 Cause of Burn

Various external sources may cause burn classified as thermal (heat-related), chemical, electrical, and radiation (Rosdahl & Kowalski 2008). In the United States, most frequent causes of burns are fire or flame (43%), scalds (34%), contact to hot objects (9%), electricity (4%), and chemicals (3%). Most (69%) burn injuries occur at home or at work (9%), and most are

accidental, with 2% due to assault by another, and 1-2% resulting from a suicide attempt. These sources can cause inhalation injury to the airway and/or lungs, occurring in about 6% (Herndon ed. 2012).

2.2.3.1 Chemical Injuries

Chemical burns appear to be uncommon in military casualties, 26 with reported incidences ranging from 0.9% to 6.5% (Cancio et al., 2005). However, the widespread use of chemicals and their ready availability creates the potential for a large number of this type of casualty. Chemical burns are caused by acids, alkalis, or organic compounds (Barillo et al., 2004). Except for white phosphorus (Barillo et al., 2004) most chemical burns should be immediately treated with copious water lavage. Wounds caused by white phosphorus, which ignites on contact with air, should be covered with water or saline until the wound can be debrided of all chemical (Barillo et al., 2004). Powdered chemical should be brushed away prior to water lavage to decrease the quantity of exposure. The process of neutralization generates heat, which can add thermal injury to the chemical insult. The duration of irrigation is variable depending on the agent. In general, alkalis require a much longer period of irrigation than acids. At a minimum, 30 minutes of continuous irrigation are required for all burns, and at least 1 hour of irrigation is necessary for alkali burns. A period of up to 6 hours of irrigation has been recommended for acid burns, and one of up to 48 hours has been suggested for ocular alkali burns. A good rule of thumb is to irrigate at least until there has been a significant decrease in pain. Chemical agents produce direct tissue damage by a variety of reactions. Acid burns tend to be superficial in depth, whereas alkali burns tend to burrow into the tissues and cause more significant destruction. Acids cause coagulation necrosis, whereas alkaline materials cause liquefaction necrosis. Hydrofluoric acid requires copious water lavage, followed by topical calcium gluconate, which, when injected into the area, may decrease the severity of the injury. Using intra-arterial calcium gluconate on wounds of the hands and feet is beneficial. Mustard agent, a chemical previously used in warfare, is rapidly absorbed by the skin, conjunctiva, and mucous membranes, and within minutes irreversibly combines with tissue proteins, damaging the lungs, eyes, and skin (Khateri et al., 2003). Over time, the exposed tissues blister and ulcerate (Cox 2007). Ophthalmic injuries are best treated with copious water irrigation. Skin should be decontaminated with 0.5%

hypochlorite. In general, skin burns from mustard are superficial and heal without difficulties; injuries to the mucosal linings are much more serious and disabling.

2.2.3.2 Electrical Injuries

Electrical injuries result from the conduction of electrical energy through tissue or from heat that is released as the current arcs through the air. Current arcs may generate temperatures as high as 3,000°C. As they arc, they ignite clothing, which results in a combination electrical and thermal injury. Injuries caused by low voltage current (<1,000V) are occasionally fatal because of immediate ventricular fibrillation. Survivors rarely have significant tissue damage. High voltage (>1,000V) current can damage tissue anywhere along its route. Electrical injuries are heat related. Electrical energy is converted to heat energy as expressed by Joule's law: power or heat equals amperage squared times resistance ($P=I^2 \cdot R$). As voltage or amperage increases, so does the heat produced. Measurement of tissue temperature experimentally reveals that it is highest directly underneath and adjacent to the contact site of the wounds. Deep tissue destruction is always greatest in areas of the body with small volume, such as the fingers, toes, wrists, or ankles. The farther away the tissue is from the contact point, the lower the current density, and the less heat is generated. Various tissues have different resistance to current flow; for example, nerves and blood vessels have low resistance, while cartilage and bone have high resistance. Because bone is highly resistant, current tends to flow at its surface, making temperature greatest at the periosteum. Thus, muscle damage is often extensive adjacent to the bone, and many times the periosteum and portions of the outer cortex of the bone may not be viable. For these reasons, the extent of electrical injury is often not well reflected in the cutaneous burn size (Cancio et al., 2005). An electrical injury may be deceiving and not initially appear severe, with only small areas visible superficially, but the casualty may have severe limb injury that will ultimately lead to amputation.

2.2.3.3 Thermal Injuries

Thermal injury accounts for approximately 95% of military burn casualties, with chemical and electrical making up the most of the remainder (Cancio et al., 2005; Kauvar et al., 2006). Burns resulting from flash fire and flame are the most common in wartime (Cancio et al., 2005; Kauvar et al., 2006). The detonation of explosive devices, such as landmines, artillery munitions, mortar

rounds, and improvised explosives, has been a significant source of combat burns (Cancio et al., 2005; Kauvar et al., 2006). The most common causes of noncombat burns in service members are related to burning waste materials, inappropriately handling ammunition and pyrotechnics, and misusing fuels. Efforts to prevent noncombat injuries continue to reduce their impact on military operations (Cancio et al., 2005; Kauvar et al., 2006). Other causes of military burn injury include exposure to hot liquids or superheated steam, typically from ship or submarine boilers, resulting in scald burns. Inhalation injury is frequently associated with superheated steam burns.

2.2.4 Epidemiology of Burn Wounds

Of the studies that reported data on childhood burns, infants and toddlers from birth through 4 years of age were shown to have a disproportionately higher number of burns (Forjuoh 2006). In many settings like Brazil, Cote d'Ivoire, and India, this age group was found to account for nearly half of all childhood burns (Vilasco & Bondurand 1995). This high number of burns in infants and toddlers was attributable largely to their total dependence on their parents and caretakers. In studies where all age groups were studied, children from birth through 4 years still comprised nearly one-third of the total number of cases (Adamo et al., 1995). The number of burns was reported to then taper off for each subsequent year until during older adolescence when burns began to increase again due to the engagement of work and improper use of flammable substances. For adults, the number of burns was found to be relatively low until among the 30–39-year age group (Vilasco & Bondurand 1995; Chien et al., 2003). The preponderance of burns seen during childhood and among the elderly in HICs was rarely seen in LMICs probably due to lack of data on the elderly. Nonetheless, a recent study in Egypt, where the elderly population is gradually expanding probably due to reduced mortality for persons between the ages of birth and five years as well as improved living conditions and medical care, among other things, found a high number of burns in persons older than 60 years (Mabrouk et al., 2003).

2.2.5 Pathophysiology of Burn Wounds

Thermal burns from dry sources (fire or flame) and wet sources (scalds) account for approximately 80% of all reported burns (<http://ameriburn.org/who-we-are/media/burn->

incidence-fact-sheet/) and can be classified based on the depth of burn (Kagan et al., 2013; Nisanci et al., 2010). In addition to local injury at the site of burn, severe thermal injury over a large area of the skin, roughly 20 % total body surface area (TBSA) or greater, results in acute systemic responses collectively known as burn shock. Burn shock is characterized by increased capillary permeability, increased hydrostatic pressure across the microvasculature, protein and fluid movement from the intravascular space into the interstitial space, increased systemic vascular resistance, reduced cardiac output, and hypovolemia requiring fluid resuscitation (Pham et al., 2008). The edema that forms in the interstitial space forms rapidly in the first 8 h following burn injury, and continues to form more slowly for at least 18h. Volume requirements for resuscitation can be estimated by the total burn size and the patient's weight (or body surface area). Additional factors influencing these needs include the presence or absence of inhalation injury, the extent of full-thickness burns, and the time since injury (Pham et al., 2008). The actual fluid infusion rate is then titrated hourly, based on the adequacy of physiological responses, such as the urine output. Following successful resuscitation, patients with larger burns then enter a more prolonged period of hyper metabolism, chronic inflammation, and lean body mass wasting, all of which may impair wound healing (Porter et al., 2013). Additionally, an increased susceptibility to infection due to altered immune status may lead to sepsis, further exacerbating systemic inflammation. Sustained hyper metabolism and inflammation impair wound healing through delayed re-epithelialization (Sommer et al., 2013). The extent of inflammation and hyper metabolism is related to the extent and depth of burn, as deeper burns show higher levels of circulating cytokines (Sakallioglu et al., 2006) and a greater hypermetabolic response. Similarly, the extent of burn is an efficient predictor of hospital length of stay (Hussain and Dunn, 2013) and mortality (Colohan, 2010). According to one model, the burn wound can be divided into three zones based on the severity of tissue destruction and alterations in blood flow (Nisanci et al., 2010; Kowalske, 2011). The central part of the wound, known as the zone of coagulation, is exposed to the greatest amount of heat and suffers the most damage. Proteins denature above 41 °C (106 °F), so excessive heat at the site of injury results in extensive protein denaturation, degradation, and coagulation, leading to tissue necrosis. Around the central zone of coagulation is the zone of stasis, or zone of ischemia, which is characterized by decreased perfusion and potentially salvageable tissue (Nisanci et al., 2010). In this zone, hypoxia and ischemia can lead to tissue necrosis within 48 h of injury in the absence of

intervention (Tan et al., 2013). The mechanisms underlying apoptosis and necrosis in the ischemic zone remain poorly understood, but appear to involve immediate autophagy within the first 24 h following injury and delayed-onset apoptosis around 24 to 48 h post-burn (Tan et al., 2013). Other studies have shown apoptosis to be active as early as 30 min post-burn (Singer et al., 2008) depending on the intensity of the burn injury. Oxidative stress may play a role in the development of necrosis, as preclinical studies have demonstrated promising reductions in necrosis with systemic antioxidant administration (Deniz et al., 2013). At the outermost regions of the burn wound is the zone of hyperemia that receives increased blood flow via inflammatory vasodilation and will likely recover, barring infection or other injury (Hettiaratchy and Dziewulski, 2004).

2.3 Wound Healing

Wound healing is a complex process in which the skin and the tissues under it repair themselves after injury (Nguyen et al., 2009). Wound healing can be depicted in a discrete timeline of physical attributes (phases) constituting the post-trauma repairing process.

2.3.1 Normal Wound Healing Process

The sequence of events in normal wound healing has been widely studied and described in literature (Gosain & Dipietro 2004). Wound healing is a complex biological process that takes place in all tissues in all organs of the body. Various cell types, including keratinocytes, neutrophils, macrophages, lymphocytes, fibroblasts and endothelial cells, are involved in this process. The necrotic tissue is either removed by scavenger cells or separated from living tissue by the process of phagocytosis. The wound healing process consists of four phases: hemostasis, inflammation, proliferation and remodeling (Velnar et al., 2009) (**Figure 2.13**).

2.3.1.1 Coagulation and Hemostasis

The initial step assists in the protection of the vascular system to maintain the functionality of the organ. The clot formed as a result of coagulation provides a matrix for the cells involved in subsequent steps of hemostasis and inflammation (Velnar et al., 2009). Various pro-inflammatory cytokines and growth factors are released by the clot and wound tissue.

Inflammatory cells then migrate to the wound site by the process of chemotaxis and promote the inflammatory phase (Gosain and DiPietro 2004; Campos et al., 2008).

2.3.1.2 Inflammation

The goal of the inflammatory phase is to fight potential bacterial contamination of the wound and to activate cytokine secretion (Velmar et al., 2009; Braiman-Wiksmann et al., 2007). Uncontrolled inflammation can destroy the early migratory effect, leading to an arrest of the healing process.

2.3.1.3 Proliferation

The proliferation phase overlaps with the preceding inflammatory phase. It represents a proliferation of both epithelial and dermal elements which results in re-epithelialization of the wound and laying down of the primary extracellular matrix. Epidermal stem cells and bone marrow derived stem cells also play a role during this phase. Angiogenesis occurs secondary to endothelial progenitor cells, a derivative of hematopoietic stem cells (Rea et al., 2009).

2.3.1.4 Wound Remodeling Leading to Scar Formation

This phase marks the final step in tissue remodeling and differentiation leading to recovery of the skin and its aesthetic restoration (Diegelmann & Evans 2004). Reconstruction of the dermis occurs by reorganization of the matrix collagen (Braiman-Wiksmann et al., 2007), fibroblasts differentiation into myofibroblasts, leading to wound contraction and closure.

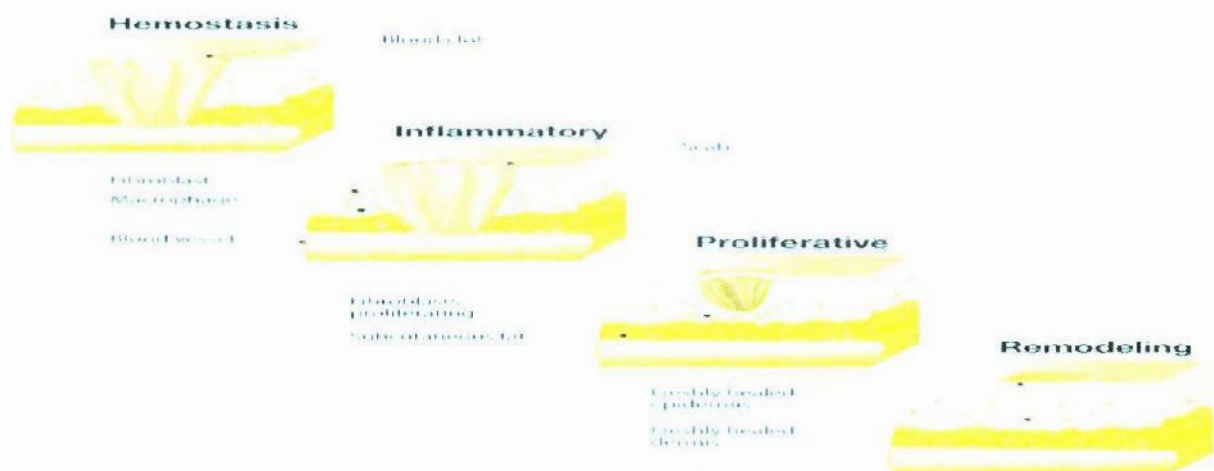


Figure 2.13: Four phases of wound healing.

2.3.2 Assessment of the Wound

Impaired wound healing occurs secondary to disordered collagen formation and underlying predisposing conditions. In order to effectively manage chronic wounds, periodic assessment of the healing process is necessary (Mullins et al., 2005). The insights gained from this type of assessment are expected to facilitate the development of novel therapies by stratifying their specific contributions to the wound healing process in time and stage-specific manner (Braiman-Wiksman et al., 2007). Hence, a standardized and reproducible model is required to obtain information about the wound healing process as well as to better understand the pathology and improve medical technologies. Instruments to assess wound healing can help to enhance communication among clinicians by defining a common language and standardizing assessment of wound characteristics (Mullins et al., 2005). Because healing is a dynamic process, it is difficult to evaluate and requires consistent measurements. A complete assessment of the wound must include the size, associated attributes, host factors and environmental factors, all of which impact optimal wound management. In addition, demographics and quality of care also provided aid in assessing the repair process. The assessment of the histological state of the healing wound is important in clinical practice for postoperative patient management (Sultana et al., 2009). Histological evaluation should include the basic components of the healing process including angiogenesis, inflammation, fibroplasia and restoration of the connective tissue matrix, wound contraction and remodeling, epithelialization and differentiation. Comparison of histologic patterns with the known physiologic variation in tissue morphology assists in qualitative derivation of the diagnosis. The degree of changes observed when scored on an ordinal scale, namely, low, medium or high grade, provides a semi-quantitative score. On the other hand, the exact quantitative measurement in terms of the absolute number of cells and areas of tissue gives a quantitative score (Lemo et al., 2010). A quantitative scoring system, while being highly specific and standardized, is difficult to score because in most cases it is difficult to objectify the exact interval between two values (Lemo et al., 2010). Hence, semi-quantitative scoring systems remain in wide use in the world of the biomedical research.

Various studies have been conducted, and wound healing models have been proposed to understand the normal healing process and to standardize the semi-quantitative and quantitative evaluation of selected parameters of wound healing. In a study assessing wound healing in the

maxillofacial region, (Sultana et al., 2009) utilized scoring of 6 histological parameters to give a healing score. The total healing score in each case was calculated by adding the scores of individual criteria, with lower scores indicating poorer wound healing. Healing status was graded as good (16-19), fair (12-15) and poor (8-11). Using this healing score, (Sultana et al., 2009) concluded that risk factors in the study group were correlated with delayed wound healing in comparison to the control group.

While studying the overall process of wound healing, (Braiman-Wikman et al., 2007) evaluated the role of multiple processes involving the skin components including the epidermis, dermis, hypodermic, blood vessel and connective tissue. They stressed an objective assessment and quantification of wound healing. Using a quantitative assessment method, the authors provide insight into the specific defects found at various stages, which involve a variety of cells and pathways in the process of wound healing.

In their experimental model of open-skin wound healing in corticosteroid-treated and diabetic rats, (Gal et al., 2008) used both semi-quantitative and quantitative methods in a time and stage bound assessment of wound healing.

Consistent with previous studies (Gal et al., 2008) they concluded that there is only a quantitative difference between primary and secondary wound healing. In contrast to the quantitative method, the semi-quantitative scoring system can evaluate keratinization, suggesting that keratinocyte differentiation is important in wound healing. Hence, a quantitative assessment alone is not sufficient to demonstrate significant differences in skin wound healing.

2.3.3 Signaling Pathways and Molecular Mechanism Involved in Wound Healing

Following a skin injury, the damaged tissue is repaired through the coordinated biological actions that constitute the cutaneous healing response. In mammals, repaired skin is not identical to intact uninjured skin, however, and this disparity may be caused by differences in the mechanisms that regulate postnatal cutaneous wound repair compared to embryonic skin development. Improving our understanding of the molecular pathways that are involved in these processes is essential to generate new therapies for wound healing complications (Bielefeld et al., 2013).

2.3.3.1 Wnt/ β -Catenin Signaling Pathway

The first Wnt gene to be described, “int-1,” was discovered by (Nusse and Varmus, 1982). Mice infected with mouse mammary tumor virus (MMTV) were found to overexpress int-1, which is similar to MMTV gene sequences in tumors at integration sites. The wingless gene (Wg) of the *Drosophila melanogaster* is a homolog of int-1 and both together are known as Wnt. The Wnt signaling pathway has a high degree of conservation across species, with a high degree of homology across lower organisms, such as the *Drosophila* species, and higher organisms, such as monkeys and humans. The Wnt family consists of at least 19 genes, including Wnt-1, Wnt-3a, Wnt-10b, and Wnt-7a (Janda et al., 2017). The β -catenin is a cytoplasm/nuclear protein that is pivotal for transcription of cell adhesion factors and the Wnt pathway, promoting cell adhesion.

The Wnt/ β -catenin pathway is one of the main processes in the regulation of wound healing and improves wound angiogenesis and epithelial remodeling. A large number of experimental studies have reported that the Wnt family is involved in many biological processes (Igota et al., 2013), including cell proliferation, apoptosis, differentiation, and the maintenance of pluripotency in stem cells. The Wnt/ β -catenin signaling can be divided into classical and non-canonical Wnt signaling. The Wnt/ β -catenin signaling is activated when the Wnt ligand binds to the receptor/co-receptor. Some synthetically made Wnt proteins transfer to the Golgi apparatus, where they first bind to Wls, a transmembrane protein secreted by Wnt and then are escorted from the Golgi apparatus to the cell membrane. The secreted Wnt ligand binds to the Frizzled protein (Frz) family of receptors and co-receptors, including LRP-5/6, which activates different signaling pathways (**Figure 2.14**). The interaction between the Wnt protein and its cognate receptor can be blocked by numerous soluble metabolites, including the Dickkopf protein (Dkk) or the Wnt inhibitor (WIF) (Veltri et al., 2018). The stable conduction of Wnt/ β -catenin signaling pathway is dependent on the stabilization of β -catenin. Without the Wnt signal, free cytosolic β -catenin is phosphorylated by a complex of Axin, casein kinase 1a (CK1a), and glycogen synthase kinase-3 β (GSK3 β). Phosphorylated β -catenin is ubiquitinated by β -transduced repetitive protein (β -TrCP) for proteasome dependent degradation. When Wnt binds to this complex, disheveled (Dvl) proteins aggregate and the expression of the degradation complex is inhibited, thus stabilizing β -catenin in cells. The β -catenin is recruited by the cytoplasm and enters the nucleus where it activates the T-cell factor/lymphoid enhancer-binding factor (LEF/TCF) protein (Yamagami et

al., 2018). Interactions between β -catenin and LEF/TCF involve transcriptional regulators and histone-modification factors, which, in turn, mediate excessive developmental and homeostasis processes.

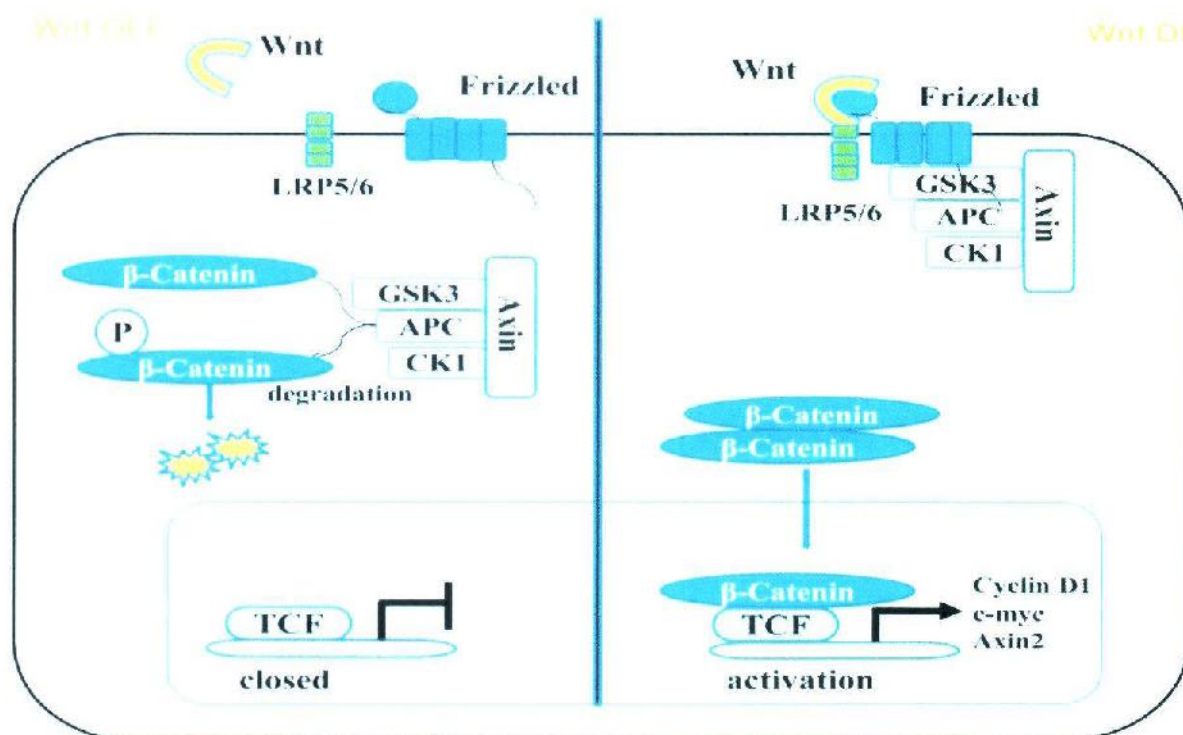


Figure 2.14: Wnt/β-catenin signaling on and off state (Zhang et al., 2018).

2.3.3.2 TGF-β pathway

Numerous growth factors and cytokines participate in wound repair. These include the TGF-βs, PDGFs, epidermal growth factors (EGFs), fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs), and various pro-inflammatory cytokines, such as the interleukins. These factors are released by various cell types as well as from sites of sequestration in the disrupted ECM (Schultz and Wysocki, 2009). TGF-βs are one of the major cytokine mediators of cutaneous wound healing. TGF-βs comprise the TGF-β1, TGF-β2 and TGF-β3 isoforms, and are part of the TGF-β superfamily that also includes BMPs, among others. During wound healing, TGF-βs are secreted by various cell types, including macrophages and fibroblasts, and they are also released from storage sites in the disrupted ECM (**Figure 2.15**). TGF-βs are secreted as inactive precursors bound to the latency-associated and latent TGF-β binding proteins. They are

subsequently activated by the action of proteases or through conformational changes induced by integrins in response to cell traction forces (Margadant and Sonnenberg, 2010).

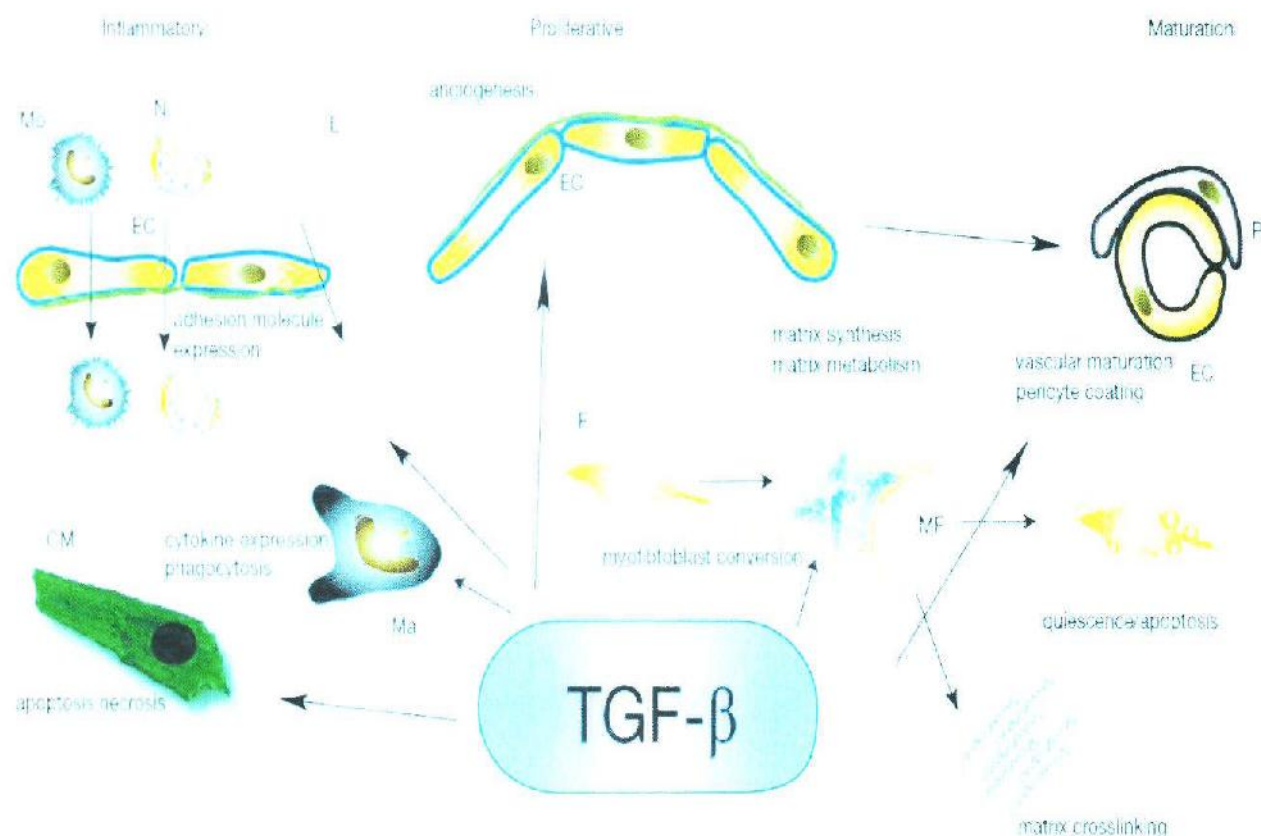


Figure 2.15: The cell biological actions of TGF- β in response to wound (Frangogiannis, 2017). Mo, monocyte; N, neutrophil; L, lymphocyte; Ma, macrophage; CM, cardiomyocyte; EC, endothelial cell; F, fibroblast; MF, myofibroblast; P, pericyte.

TGF- β s exert cell-specific effects by binding to serine/threonine kinase TGF- β receptor I and TGF- β -receptor II heterodimers, leading to the activation (phosphorylation) of Smads 2 and 3, their association with the common mediator Smad 4, and the subsequent translocation of this complex to the nucleus where they may associate with other transcription cofactors to modulate gene expression. Smad 7 is an antagonist to Smad 2/3-mediated signaling (Biernacka et al., 2011). Mechanisms of TGF- β signaling that do not involve Smads also exist, including mitogen-activated protein kinase, Akt, extracellular signal-regulated kinase, TGF- β -associated kinase I, and small GTPases, among others.

2.3.3.3 Notch Pathway

The Notch signaling pathway is a regulator of epidermal differentiation, both in the maintenance of skin homeostasis in the adult, and in embryonic development of the epidermis. It also plays a major role in other developmental and physiological processes, including angiogenesis and vascular maintenance. Notch is known to engage in crosstalk with the Wnt/ β -catenin and Hedgehog pathways (Okuyama et al., 2008), two other signaling pathways that participate in skin development and homeostasis. Notch signaling requires an interaction between a transmembrane ligand and the transmembrane Notch receptor on two adjacent cells. In mammals there are four Notch receptors, and their ligands are members of the Delta-like and Jagged families. Ligand binding causes sequential proteolytic cleavage of the Notch receptor, resulting in the liberation of the Notch intracellular domain (NICD) from the cell membrane and its translocation to the nucleus where it associates with RBP-J and other cofactors to modulate the transcription of target genes such as the Hes/Hey transcriptional repressors (Gridley, 2010).

Research has suggested that the Notch pathway participates in wound repair, and that it is required for proper healing. (Thelu et al., 2002) described expression of Jagged1 and Notch1, as well as the signaling modulator, Lunatic Fringe, in healing epidermis of wounded human skin grafted onto mice. Later studies examined the role of the Notch pathway during wound repair. Transgenic mice expressing a Notch antisense sequence that causes a 50 % reduction in Notch protein, exhibited delayed healing as assessed by surface wound size. Mice treated with the Notch ligand, Jagged, show accelerated wound closure (as assessed by surface wound size) suggesting that these effects are mediated by the Notch pathway. In vitro scratch wound assays with cultured cells, using a Notch inhibitor or activator, suggest that part of the mechanism may involve promigratory effects of Notch on fibroblast and vascular endothelial cells (Chigurupati et al., 2007). In turn, (Outtz et al., 2010) found that Notch1 hemizygous (heterozygous) mice exhibit increased collagen deposition and vascularity in healing wounds. The participation of Notch signaling in angiogenesis during wound repair was also suggested by (Caiado et al., 2008) following in vitro studies where the Notch pathway regulated the matrix-adhering and angiogenic properties of bone marrow-derived vascular precursor cells. Injection of such cells into mice caused a reduction in the size of healing wounds and also increased the density of

wound microvessels, whereas mice that were injected with cells treated with a Notch inhibitor did not exhibit these effects (Caiado et al., 2008).

2.3.3.4 Hedgehog Pathway

The hedgehog (Hh) pathway was first identified by Nüsslein-Volhard and Wieschaus, in a genetic screen in *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus, 1980). Flies deficient in Hh had developmental defects in the cuticle, displaying a layer of disorganized hair-like bristles that resembled the mammal hedgehog. Hh is a morphogen, and as such, its effect on cell fate depends on its local concentration. Hh diffuses to the extracellular matrix and thus, cells closer to the Hh-producing cells are exposed to high concentrations of Hh ligands (Briscoe and Thérond, 2013). Hh ligands (Sonic hedgehog, Shh; Indian hedgehog, Ihh; and Desert hedgehog, Dhh) are produced as 45 kDa precursor proteins, and undergo autocatalytic cleavage. The resultant N-terminal fragment has intrinsic cholesterol transferase activity, which promotes cholesterol lipidation of the active N-terminal fragment. Cholesterol modification is very important for Hh activity, promoting its retention in plasma membrane lipid rafts where Hh ligands interact with other lipids. A member of the membrane-bound O-acyltransferase (MBOAT) protein family, skinny hedgehog (SKI), mediates a second lipidation with palmitic acid. Palmitoylation is necessary for full ligand activity, as well as for long-distance movement. Release of Hh from producing cells occurs in one of three ways: a process facilitated by the protein Dispatched, through assembly in very low-density lipoproteins (VLDL), or through exosomes (Farzan et al., 2008).

The Hh pathway is a recognized maestro of the wound healing response. The wound-healing proliferative response is a coordinated reaction to liver injury that aims to overcome the loss of hepatic structure and function that results when liver cells die. Injured or fatty hepatocytes cannot mount an proliferative response to replace these cells and hence progenitor cells are crucial for sick livers to regenerate. Progenitors in the liver are sensitive to Hh. Indeed, Hh activation enhances progenitor cell viability and proliferation, whereas Hh inhibition promotes progenitor differentiation or cell death by apoptosis (Jung et al., 2010). Another conserved wound healing response that occurs after liver injury is the development of an inflammatory reaction, which is also strongly regulated by the Hh pathway. For example, hepatic NKT cells respond to Hh with improved viability and proliferation, and acquire a profibrogenic phenotype

that includes up-regulating their expression of IL-13. Hh also directly induces M2 pro-fibrogenic polarization of macrophages/Kupffer cells, further crafting a pro-fibrogenic liver microenvironment (**Figure 2.16**).

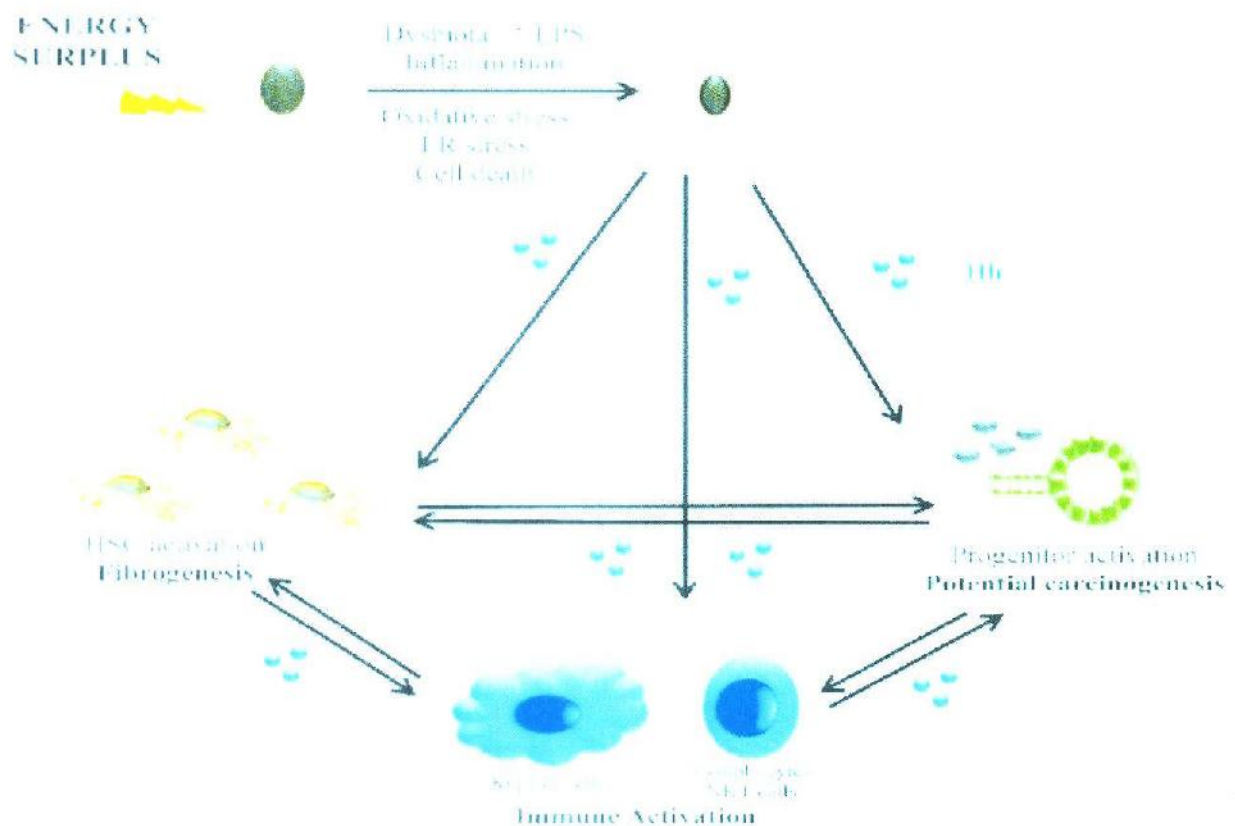


Figure 2.16: The role of Hedgehog on the wound-healing response (Machado and Diehl, 2016).

Another important player in the wound healing response is the hepatic stellate cell (HSC), the main source of myofibroblasts in the liver (Iwaisako et al., 2012). HSC not only produce the extracellular matrix necessary to maintain hepatic architecture during injury, they are a rich source of paracrine trophic substances that act on all other cell types involved in the healing response, and have recently been shown to function as progenitor cells themselves. Excessive HSC activation may lead to anomalous matrix deposition that causes progressive fibrosis. Hh enhances HSC survival by inhibiting apoptosis, promotes HSC proliferation, and stimulates HSC to undergo an epithelial to mesenchymal-like transition in order to acquire a myofibroblastic phenotype (Yang et al., 2008). Lastly, liver sinusoidal endothelial cells respond to Hh with capillarisation of hepatic sinusoids and vascular remodeling; perpetuation of this response favors the development of portal hypertension.

2.3.3.5 Crosstalk of the Pathways

During the wound healing process, the Wnt/ β -catenin signaling pathways work independently or together with other pathways to elicit an appropriate cellular response. It is becoming increasingly clear that signaling pathways do not operate in isolation, but are strongly intertwined. Although many signaling pathways have been studied extensively, the role of some signaling pathways in diabetic cutaneous ulcers (DCU) is unknown and requires investigation (Figure 2.17).

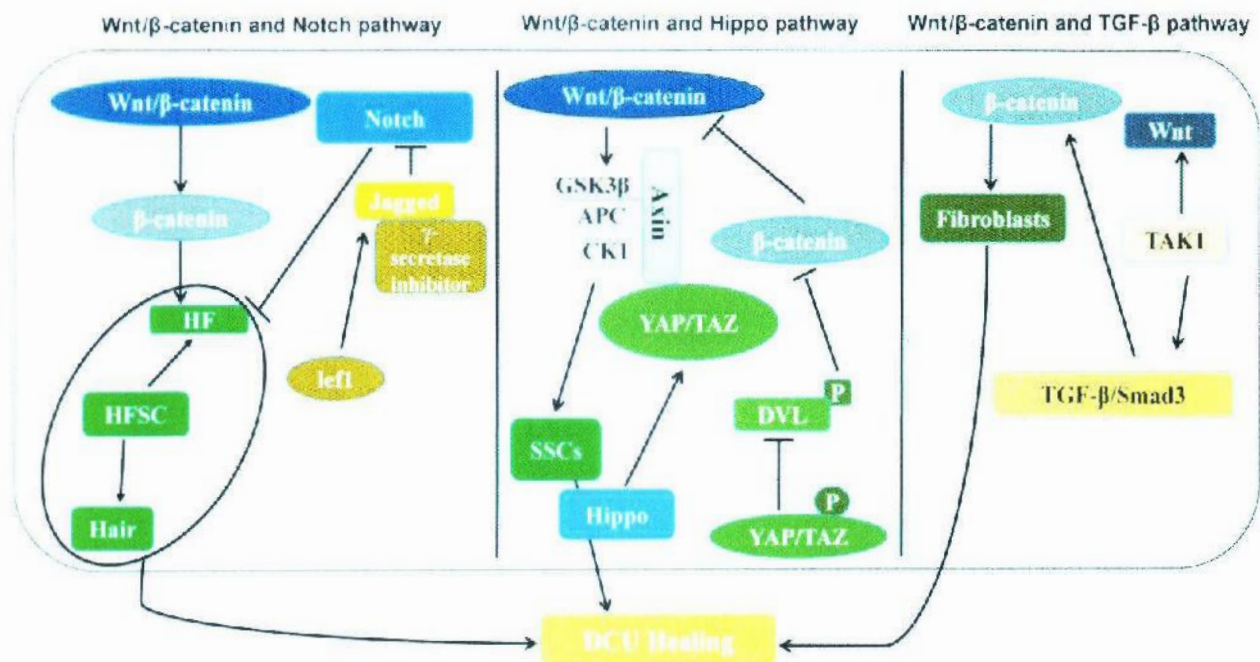


Figure 2.17: Interactions between different pathways in wound healing (Zhang et al., 2018).

The Wnt/ β -catenin pathway cooperates with the Notch pathway, to promote DCU healing. During the DCU healing process, both Wnt/ β -catenin and Notch signaling pathways are involved in the proliferative phase of wound healing, and, therefore, synergistic effects may occur in DCU regulation (Kim et al., 2017). The Wnt and Notch signaling pathways regulate HF expression. Loss of Jagged1, which is the Ligand of Notch or treatment of Notch signaling with γ -secretase inhibitors can suppress new HFs in adult epidermis regulated by β -catenin. The Notch pathway is downstream of the Wnt/ β -catenin signal and thus determines the destiny of HF. In addition, in cell culture, where HFSCs differentiate into hair and the Lefl gene is upregulated, the Wnt transcriptional target genes activate the target gene Jagged1 and directly activate Wnt/ β -catenin

and Notch signaling pathways. In contrast, in stratified IFE, β -catenin is detected in proliferating basal cells, and Notch1 is primarily expressed in the differentiated basal layer. The loss of Notch1 results in β -catenin-mediated signaling upregulation in multilayer hyperproliferating IFE, suggesting that Notch1 inhibits the Wnt/ β -catenin signaling pathway to limit its activation in the basal layer (Wang et al., 2017). The synergy and antagonism between Wnt and Notch signaling appear to influence the rate of wound healing in skin.

Recently, the Hippo pathway has been reported to be connected to Wnt/ β -catenin signaling. The Hippo signal transducer YAP/TAZ is part of the β -catenin disruption complex, which can coordinate the Wnt/ β -catenin response regulating stem cell self-renewal and tissue homeostasis (Deng et al., 2018). In cancer cells, the downregulation of Hippo signaling is linked to the upregulation of β -catenin activity. The mechanism has been proposed as a negative correlation, where Wnt/ β -catenin signaling is inhibited due to phosphorylated YAP/TAZ inhibiting the phosphorylation of Dvl and nuclear translation of β -catenin. Due to the Hippo signal being involved in the regulation of epidermal proliferation, the interaction of the Hippo and Wnt signals could have an important role in the regulation of skin regeneration and HF regeneration and requires further investigation.

The TGF- β and Smad signaling pathways and Wnt/ β -catenin signaling pathway have some synergistic effects. Several studies have confirmed the mutual regulation of Wnt/ β -catenin and TGF- β expression (Telerman et al., 2017). The Smad3 promotes the rapid nuclear translocation of β -catenin under TGF- β stimulation in mesenchymal stem cells. The TGF- β -activated kinase-1 induction enables tissues to rapidly secrete Wnt proteins, promoting fibroblast differentiation (Beljaars et al., 2017). Interaction between Wnt and TGF- β follows the opposite direction expression, such as Wnt3a-induced expression of TGF- β in mouse fibroblasts is reversed.

2.3.3.6 Role of Key Regulators

There are several key regulators which play important role in directing the fate of the damaged tissue. Some of the key regulators are discussed below:

2.3.3.6.1 Retinoic acid

Several past studies have demonstrated the efficacy of all-trans retinoic acid (RA) and its parent compound all-trans retinol (ROL, vitamin A) in wound healing. Although most studies have focused on wounds in the skin (Frosch and Czarnecki, 1989; Kitano et al., 2001), retinoid efficacy has also been demonstrated in healing of wounds in other tissues (bone, cornea, respiratory tract, upper digestive system, and gut). While retinoids have the potential of improving skin structure and function, a consequence of topical retinoid use is skin irritation. Irritated skin is characterized by redness, dryness, and flaking at the treated site. At the histological level, one sees a perivascular accumulation of mononuclear cells, with neutrophils and monocytes scattered throughout the dermis and occasional micro-abscesses in the dermis or epidermis. ROL tends to be less irritating than RA in most cases, but even with this agent, significant irritation is observed in many individuals (Kang et al., 1995). Likewise, skin irritation is also a complication with synthetic retinoidal agents currently on the market. Irritation is a major cause of non-compliance among retinoid users. In addition, excessive irritation may counteract the beneficial effects of retinoid use or actually predispose the tissue to abrasion wounding.

2.3.3.6.2 Calcium

Calcium has an established role in the normal homeostasis of mammalian skin and serves as a modulator in keratinocyte proliferation and differentiation. Gradients of calcium concentration increasing from 0.5 mM in the basal layer to > 1.4 mM in the stratum granulosum are consistent with migration patterns in response to minor abrasion (normal wear). Dermal fibroblasts require calcium but are approximately 100 times less sensitive than keratinocytes. Normal calcium metabolism in the skin is dependent on cell membrane and cytosolic calcium binding proteins (calmodulin, cadherins, etc.), but their modulation through parathyroid hormone, vitamin D or growth factors in normal or damaged tissue is not well documented. In wound repair, calcium is predominantly involved as Factor IV in the hemostatic phase, but it is expected to be required in epidermal cell migration and regeneration patterns in later stages of healing. Calcium alginate dressings are designed to liberate calcium early in the acute phase to promote hemostasis, but it is presently unclear whether the supplementary calcium influences the intracellular environment at later stages of wound repair, notably during the remodeling phase. Although experimental



studies suggest that control of calcium is obligatory in wound management, it is very little known as to how calcium in the wound bed is modulated through hormones, vitamin D, or various growth factors. Also, there is limited information as to how calcium released either from dressings, platelets, or from the circulation through the action of parathyroid hormone, growth factors or other modulators influences cell migration and remodeling in skin wounds, although experimental models suggest that management of calcium is essential in wound management (Lansdown, 2002).

2.3.3.6.3 Bone Morphogenetic Protein (BMP)

During the wound-healing process, the proliferation of fibroblasts and deposition of collagen are conducive to wound healing. However, the hyperproliferation of fibroblasts and excessive collagen deposition may lead to hypertrophic scar formation. The transforming growth factor (TGF)- β 1 cytokine is closely associated with scar formation, and overexpression of TGF- β 1 has been demonstrated to promote scar formation (Jagadeesan and Bayat, 2007). Bone morphogenetic protein-7 (BMP-7) is a member of the TGF- β superfamily. Increasing evidence suggests that BMP-7 attenuates TGF- β -induced fibrogenesis in various tissues (Weiskirchen and Meurer, 2013). (Bi et al., 2014) reported that the TGF- β 1/BMP-7 ratio regulated liver fibrosis in rats. An additional study demonstrated that maintaining BMP-7 levels and inhibiting TGF- β 1 attenuates the progression of renal fibrosis (Weiskirchen and Meurer, 2013). Furthermore, it was previously demonstrated that BMP-7 inhibits TGF- β 1-induced fibroblast-like differentiation of rat dermal papilla cells (Bin et al., 2013). Based on these reports, the authors of the current study hypothesized that BMP-7 may serve a role in promoting wound healing and attenuating scar formation.

2.3.3.6.4 Epidermal Growth Factor (EGF)

One of the first growth factors to be identified was epidermal growth factor. The discovery led to the Nobel Prize for Stanley Cohen. EGF stimulates the proliferation and migration of all types of epithelial cells (Cohen, 1983). EGF has also been found to be important for the healing of the gut mucosa and may have protective and healing effects for ulcers. Transforming Growth Factor-alpha (a completely different growth factor than TGF-beta) has a very similar action and structure as EGF; however, TGF-alpha may be more potent than EGF (Derynck, 1986). These

two growth factors (plus other members of the family) bind to the EGF receptor and initiate the signal transduction pathway described above. A mutated form of the EGF receptor that lacks the extracellular component is the oncogene v-erb-B. The loss of the extracellular binding site leads to a loss of negative feedback, chronic receptor activation, and malignant transformation.

2.3.3.6.5 Fibroblast Growth Factor (FGF)

There are currently at least nine members of the fibroblast growth factor family. Many of these growth factors have heparin-binding capabilities. There are four known FGF receptors that contain tyrosine kinase activities (FGFR-1, FGFR-2, FGFR-3, and FGFR-4) (Johnson and Williams, 1993). Another membrane protein binds FGFs but lacks kinase activity. The first two members of the FGF growth factor family to be discovered were acidic and basic FGF (aFGF or FGF-1, and bFGF or FGF-2, respectively). The majority of the healing studies have involved these two growth factors. Acidic and basic FGF have profound angiogenic activities in addition to being mitogenic for fibroblasts. One of the more recently discovered fibroblast growth factors, keratinocyte growth factor (KGF or FGF-7), has been found to have profound stimulatory effects on keratinocyte growth (Rubin et al., 1989).

2.3.3.6.6 Platelet-Derived Growth Factor (PDGF)

One of the earlier growth factors to be discovered was PDGF, a growth factor that is a product of not only platelets but macrophages and many other cell types. PDGF must exist as a dimer consisting of either A and/or B chains (AA, AB, or BB). The most common form in the human platelet is the AB form (70% AB, 30% BB) (Hart et al., 1990). Like many growth factors, abnormal PDGF regulation and feedback may lead to cell transformation. The B chain has been found to be the c-sis proto-oncogene and is very similar to the transforming protein produced by the simian sarcoma virus. There are two specific PDGF receptors: alpha and beta. The alpha receptor can bind all three isoforms (AA, AB, or BB). The beta receptor binds the BB homodimer with high affinity, the AB with lower affinity, and does not bind AA. The differential binding contributes to the varying effects of the different isoforms on cellular function. PDGF is chemotactic for fibroblasts, smooth muscle cells, and possibly monocytes, and neutrophils. PDGF is a mitogen (increases proliferation) for smooth muscle cells and fibroblasts. It also has profound effects on the extracellular matrix (Heldin et al., 1993). At least two other proteins fit

in the PDGF family. Vascular endothelial growth factor (VEGF) is a growth factor that has homology with PDGF and is a potent angiogenic factor.

2.3.3.6.7 Insulin-like Growth Factors (IGF-I, IGF-II)

The insulin-like growth factors are similar in structure to proinsulin and have considerable overlapping functions. IGF-I (also known as somatomedin C) has profound effects on stimulating growth, especially the secondary growth characteristics of adolescence. It also is very important in promoting protein synthesis. The current studies suggest that the anabolic mechanisms of growth hormone act through increases in serum levels of IGF-I. IGF-I also increases the proliferation of many cell types, including fibroblasts; however, it frequently needs to be combined with another growth factor such as PDGF or FGF (Stiles et al., 1979). IGF-II is felt to be more active in fetal growth; however, it has been found to have similar effects as IGF-I and can also improve healing. The IGF-I receptor has a structure that resembles the alpha/beta dimer structure of the insulin receptor. The IGF-II receptor has a totally different structure and has been found to be identical to the mannose-6-phosphate (M6P) receptor. Insulin, IGF-I, and IGF-II preferentially bind their own receptor but can bind to either of the other types of receptors (Nissley and Lopaczynski, 1991). Interestingly, the IGF-II/M6P receptor also binds mannose moieties on latent TGF-beta and may play a role in activating TGF-beta.

2.3.3.6.8 microRNA

microRNAs that are implicated in defining biological outcomes in response to a change in the state of tissue oxygenation are referred to as oxymiRs. OxymiRs can be directly influenced by changes in tissue oxygenation or indirectly by oxygen- sensitive transcription factors, metabolites, pH, etc. or may also affect other oxygen- sensing pathways. miRNAs that are sensitive to hypoxia are classified as "hypoxymiRs". HIF is one of the most important hypoxia sensors in our body and a number of hypoxymiRs are induced by HIF stabilization (Loscalzo, 2010). Some miRNAs also influence HIF stabilization. Induction of miR-424 is known to stabilize HIF1 α , while miRs that target the HIF1 α transcript such as miR-20b, miR-199a, and miR-17~92 are repressed to increase HIF1 α expression and transcription. Chronic non-healing wounds are characterized by ischemia/hypoxia. HypoxymiRs have therefore been a strong focus to identify dysregulated targets for possible therapeutic intervention. miR-210, miR-21, and miR-

203 are the most studied hypoxymiRs with respect to wound healing. The key oxymiRs that are differentially expressed during the different stages of wound healing are as follows (Figure 2.18):

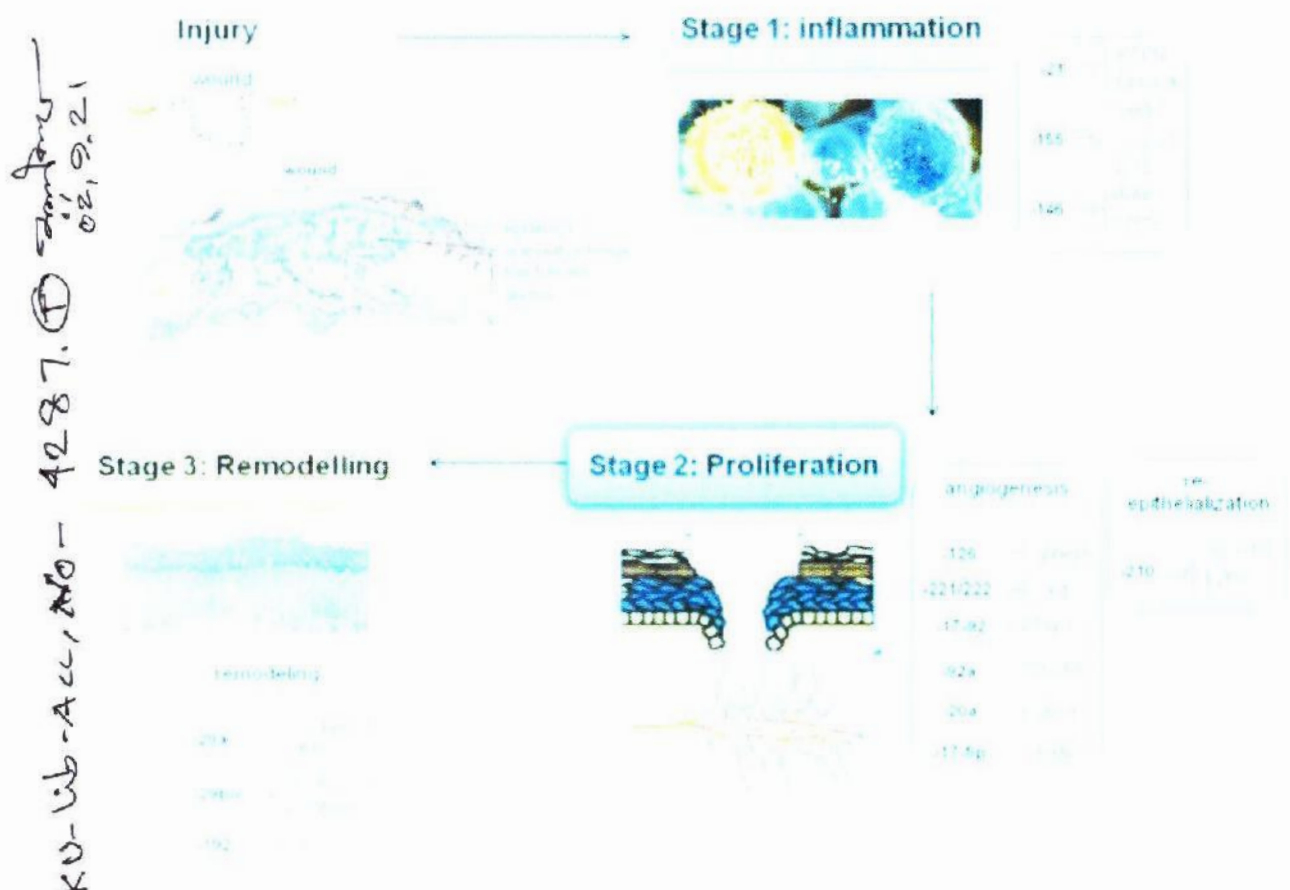


Figure 2.18: MicroRNAs involved in different stages of wound healing (Banerjee and Sen, 2015).

2.4 Amniotic Membrane (AM)

The amnion is a thin semi-transparent tissue forming the innermost layer of the fetal membrane. As fresh amnion carries risk of contaminations and disease transmission, amnion is collected from placentae of selected and screened donors. Various preservation methods have been introduced, including cryopreservation in liquid nitrogen, preservation in silver nitrate, storage in antibiotics solution, glycerol-preserved sheets, dried sheets and gamma-irradiated sheets (Halim et al., 2010). It has been claimed to be one of the most effective biological skin substitutes used in burn wounds, with efficiency of maintaining low bacteria count. It also has advantages of

reducing loss of protein, electrolytes and fluids, decreasing the risk infection, minimizing pain, acceleration of wound healing and good handling properties (Bujang et al., 2010).

2.4.1 Structure of AM

AM is the innermost lining of the fetal membrane that is in contact with the developing fetus; serving as a natural barrier to protect the fetus from infections and trauma because of the lack of a fetal immune system. From embryological aspect AM develop from extra-embryonic tissue and consist of a foetal component (the chorionic plate) and a maternal component (the deciduas). At histological level AM is a thin, tough, transparent, avascular composite membrane composed of three major layers: a single epithelial layer, a thick basement membrane, and an avascular mesenchymal layer. The amniotic epithelial cell layer is a single layer of flat, cuboidal and columnar cells that are in direct contact with the amniotic fluid consisting mainly of collagen (Ilancheran et al., 2009).

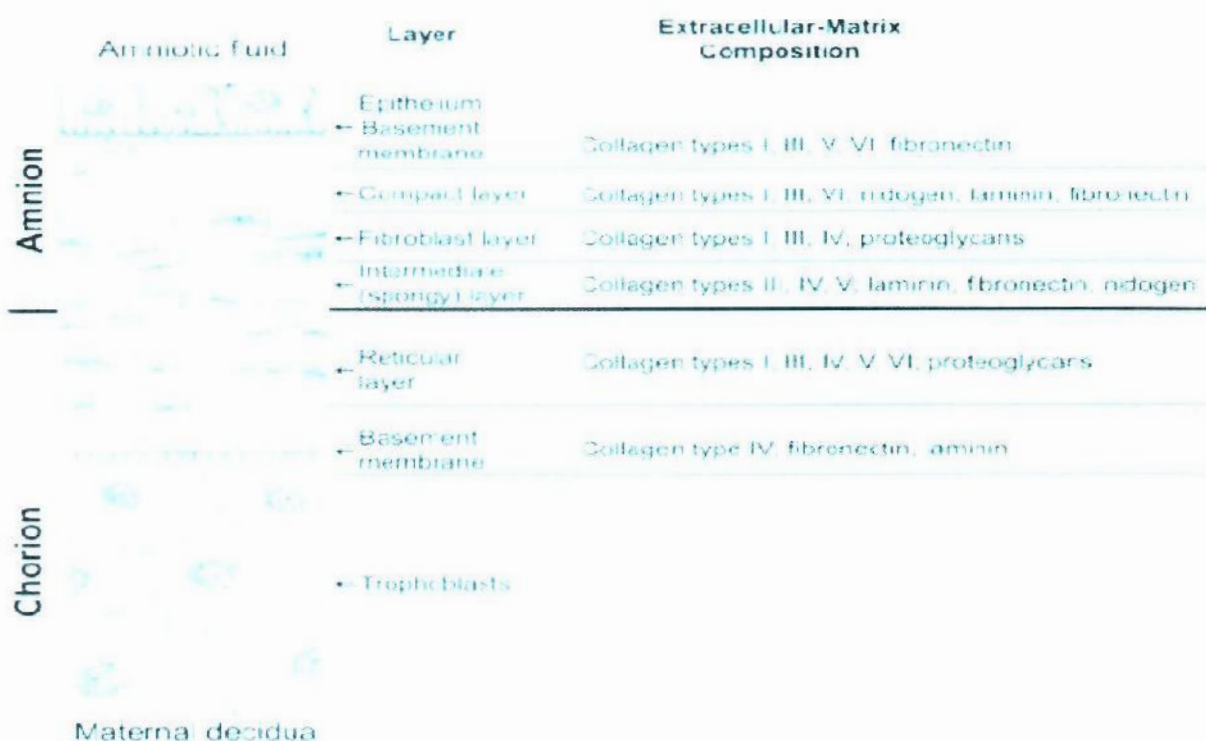


Figure 2.19: Layer and extracellular matrix composition of amniotic membrane (Pipino et al., 2013).

At ultrastructure level mesenchymal layer can be further divided in to compact, fibroblast and spongy layer. Two types of cells, with different embryological origins, present in AM; one is

amniotic epithelial cells (AECs) derived from embryonic ectoderm and amnion mesenchymal cells from embryonic mesoderm (Blackburn, 2003). The extracellular matrix mainly consists of collagen (types I, III, IV, V, VI), laminins, reticular fibers, proteoglycans, glycoproteins, cell-signaling proteins (such as cytokines), and growth factors that are essential to the healing process (Figure 2.19). There are no nerves, muscles, or lymphatics in the amniotic membrane; instead, the nutrients it requires are supplied directly by diffusion out of the amniotic fluid and/or from the underlining decidua.

2.4.2 Burn Related Properties of AM

Burn related properties of amniotic membrane include historical use of amnion for burn related casualties as well as advantages of using amnion in burn treatment.

2.4.2.1 Historical Perspective: Amnion for Burns

Human amnion has been used for centuries as a biological wound dressing. In China and Japan, amnion is considered a potent medication for the treatment of many diseases and is believed to impart the 'magical strength of youth' (Tyszkiewicz et al., 1999). In western medicine, John Staige Davis introduced the use of fetal membrane as a permanent skin transplant at the beginning of the last century. William Thornton, a final-year medical student at Johns Hopkins Hospital in 1910, suggested this concept. However, it soon became clear that amnion could only be used as a temporary biological wound dressing.

Human amnion was first used as a biological burn dressing by Sabella and Stem in 1913 (Subrahmanyam, 1995). Since then, amnion has been widely used to treat superficial and partial-thickness burns at many centers worldwide (Subrahmanyam, 1995). The versatility and reliability of human amnion as a biological dressing resulted in the extension of its application to various clinical conditions in the plastic surgery field. (Ward et al., 1989) used this biological dressing in the treatment of chronic ulcers and pressure sores. Moreover, skin graft donor sites can be effectively treated using human amniotic membrane. Recently, radiation-induced ulcers have been effectively treated using human amniotic membrane as shown by (Gajiwala and Sharma, 2003). Thus, human amnion has the potential to become an attractive biomaterial that is cheap and widely available.

2.4.2.2 Advantages of Amnion in Burn Treatment

A crucial and important factor for successful treatment of burns is the early covering of the burned area with skin substitutes. The importance of the amnion membrane as a biological dressing for burns amounts to: a barrier to bacterial colonization, hastens the epithelialization, and control of water loss.

2.4.2.2.1 Minimizing Fluid/Water Loss

The amnion is made of five distinctive layers: epithelial, basement membrane, connective tissue, fibroblasts, and a spongy layer. This structure resembles the stratum corneum in the human skin epidermis, which is a natural vapor barrier (Niknejad et al., 2008). By acting as a vapor barrier, the amnion can prevent excessive evaporation from the wound surface thus directly and markedly reducing the insensible fluid loss, which, in turn, reduces the amount of replacement fluid required for burn injuries (Gump and Kinney, 1970). It is worth to note that the membrane does not influence the fluid shift from the plasma into the interstitial compartment in the extracellular space. An additional advantage of preventing evaporation of the wound is that the temperature regulation mechanism is not overly affected and, in turn, the caloric requirements needed to maintain temperature are also reduced accordingly. There are multiple lines of evidence in the literature that support the aforementioned properties of the amnion as a burn dressing. Its application to third-degree burns and granulated wound beds caused the membrane to disintegrate. Therefore, amnion is less effective for the treatment of third-degree burns as opposed to first- and second-degree burns.

2.4.2.2.2 Pain Reduction

Pain reduction in burn wounds is a well-recognized advantage of the amnion that has been observed by many researchers (Subrahmanyam, 1995; Mohammadi et al., 2009; Mostaque and Rahman, 2011). Application of amnion to burn surfaces can protect the exposed nerve endings from external irritants such as clothing and even those as soft as moving air. Amnion has good adherence to wound surfaces and is able to maintain a moist environment, which contributes to this advantage. (Sharma et al., 1985) in their observation of patients with partial-thickness burns, demonstrated that amnion application completely alleviated pain (Sharma et al., 1985).

(Ravishanker et al., 2003) used glycerol-preserved amniotic membrane and noted that it reduced the pain score in adults and quieted a distressed child.

2.4.2.2.3 Good Wound Adherence

Amnion very firmly adheres to wound beds. The bonding that is established between the amnion and the wound surface is biological, not mechanical, and is considered to be due to the fibrin-elastin formation at the wound-dressing interface (Burlison and Eiseman, 1972). This property, which is shared with other biological dressings, helps to protect the fragile tissue at the burn wound surface, especially the injured cells that have the potential either to recover or to be converted into a deeper burn depth. The strong bond and adherence can also maintain the moisture level over the wound surface, which can accelerate re-epithelialization and protect the newly formed thin epithelium from trauma. The amnion will become dry and readily peel off once the wound is completely healed.

2.4.2.2.4 Less Frequent Dressing

In a study comparing radiation-sterilized, oven-dried amniotic membrane against topical silver sulfadiazine for the treatment of second-degree burns, (Mostaque and Rahman, 2011) demonstrated that the amniotic membrane is a better treatment option because its use reduces the length of hospital stay and the number of required dressing changes. Epithelialization of the wound is also quicker. The use of amniotic membrane is painless and odorless. The procedure is easy and comfortable for the doctor, and it is well accepted. Most of the patients remain ambulatory during treatment (Ravishanker et al., 2003; Mostaque and Rahman, 2011).

2.4.2.2.5 Neovascularization

Human amnion has inherent angiogenic properties that increase the rate of healing and epithelialization. The vascular response of amnion on the wound bed has been extensively studied using standard histological and immune histological staining techniques. In a study of the use of amnion in the treatment of chronic leg ulcers, (Faulk et al., 1980) noted that the most striking effect of amnion was the development of new vessels. This was observed grossly, histopathologically, and immune histologically (Faulk et al., 1980).

2.4.2.2.6 Faster Wound Healing

Anecdotally, amnion has been demonstrated to speed up the epithelialization process, which results in faster wound healing in superficial and partial-thickness burns. For example, Miller et al. demonstrated that partial-thickness burn wounds healed faster than controls when protected by immediate allografting. However, the routine use of allografts is limited to special centers as it requires a well-organized skin bank. On the other hand, xenografts (e.g., porcine skin) are inferior to allografts, more expensive and not readily available in many countries. Clinical trials and animal experiments have demonstrated that amnion is superior to both allograft and xenograft materials (Colocho et al., 1974). It was demonstrated that amniotic membrane application to partial-thickness burns is associated with minimal oozing, and healing occurs without the need for autografting (Sharma et al., 1985). The membrane promotes healing by accelerating the migration of fibroblasts and the development of collagen during the first 6–8 days of repair (Sharma et al., 1985). The amniotic membrane can also provide a good moisture balance that allows rapid epithelialization to occur. One other suggested mechanism for the observed rapid healing is the inhibition of proteinase activity, which, in turn, reduces inflammatory responses because of the decreased infiltration of polymorphonuclear leukocytes.

2.4.2.2.7 No Rejection Phenomenon

Almost all biological dressings or skin substitutes are predisposed to early graft reaction with the amnion membrane tissue being the only exception. As human amniotic membrane is embryologically derived from fetal ectoderm, it carries the advantages of fetal skin allografts. Therefore, it can fulfill some of the functions of the skin that are destroyed in burn injuries; however, it is different than skin allografts, and the vascularization and rejection of the membrane does not occur when the amnion is directly placed over the wound surface (Colocho et al., 1974). Faulk associated the feto-maternal relationship of the fetus with the existence of a parasite that successfully avoids host rejection (Faulk and Galbraith, 1979). Later, (McIntyre and Faulk, 1979) demonstrated that a glycoprotein that is found in trophoblasts and amniochorion is very important in suppressing the detection of the fetus as “foreign” to the mother by acting on maternal lymphocytes and preventing rejection throughout pregnancy. The lack of human leukocyte antigen (HLA) A, BC and DR surface antigens and beta-2- microglobulin expression

in human amniotic epithelial cells could further contribute to the lower inflammatory response and lack of rejection phenomenon observed in this type of allografting.

2.4.2.2.8 Anti-bacterial Properties

Microbial invasion into the burn wound has been recognized as one of the most common obstacles in the recovery and healing process for burn injuries, especially major burns with a deeper burn thickness. The current strategy employed at most institutions is to maintain strict environmental control in the burn unit, early removal/debridement of dead/non-viable burn eschar and the use of topical antimicrobial agents. Human amniotic membrane can provide a mechanical barrier to microbial invasion, and when used, has produced a lower rate of burn wound infection compared with other types of dressings (Sharma et al., 1985). There are several possible mechanisms for the observed antibacterial effect of amniotic membranes. Many authors agree that amnion is an effective biological dressing, as it adheres intimately onto the contour of the wound and inevitably lies in close opposition to the wound surface (Gruss and Jirsch 1978). It has been noted that the degree of microbial or bacterial contamination is inversely proportional to the number of leucocytes beneath the membrane (Gruss and Jirsch 1978). It is important to have a dressing that can be closely approximated to the wound surface with minimal dead space to gain this advantage. Amnion is better than other biological dressings in this manner as it is thinner and more pliable. It has also been demonstrated that the surface pH falls after amniotic membrane application and that the temperature of the underlying wound bed rises. Both of these changes promote phagocytosis and reduce the exudation of interstitial fluid.

2.4.2.2.9 Better Scarring

The use of amnion dressing in the treatment of superficial burns and superficial dermal burns has been observed to produce faster healing and superior cosmetic results for the resultant scars. (Pigeon, 1960) also noted that the use of amnion resulted in an absence of the abnormal pigmentation usually found with healed burn scars. An animal study investigating the resultant scarring from deep dermal burns demonstrated that amniotic membrane produces reduced scar tissue as compared with standard paraffintulle dressings when assessed histopathologically by significantly inhibiting alpha smooth muscle actin (α -SMA). The amniotic membrane treatment

led to a significant reduction in the amount of scar tissue generated in response to an experimental burn compared with moist wound dressings (Fraser et al., 2009).

2.5 Collagen

Collagen is the main structural protein in the extracellular space in the various connective tissues in the body. As the main component of connective tissue, it is the most abundant protein in mammals (Di Lullo et al., 2002), making 25% to 35% of the whole-body protein content. Collagen consists of amino acids wound together to form triple-helices of elongated fibrils. It is mostly found in fibrous tissues such as tendons, ligaments, and skin. Depending upon the degree of mineralization, collagen tissues may be rigid (bone), compliant (tendon), or have a gradient from rigid to compliant (cartilage). It is also abundant in corneas, blood vessels, the gut, intervertebral discs, and the dentin in teeth. In muscle tissue, it serves as a major component of the endomysium. Collagen constitutes one to two percent of muscle tissue and accounts for 6% of the weight of strong, tendinous, muscles (Sikorski, 2001). The fibroblast is the most common cell that creates collagen. Gelatin, which is used in food and industry, is collagen that has been irreversibly hydrolyzed. Collagen has many medical uses in treating complications of the bones and skin.

2.5.1 Collagen Types

Over 90% of the collagen in the human body is type I. However, as of 2011, 28 types of collagen have been identified, described, and divided into several groups according to the structure they form (Ricard-Blum, 2011):

- Fibrillar (Type I, II, III, V, XI)
- Non-fibrillar
 - FACIT (Fibril Associated Collagens with Interrupted Triple Helices) (Type IX, XII, XIV, XIX, XXI)
 - Short chain (Type VIII, X)
 - Basement membrane (Type IV)
 - Multiplexin (Multiple Triple Helix domains with Interruptions) (Type XV, XVIII)
 - MACIT (Membrane Associated Collagens with Interrupted Triple Helices) (Type XIII, XVII)

- Other (Type VI, VII)

The five most common types are:

- Type I: skin, tendon, vasculature, organs, bone (main component of the organic part of bone)
- Type II: cartilage (main collagenous component of cartilage)
- Type III: reticulate (main component of reticular fibers), commonly found alongside type I
- Type IV: forms basal lamina, the epithelium-secreted layer of the basement membrane
- Type V: cell surfaces, hair, and placenta

2.5.2 Medical Uses of Collagen

Due to several properties of collagen, they are considered for various applications in biomedical sciences.

2.5.2.1 Cardiac Applications

The collagenous cardiac skeleton which includes the four heart valve rings, is histologically, elastically and uniquely bound to cardiac muscle. The cardiac skeleton also includes the separating septa of the heart chambers – the inter-ventricular septum and the atrio-ventricular septum. Collagen contribution to the measure of cardiac performance summarily represents a continuous torsional force opposed to the fluid mechanics of blood pressure emitted from the heart. The collagenous structure that divides the upper chambers of the heart from the lower chambers is an impermeable membrane that excludes both blood and electrical impulses through typical physiological means. With support from collagen, atrial fibrillation never deteriorates to ventricular fibrillation. Collagen is layered in variable densities with cardiac muscle mass. The mass, distribution, age and density of collagen all contribute to the compliance required to move blood back and forth. Individual cardiac valvular leaflets are folded into shape by specialized collagen under variable pressure. Gradual calcium deposition within collagen occurs as a natural function of aging. Calcified points within collagen matrices show contrast in a moving display of blood and muscle, enabling methods of cardiac imaging technology to arrive at ratios essentially stating blood in (cardiac input) and blood out (cardiac output).

2.5.2.2 Cosmetic Surgery

Collagen has been widely used in cosmetic surgery, as a healing aid for burn patients for reconstruction of bone and a wide variety of dental, orthopedic, and surgical purposes. Both human and bovine collagen is widely used as dermal fillers for treatment of wrinkles and skin aging. Some points of interest are:

- When used cosmetically, there is a chance of allergic reactions causing prolonged redness; however, this can be virtually eliminated by simple and inconspicuous patch testing prior to cosmetic use.
- Most medical collagen is derived from young beef cattle (bovine) from certified BSE-free animals. Most manufacturers use donor animals from either "closed herds", or from countries which have never had a reported case of BSE such as Australia, Brazil, and New Zealand.

2.5.2.3 Bone Grafts

As the skeleton forms the structure of the body, it is vital that it maintains its strength, even after breaks and injuries. Collagen is used in bone grafting as it has a triple helical structure, making it a very strong molecule. It is ideal for use in bones, as it does not compromise the structural integrity of the skeleton. The triple helical structure of collagen prevents it from being broken down by enzymes, it enables adhesiveness of cells and it is important for the proper assembly of the extracellular matrix (Cunniffe and O'Brien, 2011).

2.5.2.4 Tissue Regeneration

Collagen scaffolds are used in tissue regeneration, whether in sponges, thin sheets, or gels. Collagen has the correct properties for tissue regeneration such as pore structure, permeability, hydrophilicity, and being stable in vivo. Collagen scaffolds are also ideal for the deposition of cells such as osteoblasts and fibroblasts, and once inserted, growth is able to continue as normal in the tissue (Oliveira et al., 2009).

2.5.2.5 Reconstructive Surgical Uses

Collagens are widely employed in the construction of the artificial skin substitutes used in the management of severe burns and wounds (Gould, 2016). These collagens may be derived from bovine, equine, porcine, or even human sources; and are sometimes used in combination with silicones, glycosaminoglycans, fibroblasts, growth factors and other substances.

2.5.2.6 Wound Care

Collagen is one of the body's key natural resources and a component of skin tissue that can benefit all stages of the wound healing process. When collagen is made available to the wound bed, closure can occur. Wound deterioration, followed sometimes by procedures such as amputation, can thus be avoided.

Collagen is a natural product and is thus used as a natural wound dressing and has properties that artificial wound dressings do not have. It is resistant against bacteria, which is of vital importance in a wound dressing. It helps to keep the wound sterile, because of its natural ability to fight infection. When collagen is used as a burn dressing, healthy granulation tissue is able to form very quickly over the burn, helping it to heal rapidly (Singh et al., 2011).

Throughout the 4 phases of wound healing, collagen performs the following functions in wound healing:

- **Guiding function:** Collagen fibers serve to guide fibroblasts. Fibroblasts migrate along a connective tissue matrix.
- **Chemotactic properties:** The large surface area available on collagen fibers can attract fibrogenic cells which help in healing.
- **Nucleation:** Collagen, in the presence of certain neutral salt molecules can act as a nucleating agent causing formation of fibrillar structures. A collagen wound dressing might serve as a guide for orienting new collagen deposition and capillary growth.
- **Hemostatic properties:** Blood platelets interact with the collagen to make a hemostatic plug.

2.6 Epithelial Sheath

Epithelial sheath is considerably a recent and novel concept of covering the wound area along with other wound healing agents which itself may be composed of biomaterials. It may serve the dual function of wound healing and protecting the wound area from external environment.

For many years, conventional wound dressing (i.e. cotton gauze) and topical antibacterial agents (i.e. SSD) were used to protect the wounds from trauma, and environmental infection and provide their stability. However, these dressing materials have several limitations including adherence to wound, resulting in ischemia and/or necrosis, occurrence of a foreign-body reaction, and irritation, or allergic reaction. In addition, SSD as the topical gold standard antibacterial agents for burned wounds has several disadvantages including the nephro/hepatotoxicity of silver as a heavy metal, argyria and dyschromia (Elliott, 2010).

Newer wet dressing methods such as epithelial sheath, hydrogels, alginate, and hydrocolloids have been introduced in order to overcome the conventional dressing drawbacks. The moisture suppresses scar formation, allowing efficient keratinocyte migration and re-epithelialization. Moreover, moisture decreases the amount of lost dermis and adnexal structures, thus improving the cosmetic outcome of the injury (Sood et al., 2014). Poor physical stability of these wet dressing materials creates a new challenge for researchers to use them alone on wounds. In the recent years, new biodegradable natural materials have been fabricated to protect burn wounds. Collagen-chitosan scaffold as epithelial sheath is one of these materials which encourage deposition and organization of the newly formed collagen fibers and granulation tissue in the wound bed (Singh et al., 2011). Moreover, the collagen fibers in the collagen-chitosan based dressings can be degraded by the matrix metalloproteinases (MMPs) found in the wound exudates, as an alternative source of natural collagen available for normal wound healing.

2.6.1 Chitosan as Part of Epithelial Sheath

Chitosan is a linear polysaccharide composed of randomly distributed β -(1 $\rightarrow$ 4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is made by treating the chitin shells of shrimp and other crustaceans with an alkaline substance, like sodium hydroxide.

Chitosan has a number of commercial and possible biomedical uses. It can be used in agriculture as a seed treatment and biopesticide, helping plants to fight off fungal infections. In winemaking, it can be used as a fining agent, also helping to prevent spoilage. In industry, it can be used in a self-healing polyurethane paint coating. In medicine, it is useful in bandages to reduce bleeding and as an antibacterial agent; it can also be used to help deliver drugs through the skin. More controversially, chitosan has been asserted to have use in limiting fat absorption, which would make it useful for dieting, but there is evidence against this. Other uses of chitosan that have been researched include use as a soluble dietary fiber.

2.6.1.1 Medical Uses of Chitosan

Chitosan's is used within some wound dressings to stop bleeding. Such dressing first received approval for medical use in the United States in 2003 (Zhang et al., 2015). It also appears to decrease the growth of bacteria and fungus. It was also being look at as a burn dressing as of 2011 (Ducheyne et al., 2011). Chitosan dressings have been used during the wars in Iraq and Afghanistan. A study during the war found tentative evidence of success. Versions are made by a number of manufacturers in a number of formats (Zhang et al., 2015).

Chitosan hemostatic agents are often chitosan salts made from mixing chitosan with an organic acid (such as succinic or lactic acid). The hemostatic agent works by an interaction between the cell membrane of erythrocytes (negative charge) and the protonated chitosan (positive charge) leading to involvement of platelets and rapid thrombus formation (Baldrick, 2010). The chitosan salts can be mixed with other materials to make them more absorbent (such as mixing with alginate), or to vary the rate of solubility and bioabsorbability of the chitosan salt. The chitosan salts are biocompatible and biodegradable making them useful as absorbable hemostats. The protonated chitosan is broken down by lysozyme in the body to glucosamine (Baldrick, 2010) and the conjugate base of the acid (such as lactate or succinate), substances naturally found in the body.

2.7 Advantages of Using Hydrogel over Skin Graft

Despite recent advances in burn wound skin management, the death rates remain high throughout the world with a vast majority (eleven fold higher) in low-income countries. However, in particular, people of rural areas are more prone to the burden of burn related mortalities and

morbidities. Fast aid and swift treatment of burn patients are vital steps to increase the survivability by closing and protecting the burn wounds as immediately as possible to mitigate disabilities and fatalities. In most developing countries, people would like to treat burn injuries immediately at home before going to clinic and they are still struggling for a low cost and effective fast aid product to manage burn injuries in an efficient and rapid manner. Nonetheless, in developed world, commercial skin grafts such as integra and biobrane are available which have been shown to improve wound healing but they are also expensive and sometimes do not deliver optimal outcomes. So that, amnion can be clinically applied as a wound covering bioactive material for split thickness skin burn wounds as well as for children with partial-thickness facial skin burn wounds. It is used as a graft for burn wound enclosures in Bangladesh which appears to be advantageous. It has a very easy availability and the cost of the procurement of AM is comparatively low. But, amniotic membrane grafting service is available only in city areas at a very limited scale. Considering the limitations of amnion grafting in Bangladesh and to treat burn injuries in home instantly, and taking the advantages of wound healing properties of human amnion and collagen; a novel cost efficient hydrogel product can be developed which in fact, is easy to produce and store, and is physiologically effective. It is also useful for topical application. So many impoverished people who are the majority in the population of developing countries like Bangladesh will be able to have proper treatment at a very low cost. Industrial development can be possible which can make opportunity for the people. It can also open future research window as well.

CHAPTER THREE
MATERIALS AND METHODS



3.1 Working Place

The research work entitled “**Characterization and *in vivo* Evaluation of Amniotic Membrane and Collagen Based Hydrogel on Rat Model**” was designed and carried out in the Institute of Tissue Banking and Biomaterial Research (ITBBR), Atomic Energy Research Establishment (AERE), Savar, Dhaka from July, 2018 to May 2019. An outline of this research has been depicted as a flowchart and schematic diagram in **Figure 3.1**. All the chemicals and equipment used in this study are given in Appendix I and II.

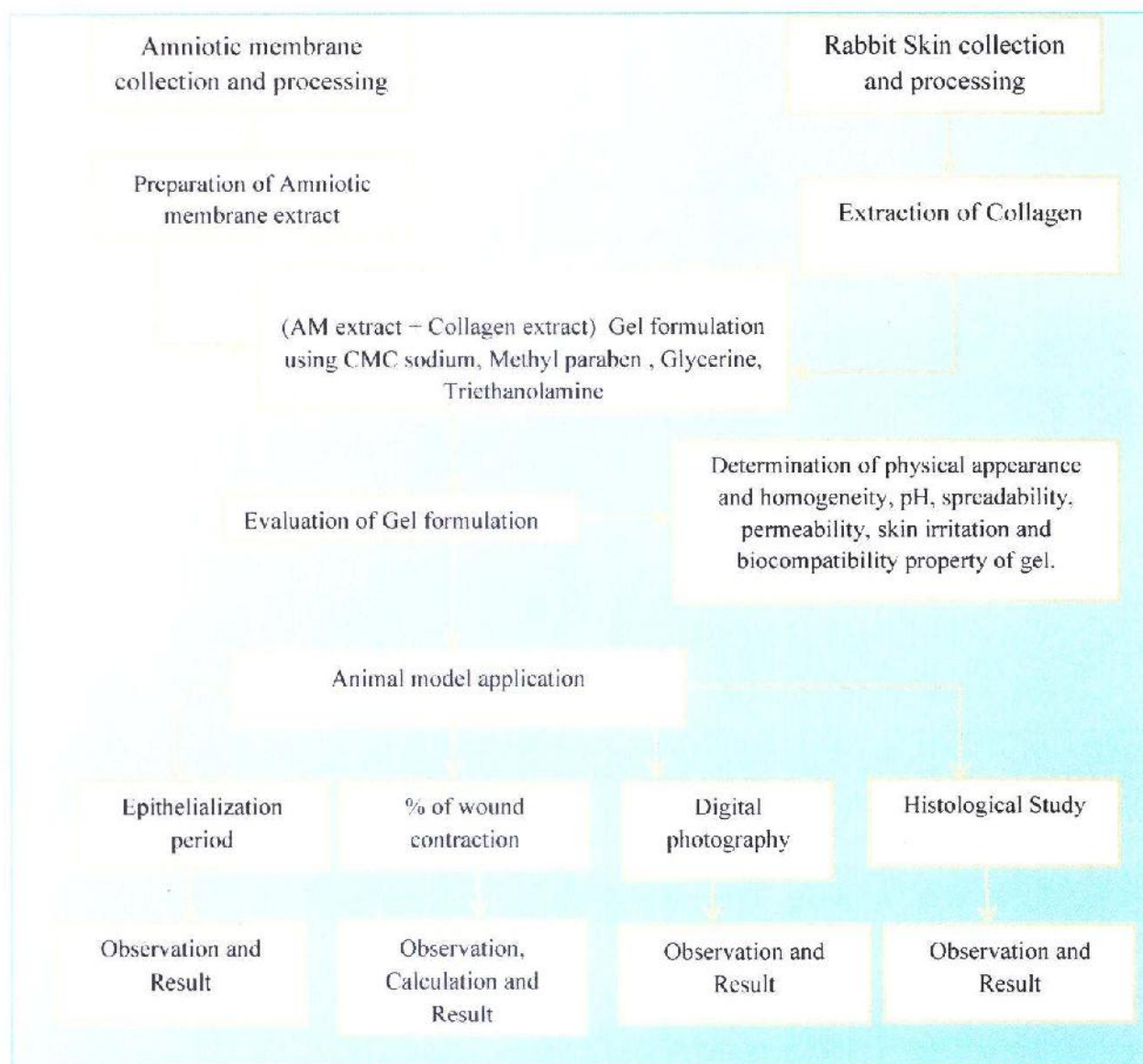


Figure 3.1: Flow chart of experimental design.

3.2 Processing of Amniotic Membrane (AM)

3.2.1 Collection of AM

Amniotic sacs were collected from sero-negative donor (HIV, Syphilis, Hepatitis B and C viruses) of cesarean deliveries or caesarean deliveries. The ages of the donors were ranged from 25 to 35 years. The tissues were collected by the written consent of the donor by following the “Human Organ / Tissue Donation and Transplantation Act” that has been passed by the National Parliament of the People’s Republic of Bangladesh. Amniotic Sacs were collected from Gonoshasthaya Samaj Vittik Medical College, Savar, Dhaka (**Figure 3.2**). After collection, the samples were stored in a sterile plastic jar containing sterile physiological solution in. Then, sample bottles were preserved in a refrigerator and were transported to Institute of Tissue Banking and Biomaterial Research (ITBBR), Savar, Dhaka.

Adequate number of plastic containers containing normal sterile saline (0.9% NaCl) was provided to the hospital before delivery of new borne. All the containers were accurately labeled with placenta sample types (caesarean), donor ID and hospital registration number. Amnion samples (glossy, translucent, and thinner) were then collected into those containers shortly after the delivery and preserved temporarily in a freezer (Lab Cold, Model: RLHE0845, UK) below -20°C .



Figure 3.2: Sample collection area.

3.2.2 Preparation of AM Extract

Frozen AM were allowed to thaw in normal room temperature (**Figure 3.3A**). Firstly, amniotic sac was taken in a tray and human AM was carefully separated from chorionic membrane by forceps (**Figure 3.3B**). Separated AM was then washed in a 500ml beaker containing sterile saline water (**Figure 3.3C**). AM was then taken into 250 ml conical flasks and appropriate amount of saline water was poured into them (**Figure 3.3D**). The conical flasks were then placed on a shaker incubator and rotated at 200 rpm for 24 hours (**Figure 3.3E**). This process is followed by changing of saline water with the same amount of saline water. Previous steps were repeated until the AM were cleaned properly (removing all the blood residues from the membrane). Membranes were then washed with saline solution containing streptomycin and penicillin. After washing each membrane was spread in a sterile petri dish and allowed to be frozen at -20°C (**Figure 3.3F**). Frozen membranes were then freeze dried at -55°C for 16 hours (Alpha1-4LD, CHRIST, Germany) (**Figure 3.3G**). Then the freeze-dried AMs (**Figure 3.3H**) were then collected and sealed into plastic bags for radiation sterilization at 25 KGY using cobalt 60 source. Irradiated AM (**Figure 3.3I**) will be put into a blender machine (**Figure 3.3J**) and AM powder will be collected in a polybag (**Figure 3.3K**) and stored in refrigerator until further use (Dillard and German, 2000).

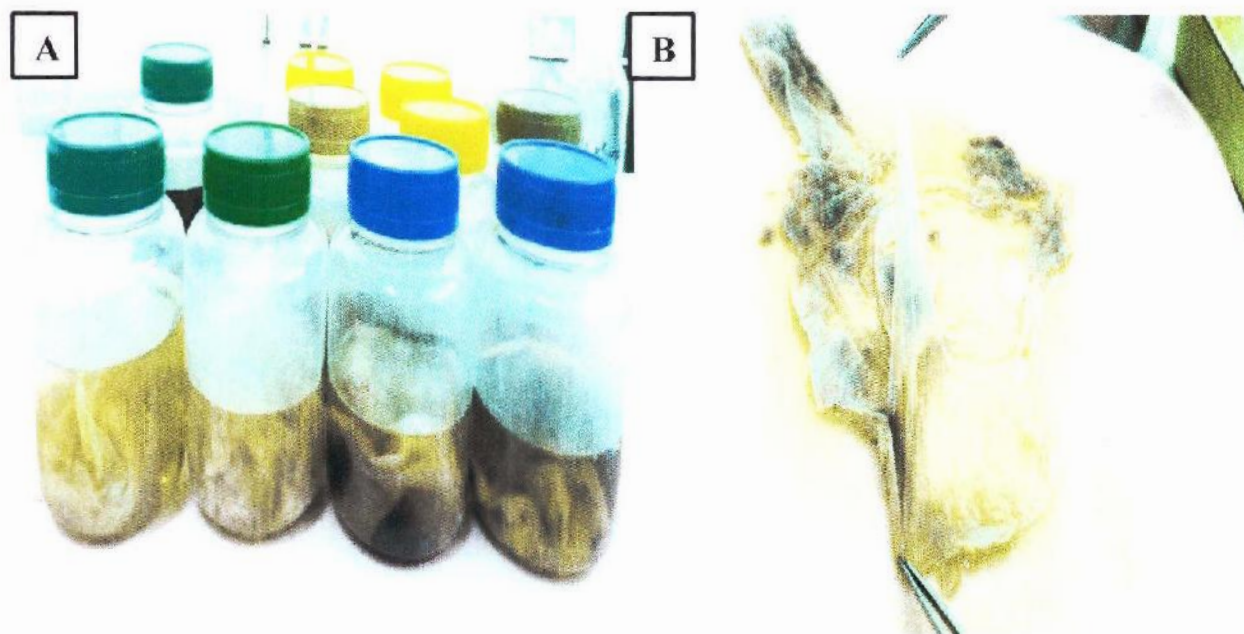


Figure 3.3: Processing of AM; (A) collected human AM, (B) separation of AM from chorion.

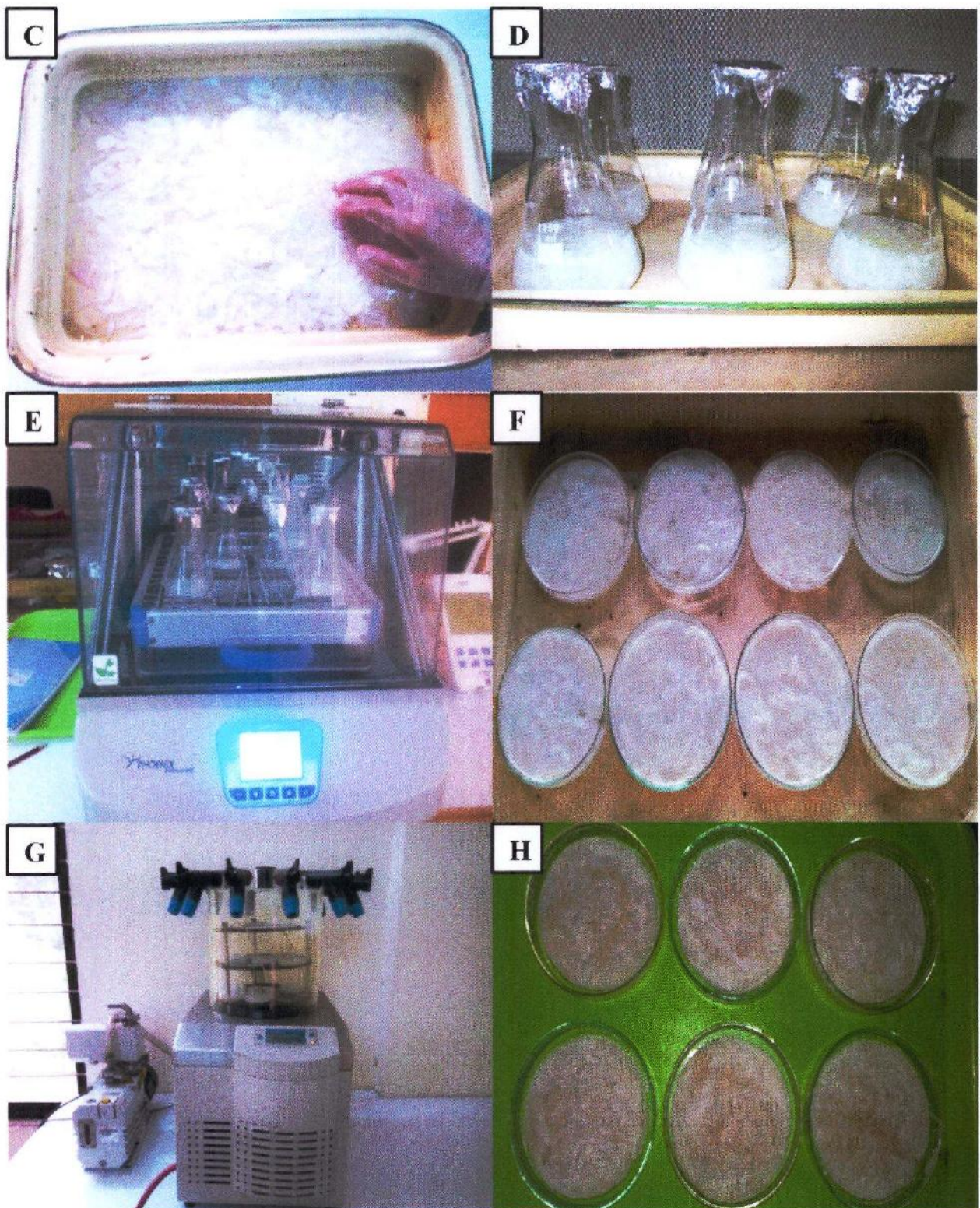


Figure 3.3: Processing of AM; (C) separated AM, (D) washing AM in saline water, (E) shaking AM in antibiotic and saline solution overnight, (F) Plated AM, (G) AM during freeze drying, (H) freeze dried AM.



Figure 3.3: Processing of AM; (I) Irradiated AM, (J) Blending AM in blender, (K) AM powder.

3.3 Extraction of Collagen from Rabbit Skin

For collagen extraction, rabbit skins were obtained from ITBBR animal facility. Pretreated rabbit skins (**Figure 3.4 A-B**) were cut into small pieces (approx. 1cm^2 each) (**Figure 3.4C**) and suspended in 0.5M acetic acid solution at a solid-to-solvent ratio of 1:10 (w/v) (**Figure 3.4D**). The sample will be shaken in a shaker incubator at 200 rpm for 24 hours at room temperature (**Figure 3.4E**). After this time, the sample will be filtered (**Figure 3.4F**) and residues will be treated in the same conditions as mentioned before in order to obtain second and third acid extractions. After the three acid extractions, the non-dissolved residues will be used for pepsin soluble collagen extraction as follows: the residues will be re-suspended in a new solution of 0.5 M acetic acid at a concentration of 1:10 (w/v) and pepsin at a concentration of 1 g/L. This solution will be placed in a shaker incubator at the same speed as described above for 48 hours.

Acid and enzymatic collagens (permeate) will be precipitated with a solution of 2.6 M sodium chloride. The precipitated material will be centrifuged for 25 minutes at a relative centrifugal force of 10000rpm. The pellet will be collected and will be re-suspended in five volumes of 0.5 M acetic acid. The sample will undergo final centrifugation at the same centrifugal force and duration as described above. The supernatant will be collected as pure collagen solution (**Figure 3.4G**). The solution will be kept at -20°C and later freeze dried (**Figure 3.4H**). Dried collagen (**Figure 3.4I**) will undergo blending (**Figure 3.4J**) and collagen powder will be collected in plastic container (**Figure 3.4K**) and stored in refrigerator until further use (Martínez-Ortiz and Hernández-Fuentes, 2015).

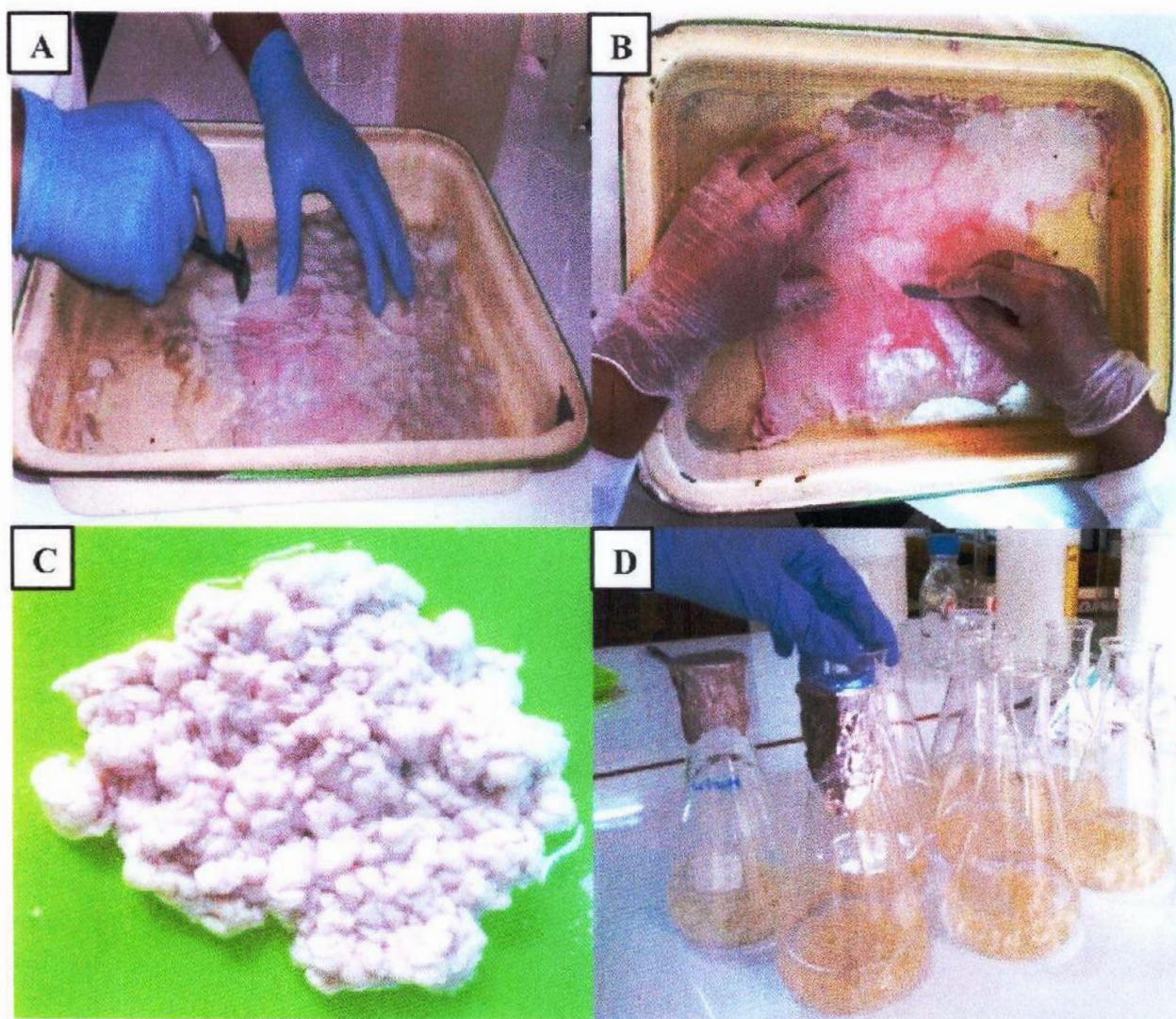


Figure 3.4: Collagen extraction; (A) Fur removal, (B) Fat removal, (C) Minced skin, (D) Minced skin suspended in acetic acid solution.

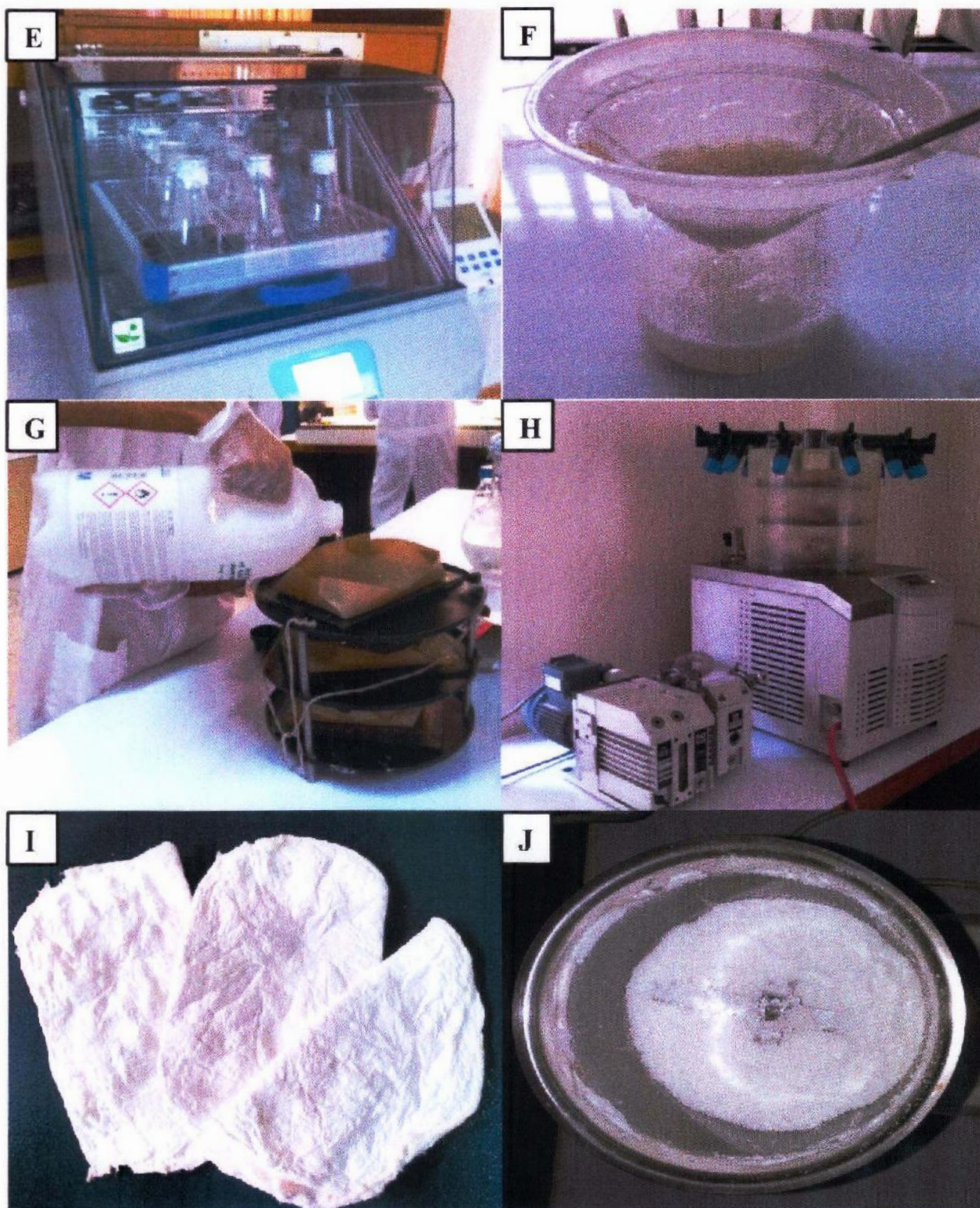


Figure 3.4: Collagen extraction; (E) Shaking minced skin in acid solution overnight, (F) Filtering collagen solution, (G) Plating pure collagen solution, (H) Freeze-drying collagen solution, (I) Freeze-dried collagen, (J) Blended collagen.

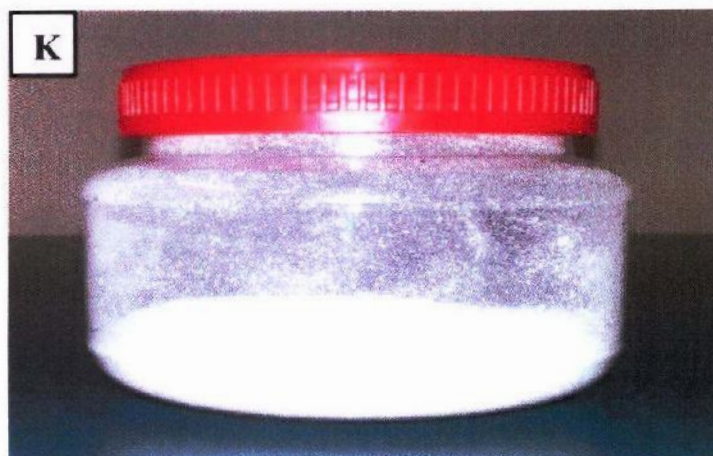


Figure 3.4: Collagen extraction; (K) Collagen powder in plastic container.

3.4 Gel Formulation

Nine types of gels (**Table 3.1**) were formulated based on the type of biomaterial and the percentage of gelling agent used according to the following procedure (Abdul et al., 2013):

3.4.1 Solution A

At first, sufficient amount of autoclaved distilled water was taken in a beaker and 1% citric acid was prepared to dissolve amniotic membrane (AM) and collagen. In case of AM gels, AM was homogenized using homogenizer machine (**Figure 3.6 A-B**) before adding other ingredients. AM/collagen was used at 2% concentrations but at 1% concentration each when used in combination. Antibacterial ingredients such as methyl paraben and propyl paraben were added at 0.02% and 0.002% concentrations respectively. Glycerin was used as moisturizing agent at 5% concentration. At last, sufficient amount of triethanolamine was added dropwise to neutralize the solution near pH 7. All those components were added under continuous stirring (**Figure 3.6 C-D**).

3.4.2 Solution B

Sufficient amount of autoclaved distilled water was taken in another beaker and different concentrations (4% / 5% / 6%) of gelling agent carboxymethyl cellulose sodium (CMC-Na) were added slowly at temperature of about 60°C. Mixing was continued until a transparent gel was formed. It was then placed to cool down up to 30°C. At last, solution A was poured onto solution B and mixed thoroughly using spatula (**Figure 3.5**).

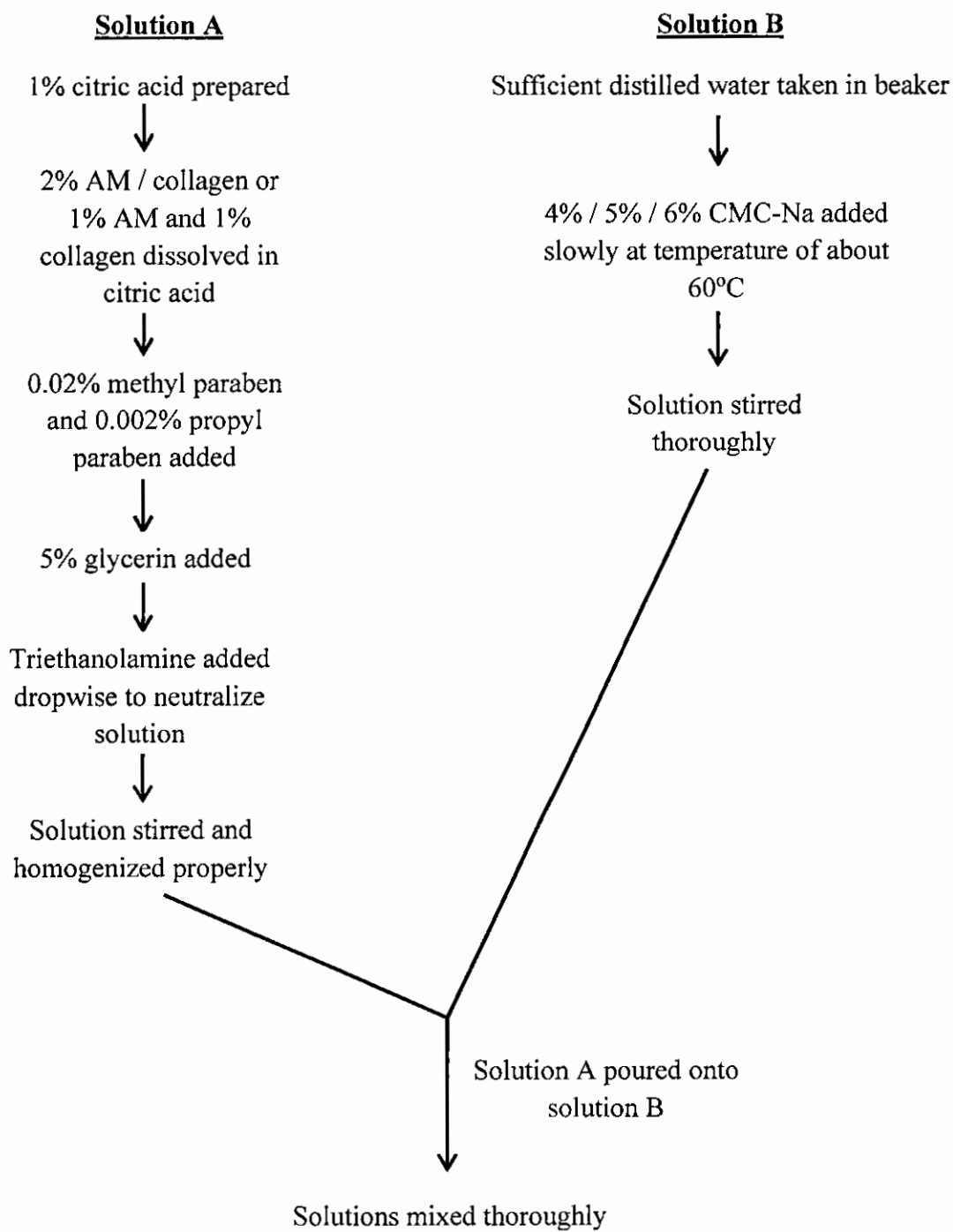


Figure 3.5: Flowchart of gel formulation.

Table 3.1: Nine types of gels

Sample ID	CMC-Na Percentage	Gel Type
F1	4%	AM
F2		AM-Collagen
F3		Collagen
F4	5%	AM
F5		AM-Collagen
F6		Collagen
F7	6%	AM
F8		AM-Collagen
F9		Collagen

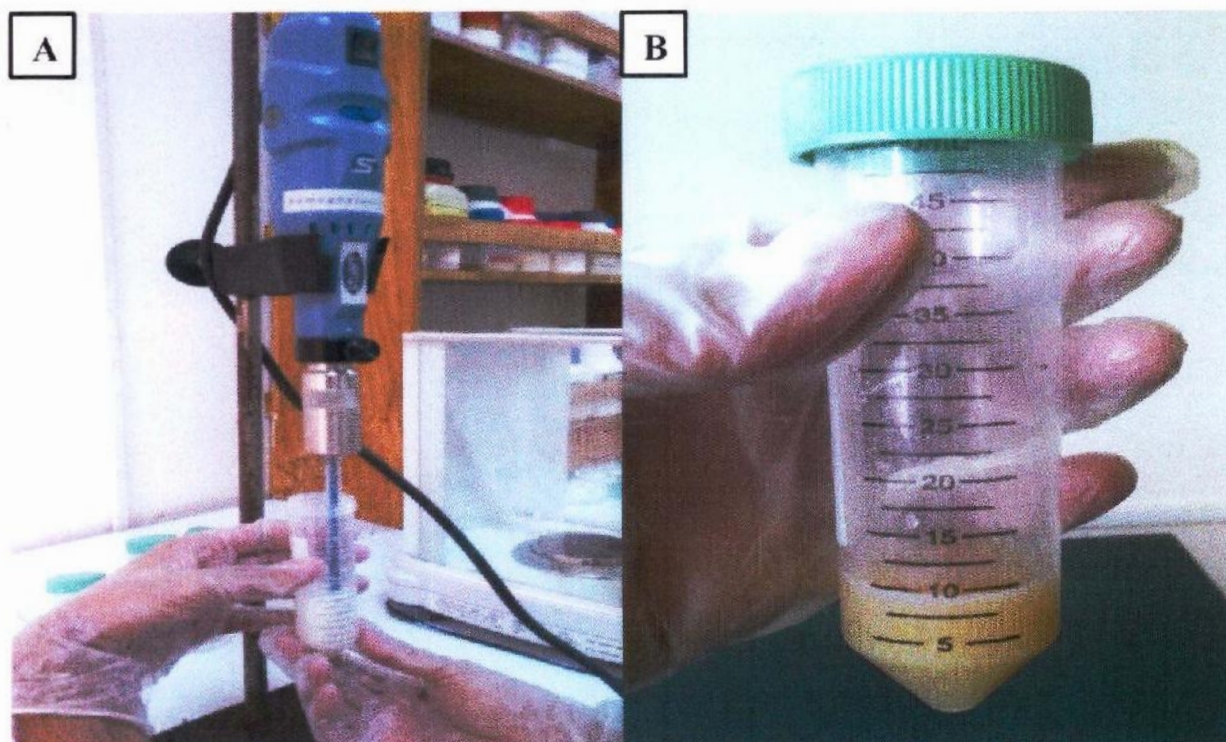


Figure 3.6: Gel formulation; (A) AM homogenization, (B) Homogenized AM.

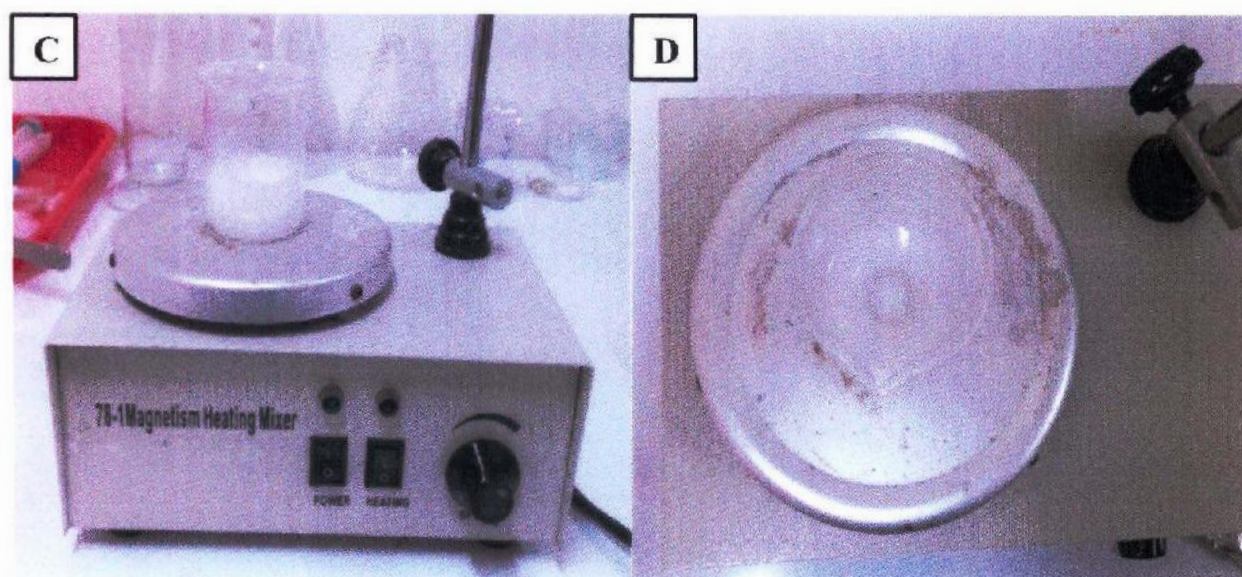


Figure 3.6: Gel formulation; (C) Solutions stirred on stirrer machine, (D) Continuous stirring leading to transparent gel formation.

3.5 Epithelial Sheath Preparation

Eleven types of epithelial sheaths (Table 3.2) were prepared (Figure 3.7 A-B) using collagen and chitosan at different concentrations. 0.5 M acetic acid was used to dissolve collagen and chitosan.

Table 3.2: Eleven types of epithelial sheaths

Sample ID	Collagen concentration	Chitosan concentration
ES1	10 mg/ml	10 mg/ml
ES2	12 mg/ml	8 mg/ml
ES3	8 mg/ml	12 mg/ml
ES4	14 mg/ml	6 mg/ml
ES5	6 mg/ml	14 mg/ml
ES6	15 mg/ml	5 mg/ml
ES7	5 mg/ml	15 mg/ml
ES8	16 mg/ml	4 mg/ml
ES9	4 mg/ml	16 mg/ml
ES10	18 mg/ml	2 mg/ml
ES11	2 mg/ml	18 mg/ml

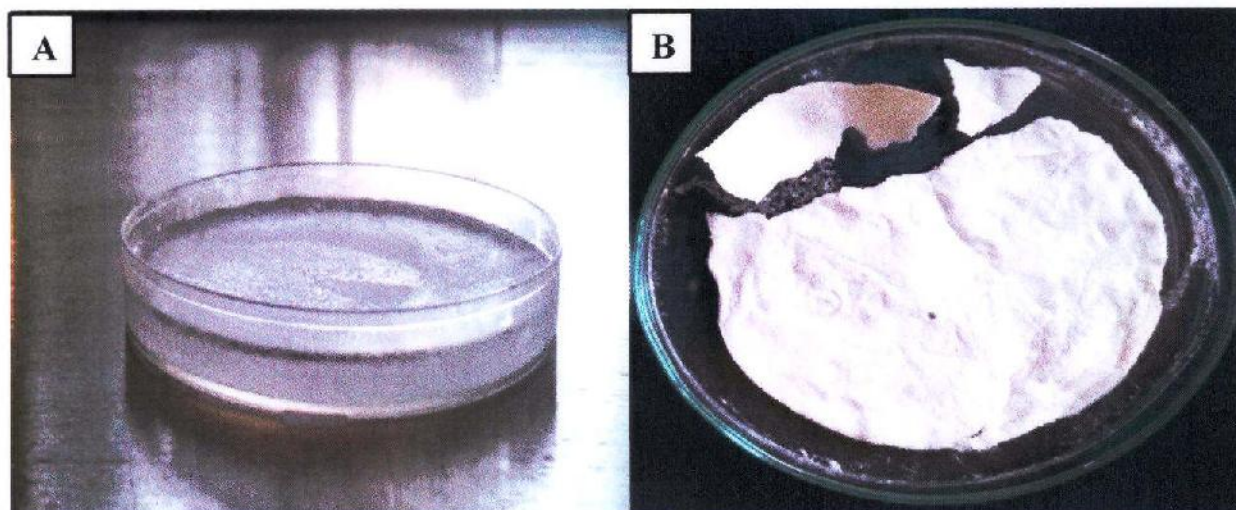


Figure 3.7: Epithelial sheath preparation; (A) Plated epithelial sheath before freeze-drying, (B) Freeze-dried epithelial sheath.

3.6 Characterization of Gel Formulations

The formulated gels (**Table 3.1**) were characterized based on several physical properties.

3.6.1 Physical Appearance and Homogeneity

The developed formulations were tested for homogeneity by visual inspection after the gel had been filled in the container. They were tested for their appearance and presence of any aggregates.

3.6.2 pH Measurements

The pH of gel formulations was determined by using electronic pH meter (HI-98107, Hanna Instruments, Italy).

3.6.3 Determination of Swelling Ratio

The pre-weighted freeze-dried gel samples (**Figure 3.8A**) were immersed in distilled water for 2 hours at room temperature. The swollen gels were removed from distilled water and weighted after removing surface water with a filter paper. The swelling ratio was calculated as follows (Bhuiyan et al., 2015):

$$\text{Swelling ratio} = [W_s - W_d] / W_d,$$

where W_s is the weight of swollen gel and W_d is the weight of the dry gel.

3.6.4 Determination of Water Absorption

The hydrogel samples were freeze-dried to constant weight (**Figure 3.8B**), immersed in distilled water at room temperature. The samples were weighed periodically after removing excess water from the surface of gel with a filter paper. Water absorption of the samples was calculated as follows (Bhuiyan et al., 2015):

$$\text{Water absorption [\%]} = [W_t - W_i] \times 100,$$

where, W_t is the weight of swollen gel sample at time 't' and W_i is the initial weight of dry gel samples.

3.6.5 Determination of Equilibrium Water Content (EWC)

The hydrogel samples freeze-dried to constant weight were immersed in distilled water at room temperature (**Figure 3.8C**). The swelling of samples was continued until a constant weight and weighed after removing surface water with filter paper. Equilibrium water content (EWC) was calculated as follows (Bhuiyan et al., 2015):

$$\text{EWC [\%]} = [(W_{eq} - W_i) / W_{eq}] \times 100,$$

where, W_{eq} is the weight of swollen gel at equilibrium and W_i is the initial weight of dry gel.

3.6.6 Determination of Gel Fraction

The hydrogel samples freeze-dried to constant weight (W_i) were immersed in distilled water in plastic containers for 2 hours to remove any soluble fraction or unreacted components. The gel samples were taken out from containers, dried in air and then in incubator at a temperature of 50°C (**Figure 3.8D**). The gel fractions of the samples were calculated gravimetrically according to the following equation (Bhuiyan et al., 2015):

$$\text{Gel fraction (\%)} = [W_d / W_i] \times 100,$$

where, W_d is the weight of dried gel after extraction and W_i is the initial weight of dried gel.

3.6.7 Spreadability

One of the criteria for a topical formulation to meet the ideal qualities is that it should possess good spreadability. It is the term expressed to denote the extent of area to which formulation readily spreads on application to skin or affected part. The therapeutic efficacy of a formulation also depends upon its spreading value. To determine the spreadability of formulation, 1 g of gel was placed within a circle of 2.3 cm diameter pre-marked on a glass plate of 20×20 cm (**Figure 3.8E**), over which a second glass plate was placed. A weight of 500 g in the form of water-filled beaker was allowed to rest on the upper glass plate for 5 min (**Figure 3.8F**). The increase in the diameter due to gel spreading was noted (Abdul et al., 2013).

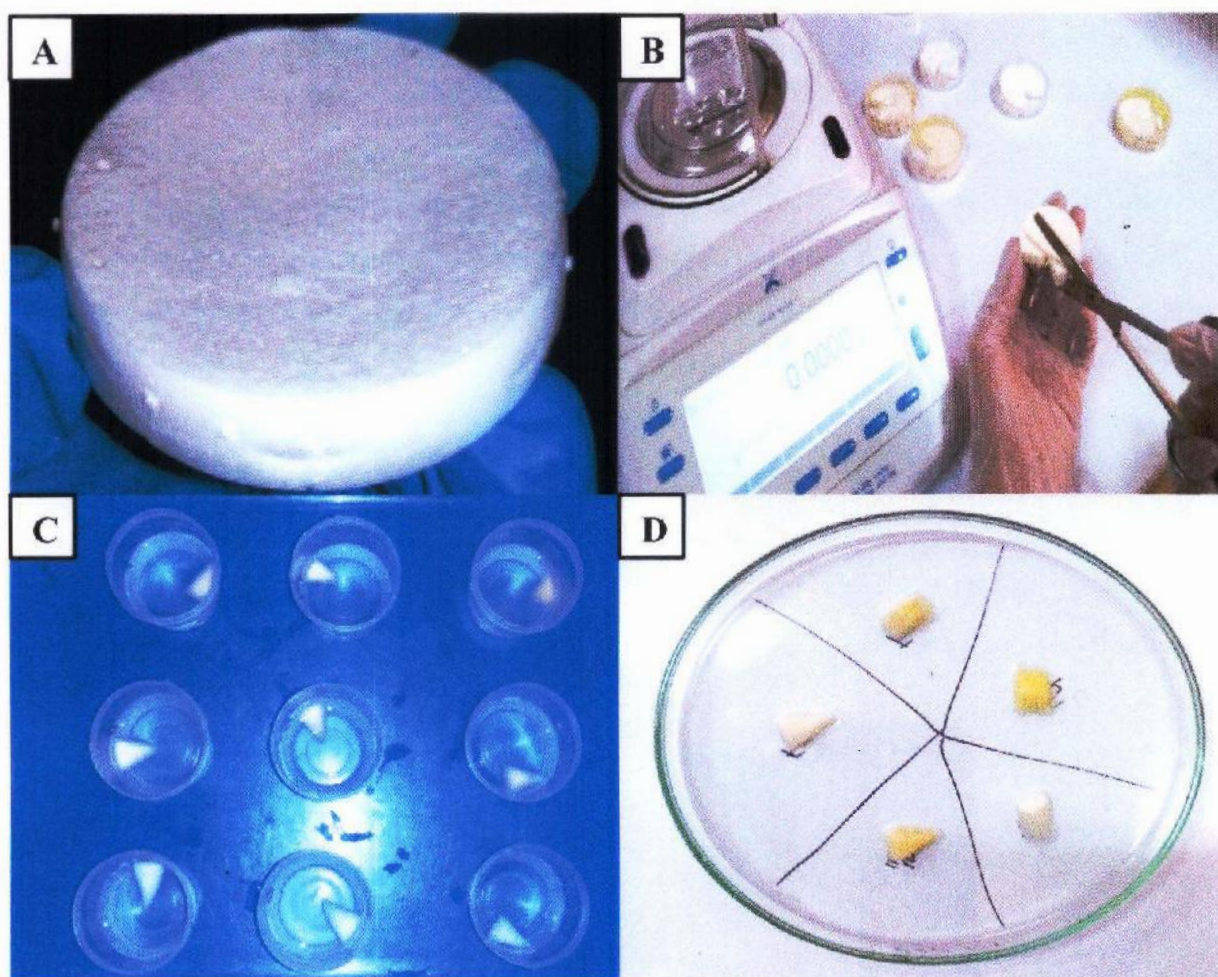


Figure 3.8: Characterization of gel formulations; (A) Freeze-dried gel sample, (B) Samples cut into small pieces and weighted, (C) Samples immersed in distilled water, (D) Dried samples after incubation.

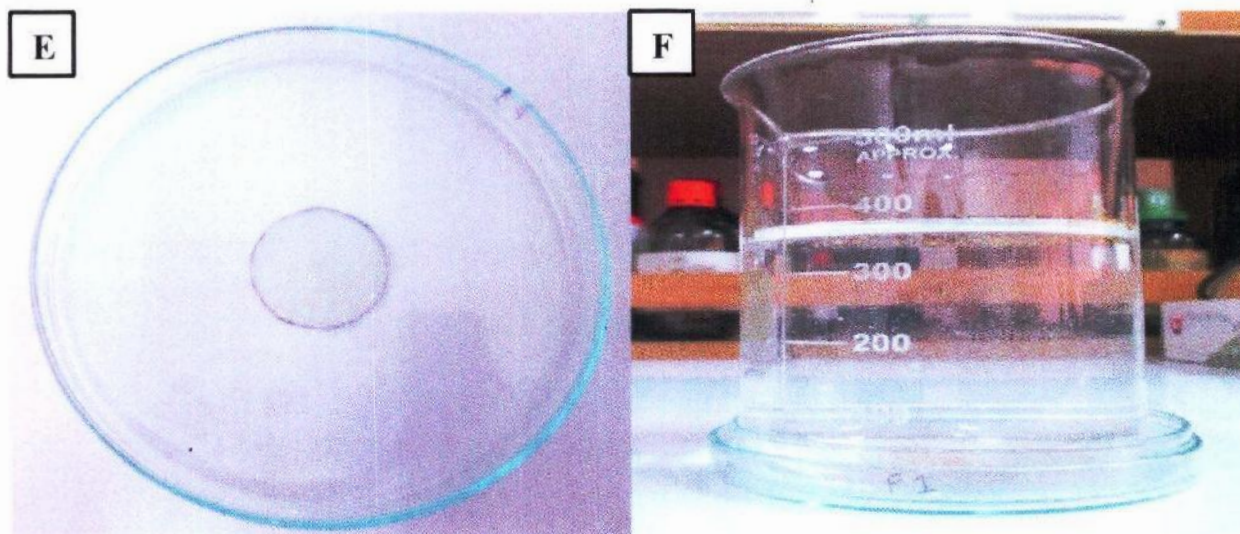


Figure 3.8: Characterization of gel formulations; (E) Gel sample placed within circle of petridish, (F) Water-filled beaker rest on upper petridish as a measure of pressure.

3.7 Characterization of Epithelial Sheaths

The prepared epithelial sheaths (**Table 3.2**) were characterized (**Figure 3.9 A-B**) based on several physical properties such as swelling ratio, water absorption, equilibrium water content using the same method as stated in 3.6.3, 3.6.4 and 3.6.5.

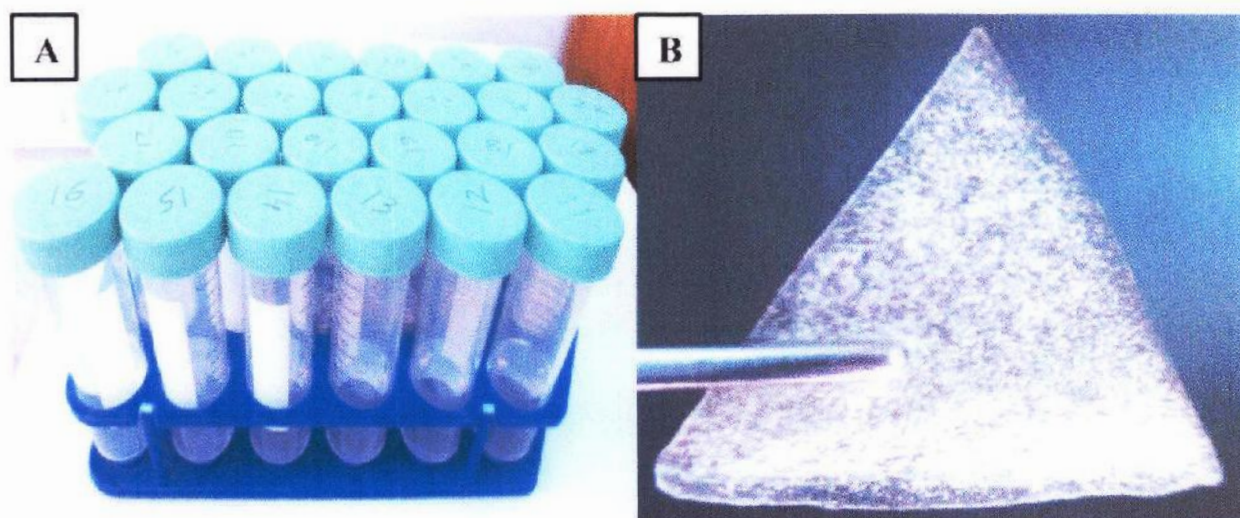


Figure 3.9: Characterization of epithelial sheaths; (A) Sheath samples immersed in distilled water, (B) Swollen sheath sample.

3.8 Characterization of Selected Gel Formulations

The final selected formulated gels based on the properties of different CMC-Na concentrated gels (Table 3.1) were characterized based on several physical and biological properties.

3.8.1 Physical Appearance, Homogeneity and pH Measurements

The final selected formulations were tested for physical appearance, homogeneity and pH (Figure 3.10) using the same method as stated in 3.6.1 and 3.6.2.



Figure 3.10: pH measurement.

3.8.2 *In vitro* Cytotoxicity Test

In vitro cytotoxicity test was performed using brine shrimp lethality bioassay method as showed in (Figure 3.11 A-E) (Bundela and Bajpai, 2008). Brine shrimps (*Artemiasalina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater and pH was adjusted at 8.5 using 0.1N NaOH under constant aeration for 48 hours. After hatching, active nauplii free from egg shells were collected from brighter portion of hatching chamber and used for the assay. The final gel samples were dissolved in artificial seawater at different concentrations and taken in a sample container where the active nauplii were inoculated. After overnight incubation, the nauplii were counted. Here, 0.5 mg/ml of Colchicines (an anti-cancer drug) was used as positive control. Sea water alone was used as negative control.

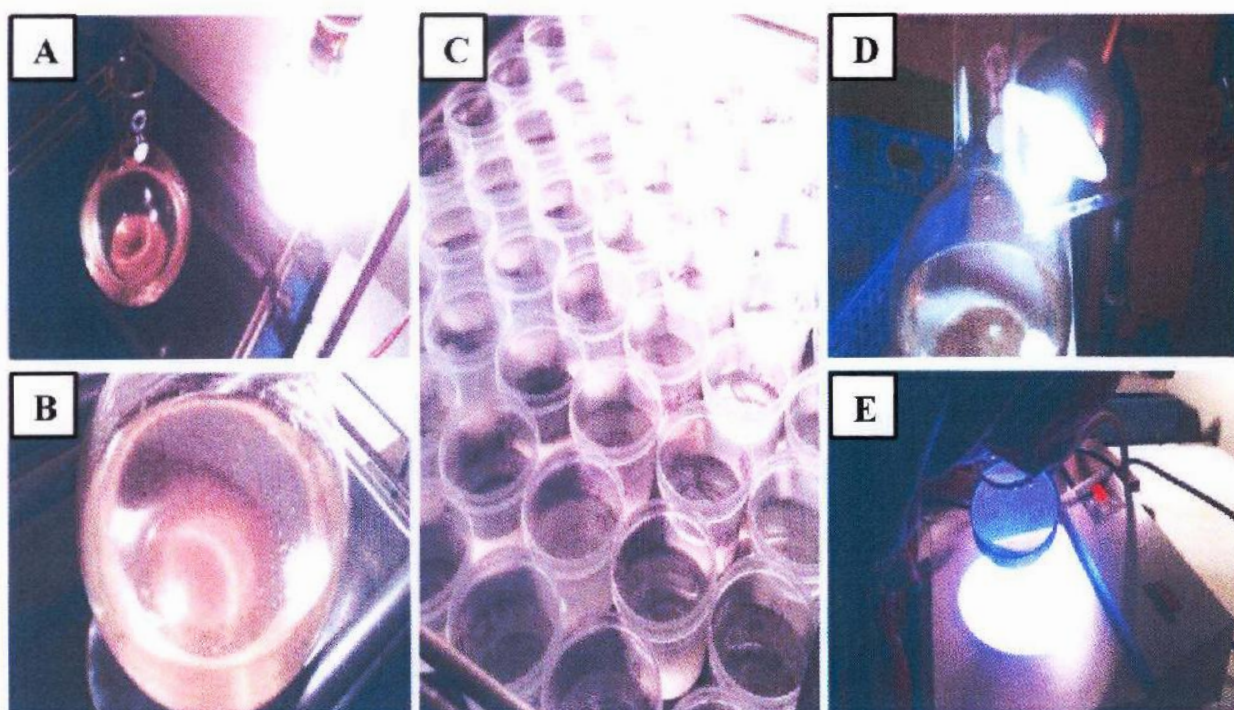


Figure 3.11: *In vitro* Cytotoxicity Test of selected gels on Brine Shrimp; (A) Hatching of Brine Shrimp, (B) Hatched Brine Shrimp (Nauplii), (C) Inoculation of Nauplii in different concentrations of gel samples, (D) Counting of Live Nauplii, (E) Counting Nauplii after inoculation.

3.8.3 Red Blood Cell Compatibility Test

Heparinized human blood was used to assay the biocompatibility of the selected gel samples (Bhowmik et al., 2017). After collecting required volume of blood, heparin was added at the concentration of 30 units per ml (**Figure 3.12**). Samples were then prepared using blood and selected gel samples at 3:1 ratio. Blood sample of the same donor was also diluted at the same ratio with distilled water and normal saline for positive and negative control respectively. After mixing the gel samples, saline, and distilled water with blood at 3:1 ratio, the mixtures were kept in an incubator for 2 h at 37°C. Then the samples were spread on glass slides and observed under an inverted microscope (20× magnification).

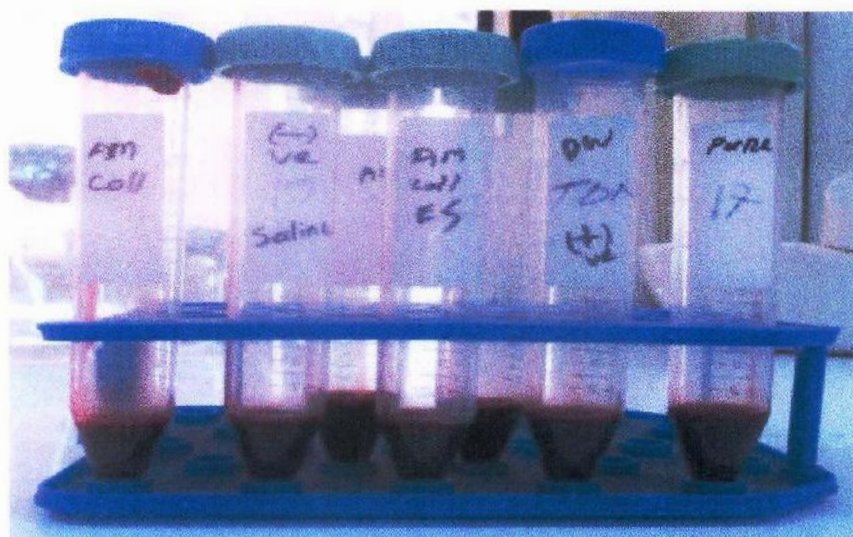


Figure 3.12: Heparinized blood samples.

3.8.4 Skin Irritation Studies

Female rats were used for this test. The intact skin was used. The dorsal hairs were shaved on the date of experiment. 4 animals were taken to perform skin irritation studies of four gel formulations – AM, AM-Collagen, Collagen and AM-Collagen-Epithelial Sheath. One rat was used in each group. The gels were used on animal for a day. Finally the treated skin was visually examined the next day for erythema and edema.

3.9 Burn Healing Experiment

3.9.1 Preparation of Animal

Animals were nurtured in Institute of Tissue Banking and Biomaterial Research (ITBBR), Savar, Dhaka-1349 for about one month before the experiment and acclimatized with environment. Animals were fed standard diet prepared in ITBBR. Total 24 Wistar rats of 100-150g body weight were taken and randomly divided into six groups 4 rats per group: 1% silver sulfadiazine (Burnsil, Beximco Pharmaceuticals) as positive control, negative control, treated with collagen, treated with AM, treated with AM-Collagen and treated with AM-Collagen-Epithelial Sheath. Each rat was anesthetized with Ketamine HCl solution 100mg/kg (Gonosastho Pharmaceuticals).

Ltd, Bangladesh) body weight by intraperitoneal injection (**Figure 3.13A**). Subsequently, the hair at dorsal region was trimmed using electric hair clipper (KM-3909, Kemei, India) and then shaved with sharp blade (**Figure 3.13B**).

Shaved area was cleansed with alcohol swab. Burns were created using piece of aluminum (2.5 cm diameter) heated to 100°C for 30 min. It was applied for 30 seconds in the shaved area of rats without pressure (**Figure 3.13C**).

The animals were treated with 1ml of gels on daily basis, topically, for a period of 16 days. The wound was left undressed to the open environment. In this model, wound contraction and wound closure time was monitored.

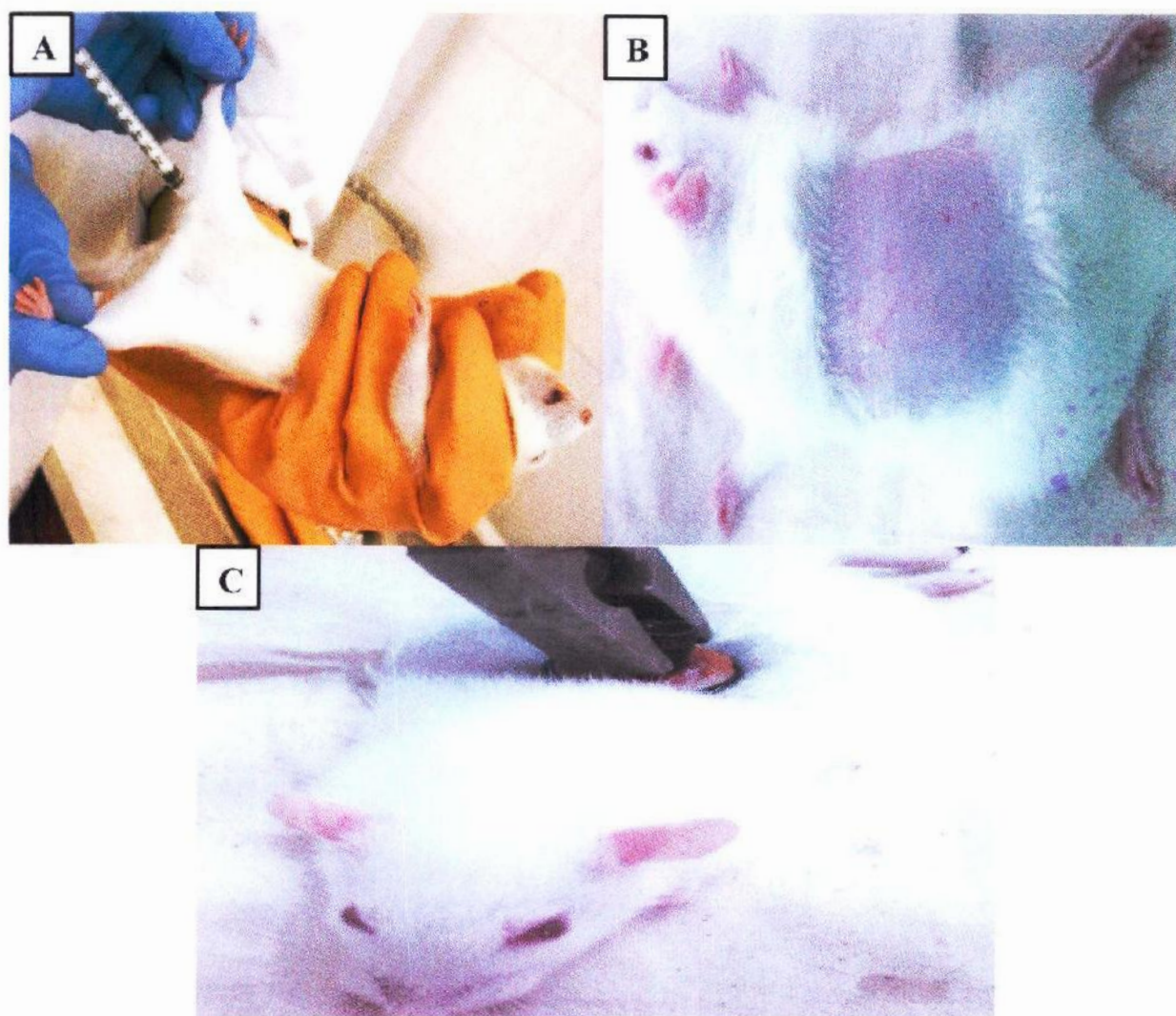


Figure 3.13: Preparation of animal; (A) Anesthesia given to rats, (B) Shaved rat, (C) Burn induction.

3.9.2 Percent Wound Contraction

After wound creation, the wound margin was traced (Figure 3.14) at 4 days intervals by millimeter measuring scale and measurements were continued up to 16 days. After every four days, healed area was calculated by subtracting initial wound area to the unhealed area (Murphy et al., 2017). The percentage of wound contraction was calculated taking the initial area of wound (625 mm²) as 100%.

$$\% \text{ Wound Contraction} = \frac{\text{Initial Wound Area} - \text{Final Wound Area}}{\text{Initial Wound Area}} \times 100$$

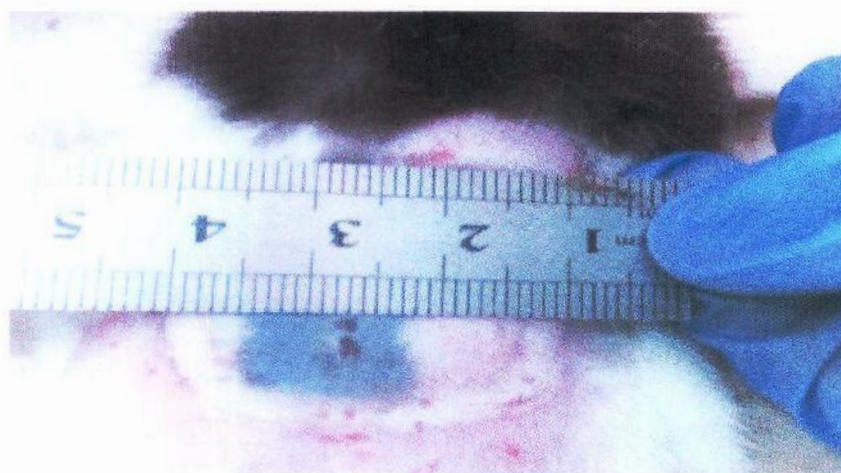


Figure 3.14: Initial wound area measurement.



3.9.3 Epithelialization Period

It was monitored by noting the number of days required for crust to fall away, leaving no raw wound behind.

3.10 Statistical Analysis

All quantitative data were presented and mean as well as standard deviations (SD) were calculated by one-way independent test using Microsoft Excel 2010. $P < 0.05$ was considered as statistically significant.

CHAPTER FOUR

RESULTS

4.1 Composition of Formulated Gels

Pathophysiologically, burn is considered one of the most severe types of wounds as it is easily susceptible to infection due to vascular necrotic tissue and loosening of epidermal integrity. Beside active ingredients, the applicability of burn wound healing gel is dependent on the properties such as pH, appearance, homogeneity and viscosity. All the nine types of gels were formulated successfully using CMC-Na salt along with 1% citric acid, propyl paraben, methyl paraben, amniotic membrane (AM), Collagen, triethanolamine and glycerin (**Table 4.1**).

Table 4.1: Composition of nine types of gels

Sample ID	F1	F2	F3	F4	F5	F6	F7	F8	F9
Gel Type	AM	AM-Collagen	Collagen	AM	AM-Collagen	Collagen	AM	AM-Collagen	Collagen
CMC-Na		4%			5%			6%	
AM Powder	2%	1%	-	2%	1%	-	2%	1%	-
Collagen Powder	-	1%	2%	-	1%	2%	-	1%	2%
Citric Acid					1%				
Methyl Paraben					0.02%				
Propyl Paraben					0.002%				
► Glycerin					5%				
Distilled Water					Up to 25 ml				
Triethanolamine	70 µl	60 µl	65µl	60 µl	65 µl	70 µl	60 µl	65 µl	65 µl

4.2 Evaluation of Formulated Gels

The formulated gels were evaluated based on several physical properties.

4.2.1 Physical Appearance, Homogeneity and pH Measurements

All formulations were found to be homogeneous in nature with good consistency. pH of all formulations was found to be in between 6.56 to 7.3 which denotes that gels were suitable to apply in skin (**Table 4.2**). Color of gels containing only AM extracts were off-white while the gels containing both AM and collagen extracts were light white. In case of collagen gels, color was found to be deep white (**Figure 4.1 A-I**).

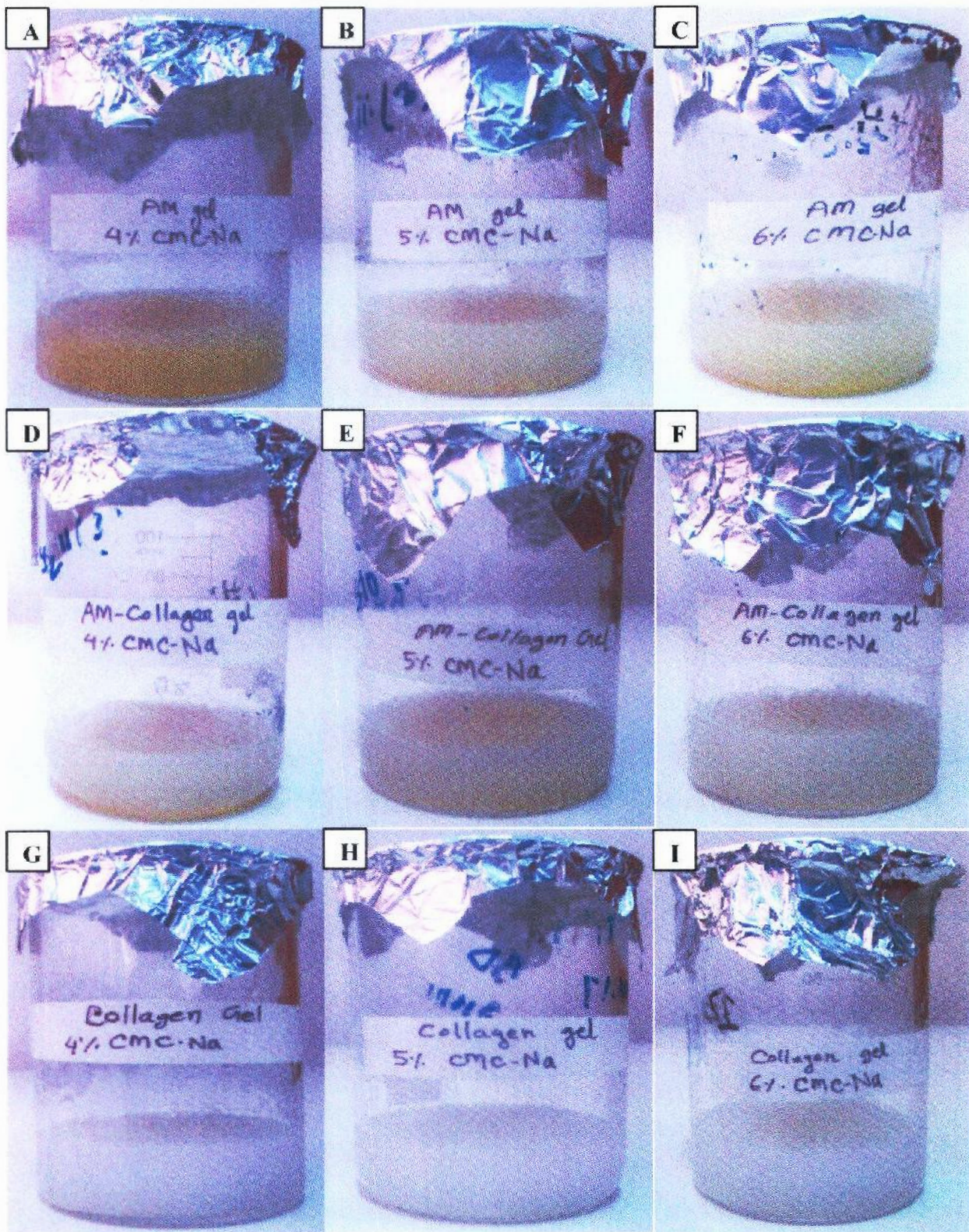


Figure 4.1: Physical appearance and homogeneity; (A-C) AM gels, (D-F) AM-Collagen gels, (G-I) Collagen gels.

Table 4.2: Physical properties of formulated gels

Sample ID	Gel Type	CMC-Na Percentage	pH	Homogeneity	Color
F1	AM	4%	7.15	Homogeneous	Off White
F2	AM-Collagen		6.56	Homogeneous	Light White
F3	Collagen		6.67	Homogeneous	Deep White
F4	AM	5%	6.85	Homogeneous	Off White
F5	AM-Collagen		7.2	Homogeneous	Light White
F6	Collagen		7.3	Homogeneous	Deep White
F7	AM	6%	6.56	Homogeneous	Off White
F8	AM-Collagen		6.86	Homogeneous	Light White
F9	Collagen		6.68	Homogeneous	Deep White

4.2.2 Other Physical Properties

Other physical properties of formulated gels were also analysed in order to find out which concentration of CMC-Na better contain the physical properties within the gels (Table 4.3).

Table 4.3: Other physical properties of formulated gels (Values $\pm$ SD)

Sample ID	Gel Type	CMC-Na (%)	Swelling Ratio	Water Absorption (%)	Equilibrium Water Content (%)	Gel Fraction (%)	Spreadability (cm)
F1	AM	4%	1.26 $\pm$ 0.10	19.23 $\pm$ 4.65	55.66 $\pm$ 2.05	82.87 $\pm$ 2.83	4.9 $\pm$ 0.44
F2	AM-Collagen		2.59 $\pm$ 0.16	22.91 $\pm$ 5.87	72.09 $\pm$ 1.24	74.26 $\pm$ 7.48	6.3 $\pm$ 0.53
F3	Collagen		1.73 $\pm$ 0.05	23.08 $\pm$ 5.41	63.43 $\pm$ 0.59	70.83 $\pm$ 7.33	5.2 $\pm$ 0.36
F4	AM	5%	1.92 $\pm$ 0.13	20.03 $\pm$ 3.97	65.66 $\pm$ 1.50	84.97 $\pm$ 0.95	4.5 $\pm$ 0.17
F5	AM-Collagen		2.11 $\pm$ 0.17	21.04 $\pm$ 4.68	67.81 $\pm$ 1.77	84.36 $\pm$ 3.62	4.77 $\pm$ 0.15
F6	Collagen		2.20 $\pm$ 0.21	22.15 $\pm$ 5.62	68.67 $\pm$ 2.18	80.47 $\pm$ 3.14	4.7 $\pm$ 0.2
F7	AM	6%	2.21 $\pm$ 0.07	23.70 $\pm$ 1.27	68.85 $\pm$ 0.62	87.30 $\pm$ 1.92	3.9 $\pm$ 0.26
F8	AM-Collagen		2.30 $\pm$ 0.14	25.85 $\pm$ 4.05	69.62 $\pm$ 1.31	87.05 $\pm$ 4.76	4.3 $\pm$ 0.26
F9	Collagen		2.14 $\pm$ 0.51	25.12 $\pm$ 3.55	67.66 $\pm$ 5.05	81.72 $\pm$ 3.02	4.1 $\pm$ 0.26

Table 4.3 shows that 6% gels contain better physical properties than others. Considering gel type, the gels combining both amniotic membrane and collagen show better physical properties in most instances than other gel types.

The following graphs illustrate that the 6% gels supersede rest of gels in case of swelling ratio, water absorption, equilibrium water content, gel fraction and spreadability (Figure 4.2 A-E).

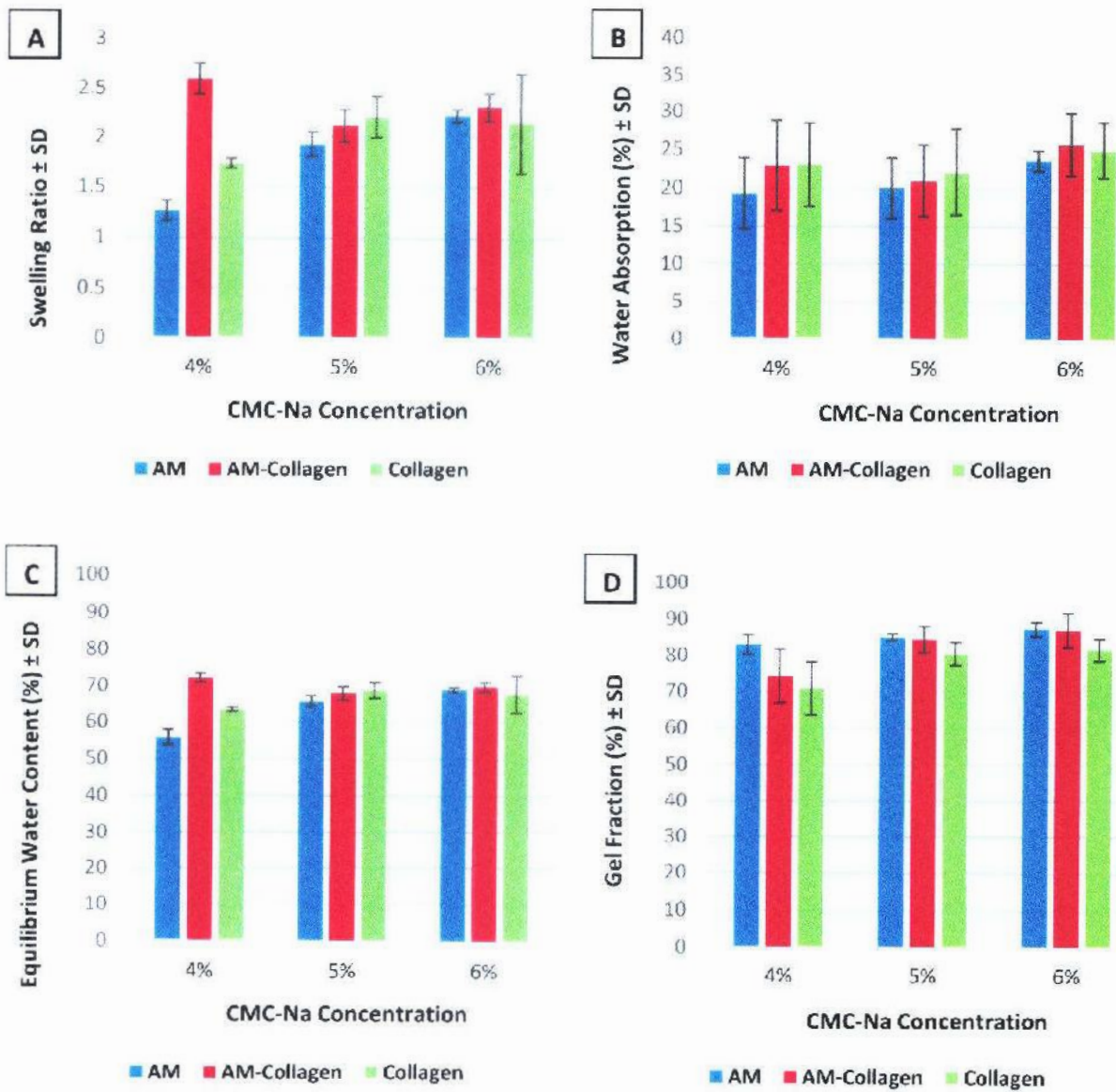


Figure 4.2: Physical properties of different gel samples at different CMC-Na concentrations; (A) Swelling ratio, (B) Water absorption (%), (C) Equilibrium water content (%), (D) Gel fraction (%).

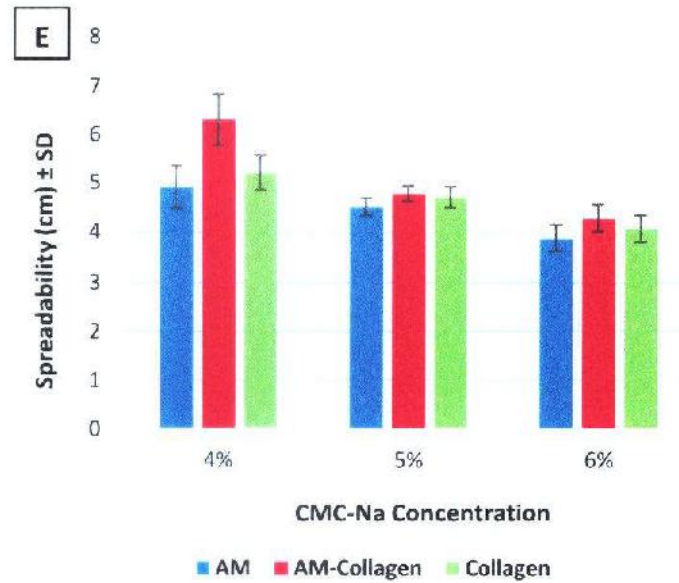


Figure 4.2: Physical properties of different gel samples at different CMC-Na concentrations; (E) Spreadability (cm).

Spreadability of 6% gels is lower (Figure 4.2E) because high concentration gels tend to be thicker and spread less which is better illustrated in the following figures (images taken in gel documentation system) (Figure 4.3 A-I).

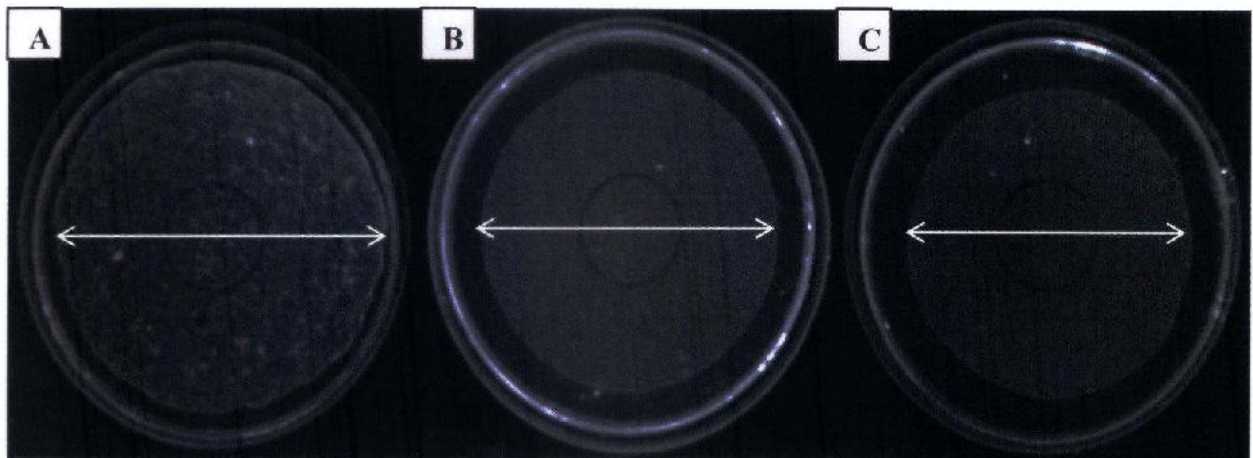


Figure 4.3: Illustration of spreadability of different gel samples at different CMC-Na concentrations; (A) 4% AM gel, (B) 4% AM-Collagen gel, (C) 4% Collagen gel. [N.B: White arrow shows the diameter covered by gels upon pressure]

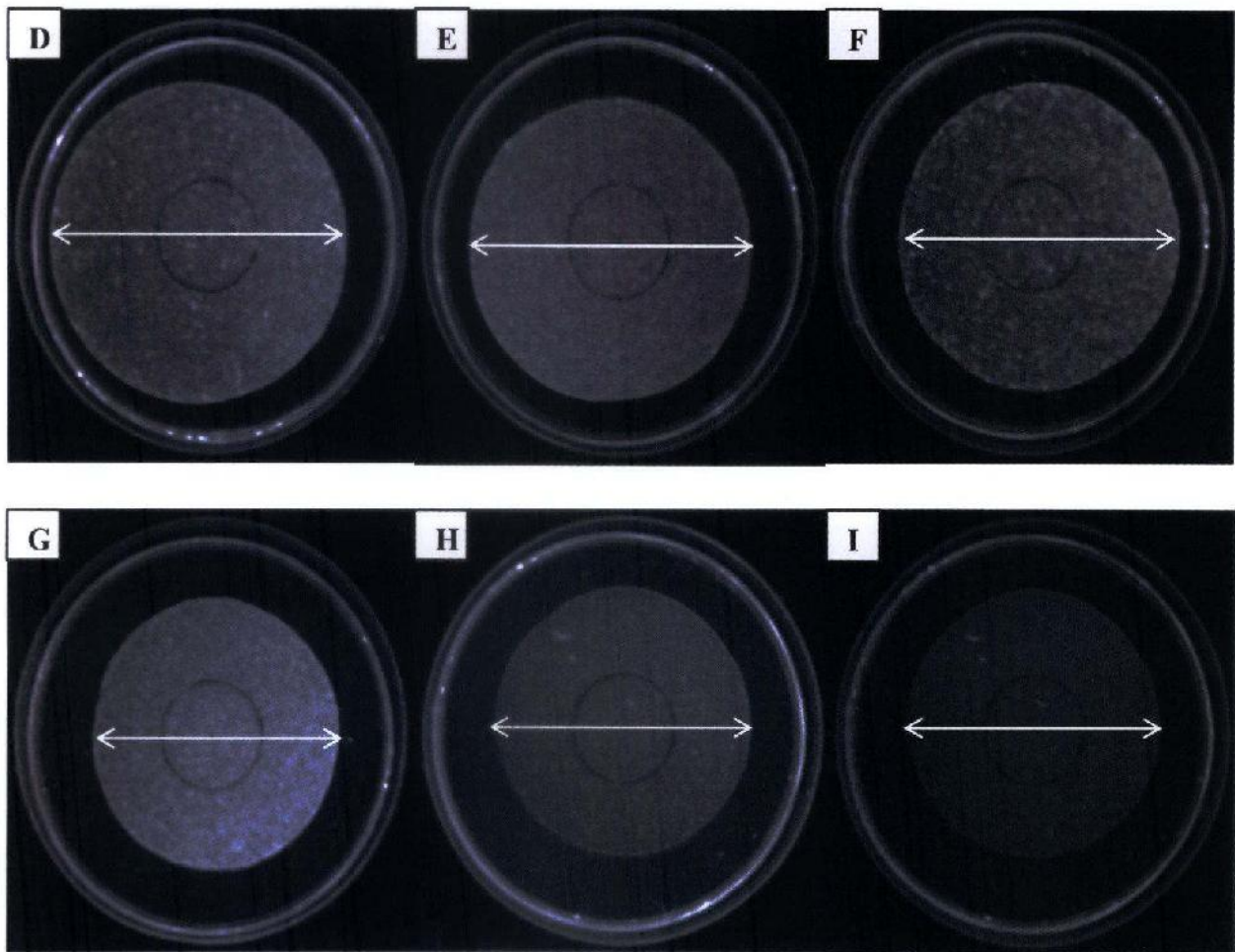


Figure 4.3: Illustration of spreadability of different gel samples at different CMC-Na concentrations; (D) 5% AM gel, (E) 5% AM-Collagen gel, (F) 5% Collagen gel, (G) 6% AM gel, (H) 6% AM-Collagen gel, (I) 6% Collagen gel.

4.2.3 Selection of Optimum Gels and Interpretation

Based on **Figure 4.2** and **Figure 4.3**, it can be concluded that 6% gels showed relatively better physical properties than 4% and 5% gels. In case of spreadability, lower values of 6% gels indicate that they spread less and tend to stick to the wound area over a longer period of time than 4% and 5% gels which is eventually expected for topical application. Again, 6% concentration can be regarded as threshold thickness level above which appropriate gel texture cannot be maintained and it is also difficult to prepare gels above 6% concentration. Moreover, pH can be maintained properly as higher concentration of CMC-Na helps to neutralize relatively acidic condition of solution A (**Figure 3.5**).

4.3 Evaluation of Epithelial Sheaths

The prepared epithelial sheaths were evaluated based on several physical properties such as swelling ratio, water absorption (%) and equilibrium water content (%) (Table 4.4).

Table 4.4: Physical properties of prepared epithelial sheaths (Values $\pm$ SD)

Sample ID	Collagen concentration (mg/ml)	Chitosan concentration (mg/ml)	Swelling Ratio (%)	Water Absorption (%)	Equilibrium Water Content (%)
ES1	10	10	30.66 $\pm$ 7.08	22.88 $\pm$ 4.73	96.73 $\pm$ 0.74
ES2	12	8	20.61 $\pm$ 3.75	13.76 $\pm$ 2.25	95.28 $\pm$ 0.77
ES3	8	12	19.04 $\pm$ 0.48	11.02 $\pm$ 1.40	95.01 $\pm$ 0.12
ES4	14	6	20.46 $\pm$ 2.50	12.76 $\pm$ 1.67	95.30 $\pm$ 0.56
ES5	6	14	18.53 $\pm$ 2.21	12.91 $\pm$ 1.62	94.83 $\pm$ 0.61
ES6	15	5	19.45 $\pm$ 3.10	13.66 $\pm$ 3.47	95.03 $\pm$ 0.81
ES7	5	15	18.02 $\pm$ 1.97	13.65 $\pm$ 3.27	94.71 $\pm$ 0.53
ES8	16	4	10.85 $\pm$ 2.03	8.04 $\pm$ 1.65	91.38 $\pm$ 1.58
ES9	4	16	20.69 $\pm$ 1.99	13.28 $\pm$ 1.02	95.37 $\pm$ 0.41
ES10	18	2	20.15 $\pm$ 0.51	17.11 $\pm$ 1.28	95.27 $\pm$ 0.12
ES11	2	18	12.94 $\pm$ 0.83	8.51 $\pm$ 0.98	92.81 $\pm$ 0.42

It is obvious from the above table that ES1 sample [collagen concentration (10 mg/ml) and chitosan concentration (10 mg/ml)] contains better physical properties than rest of the epithelial sheath samples. This is better illustrated in the following graphs (Figure 4.4, 4.5 and 4.6):

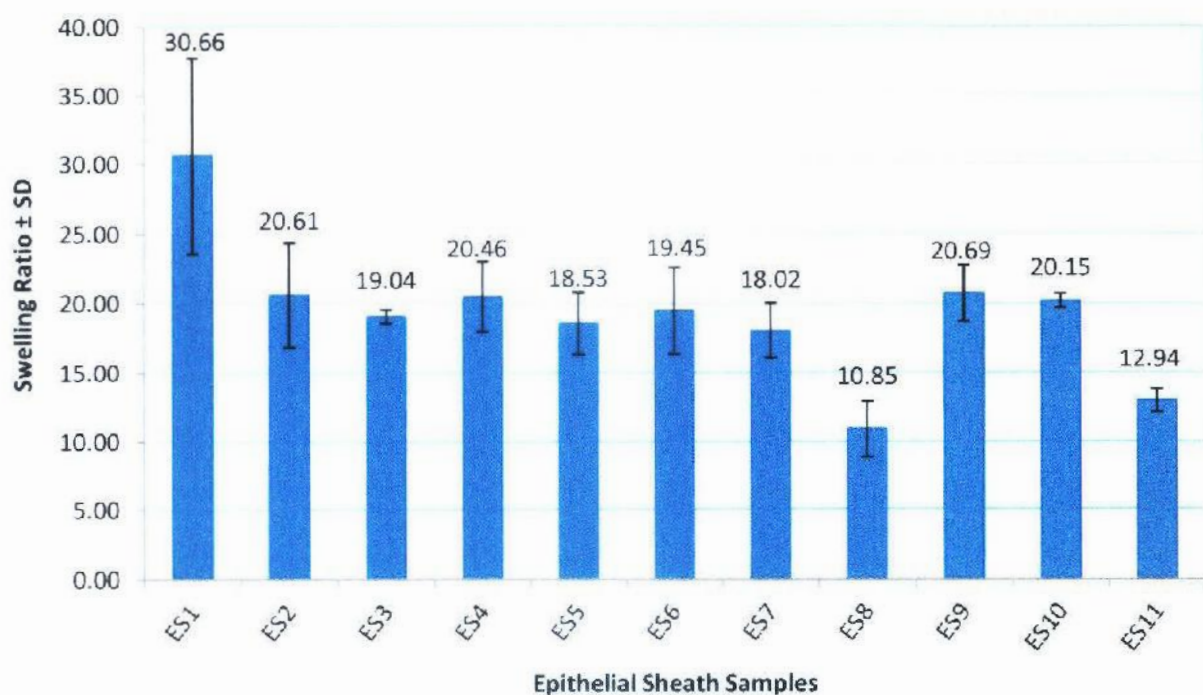


Figure 4.4: Swelling ratio of different epithelial sheath samples.

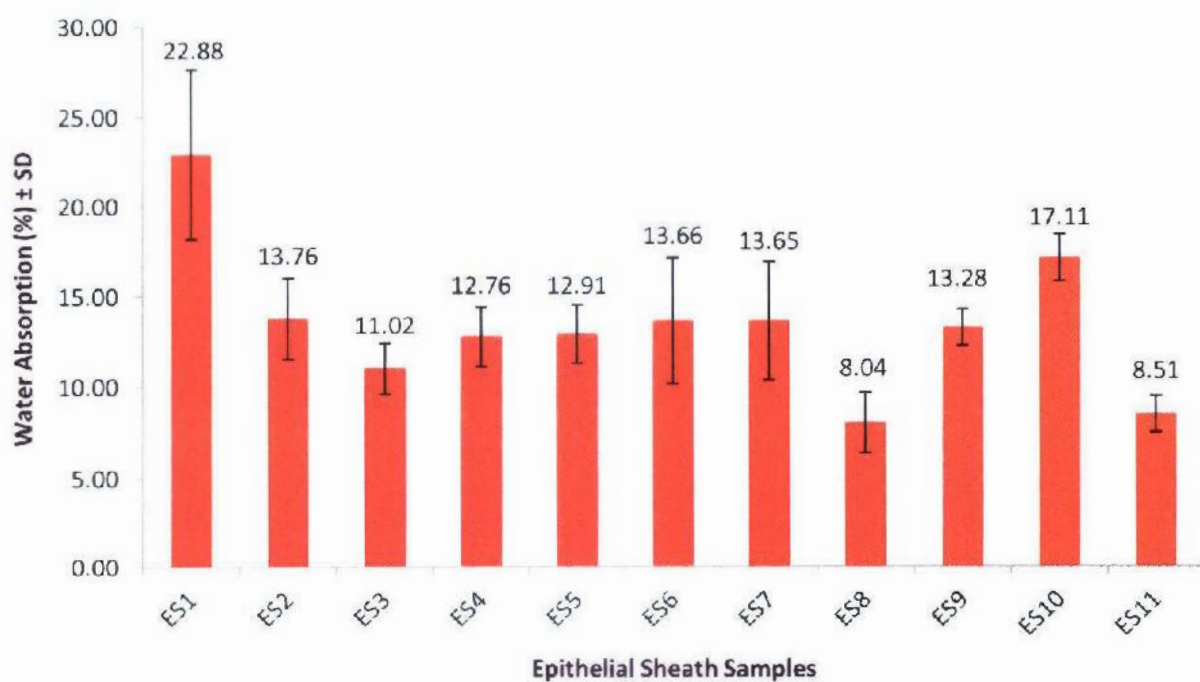


Figure 4.5: Water absorption (%) of different epithelial sheath samples.

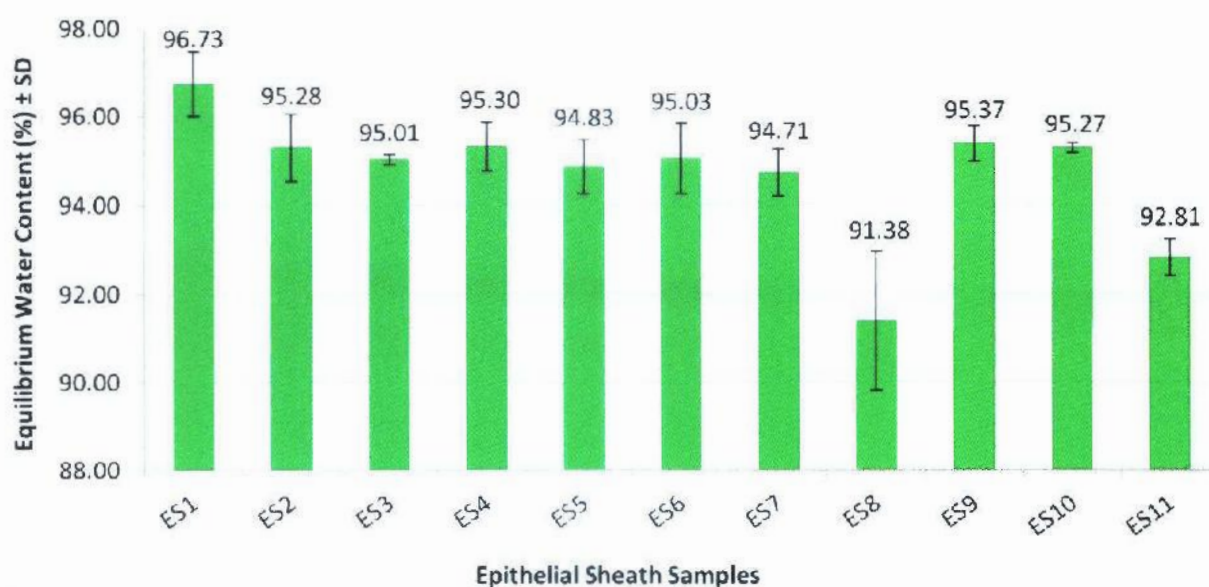


Figure 4.6: Equilibrium water content (%) of different epithelial sheath samples.

Based on the above illustrations (**Figure 4.4, 4.5 and 4.6**), ES1 sample [collagen concentration (10 mg/ml) and chitosan concentration (10 mg/ml)] can be selected for final wound healing applications. It may be due to the reason that the equal concentration of collagen and chitosan imparts the optimum wound healing properties into the epithelial sheath sample.

4.4 Composition of Final Selected Gels

Selected gels of 6% concentration of 3 different gel types were formulated successfully for final wound healing applications (**Table 4.5**).

Table 4.5: Composition of final selected gels

Gel Type	AM	AM-Collagen	Collagen
CMC-Na		6%	
AM Powder	2%	1%	-
Collagen Powder	-	1%	2%
Citric Acid		1%	
Methyl Paraben		0.02%	
Propyl Paraben		0.002%	
Glycerin		5%	
Distilled Water		Up to 100 ml	
Triethanolamine	235 µl	240 µl	245 µl

4.5 Evaluation of Final Selected Gels

The final selected gels were evaluated based on several physical and biological properties.

4.5.1 Physical Appearance, Homogeneity and pH Measurements

All final formulations were found to be homogeneous in nature with good consistency. pH of final formulations was found to be in between 6.95 to 7.04 which implies that gels were suitable for skin applications (**Table 4.6**). Color of gels containing only AM extracts were off-white while the gels containing both AM and collagen extracts were light white. In case of collagen gels, color was found to be deep white (**Figure 4.7 A-F**).

Table 4.6: Physical properties of final formulated gels

Gel Type	CMC-Na Percentage	pH	Homogeneity	Color
AM	6%	6.95	Homogeneous	Off White
AM-Collagen		6.96	Homogeneous	Light White
Collagen		7.04	Homogeneous	Deep White

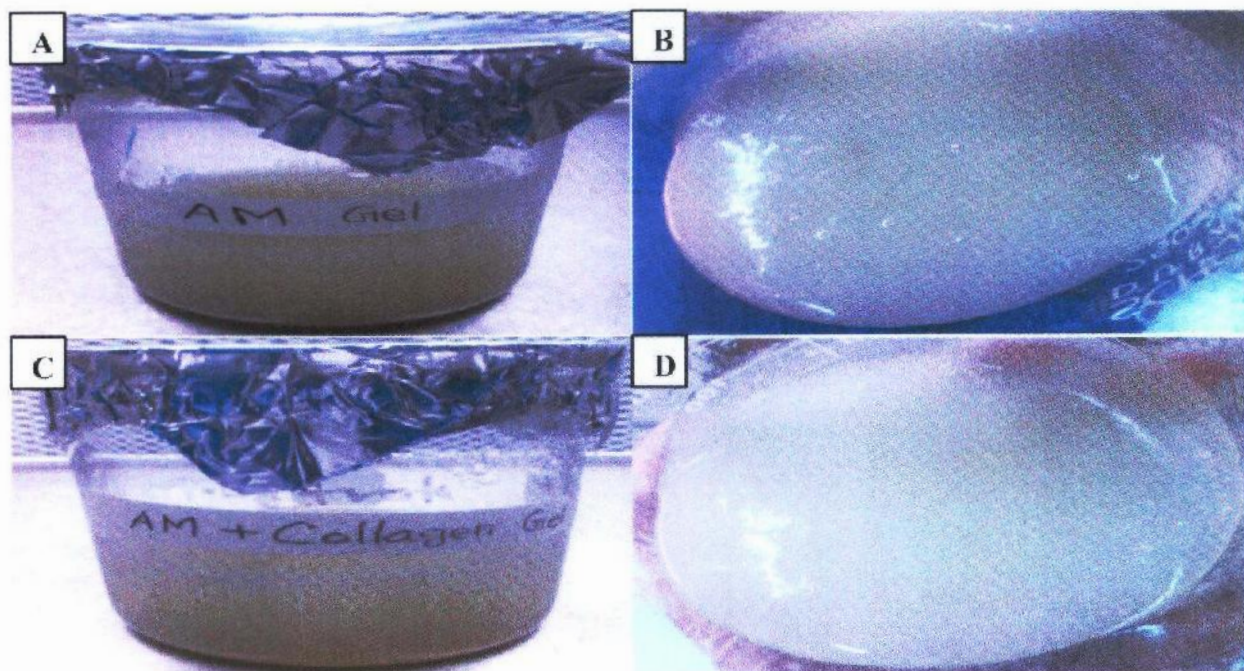


Figure 4.7: Homogeneity and physical appearance; (A-B) AM gel, (C-D) AM-Collagen gel.

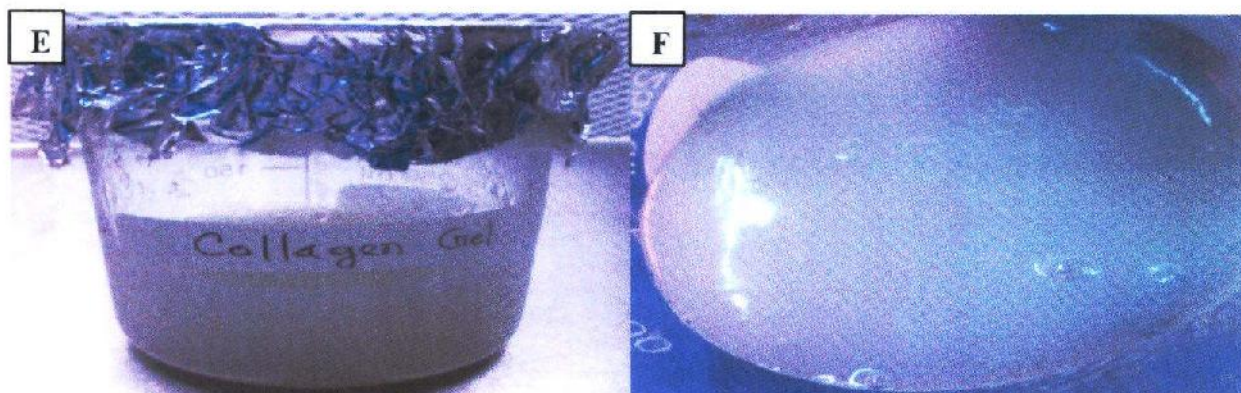


Figure 4.7: Homogeneity and physical appearance; (E-F) Collagen gel.

4.5.2 *In vitro* Cytotoxicity Test

The brine shrimp bioassay method was used to observe the cytotoxic effect of the final formulated gel samples as depicted in **Table 4.7**. The gel samples were dissolved in artificial sea water at different concentrations in which 20 nauplii (larvae of brine shrimp in number) were inoculated in each vessel. Colchicines was used as a positive control at certain dose (0.5 mg/ml). Sea water alone was used as negative control.

Table 4.7: Number and percentage of mortality of nauplii after the cytotoxicity test

Sample Name	Dose (mg/ml)	No. of Nauplii Present after Incubation	Mortality (%)
(+) ve control	0.5	0	100
(-) ve control	-	20	0
AM	0.25	20	0
	0.5	20	0
	0.75	20	0
	1	18	10
Collagen	0.25	20	0
	0.5	20	0
	0.75	19	5
	1	18	10
AM-Collagen	0.25	20	0
	0.5	20	0
	0.75	18	10
	1	17	15
AM-Collagen-ES	0.25	20	0
	0.5	20	0
	0.75	18	10
	1	17	15

The results suggested that there was no death of the nauplii, not even at higher doses of gel samples except AM-Collagen gel and the combination of AM-Collagen gel and epithelial sheath. In the case of AM-Collagen and AM-Collagen-ES samples, there was no death of the nauplii at low doses (0.25 and 0.50 mg/ml). Furthermore, there was very small rate of death of the nauplii at high dose (0.75 and 1 mg/ml) of AM-Collagen and AM-Collagen-ES samples. To be exact, the rate of death of nauplii at higher dose (1 mg/ml) of the samples was only 15%. A number of causes may attribute to the death of nauplii such as, the cytotoxic effect of the samples, formation of viscous layer on the gills of nauplii, and the reduction in the dissolved oxygen concentration of the artificial sea water. However, in the present study, the possibility of nauplii death due to cytotoxicity of the gel samples is very low as the number of death was zero at lower concentrations, suggesting no cytotoxic effect of the samples. Hence the most possible causes for the death of nauplii are (1) the formation of the viscous layer on the gills of the nauplii as the highly concentrated solution of the gel samples led to high viscosity, and (2) the formation of a gel-like structure on the gills which eventually inhibited oxygen dissolution.

4.5.3 Red Blood Cell Compatibility Test

Inverted microscopic analysis was carried out to observe the red blood cell morphology by magnifying the designed slides (**Figure 4.8 A-G**). RBC undergoes lysis or coagulation in contact with a non-biocompatible agent. In our study, less than 2% hemolysis was observed for the gel samples and the red blood cells remained intact when incubated with the gel samples in 3:1 ratio which indicated biocompatibility of the gel samples (**Figure 4.8 A-D**). Similar results were found in the case of saline water incubation (negative control). On the other hand, cell damage occurred by distilled water (positive control) incubation with the same ratio (**Figure 4.8G**), which was due to osmotic shock and gave us an idea of the morphology of RBC in contact with a non-biocompatible agent. The pure blood and negative control showed the intact morphology of RBC (**Figure 4.8 E-F**).

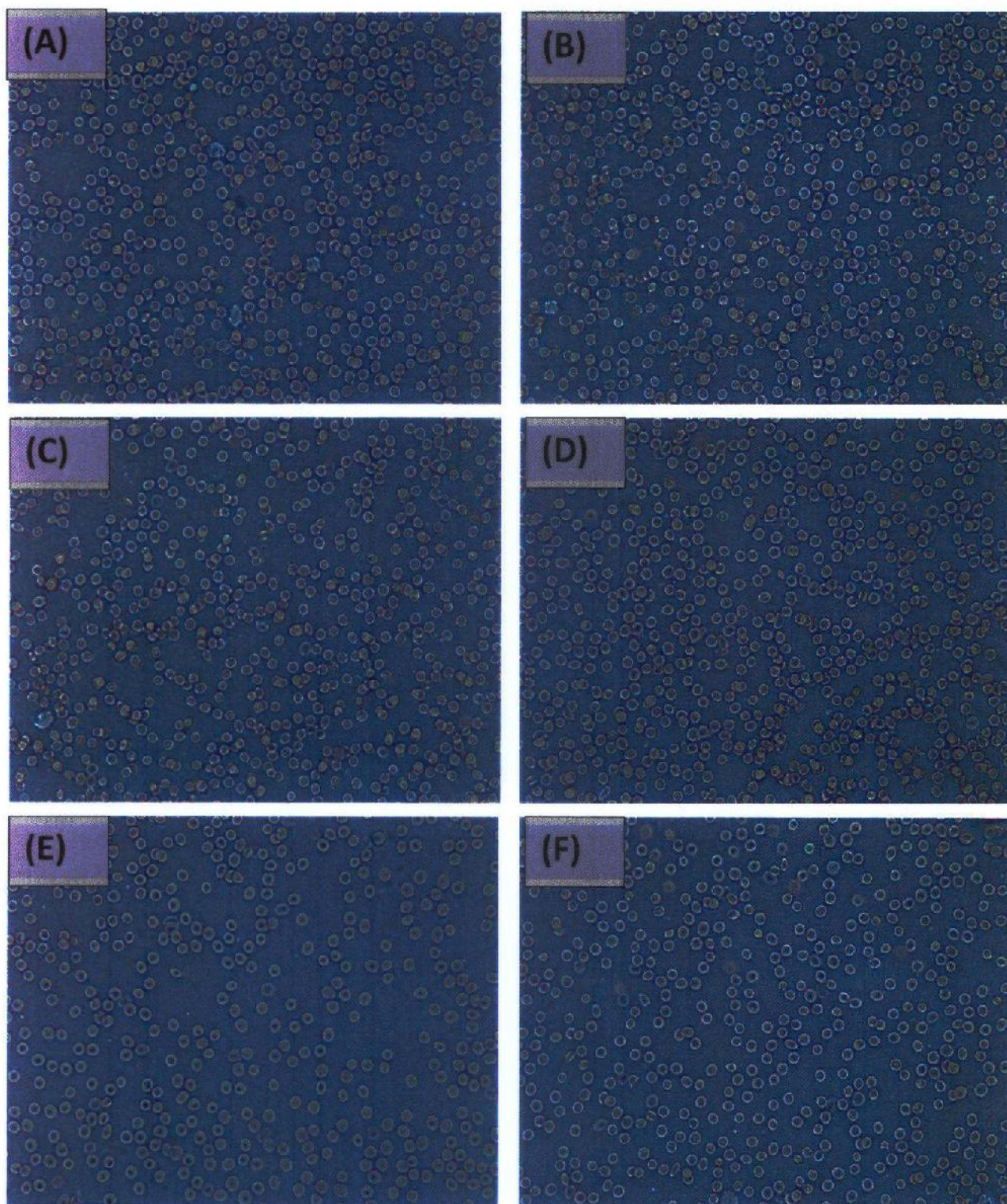


Figure 4.8: Microscopic view of blood incubated with gel samples at 20 $\times$ magnification; (A) AM gel, (B) AM-Collagen gel, (C) AM-Collagen-ES gel, (D) Collagen gel, (E) Pure blood, (F) Negative control.

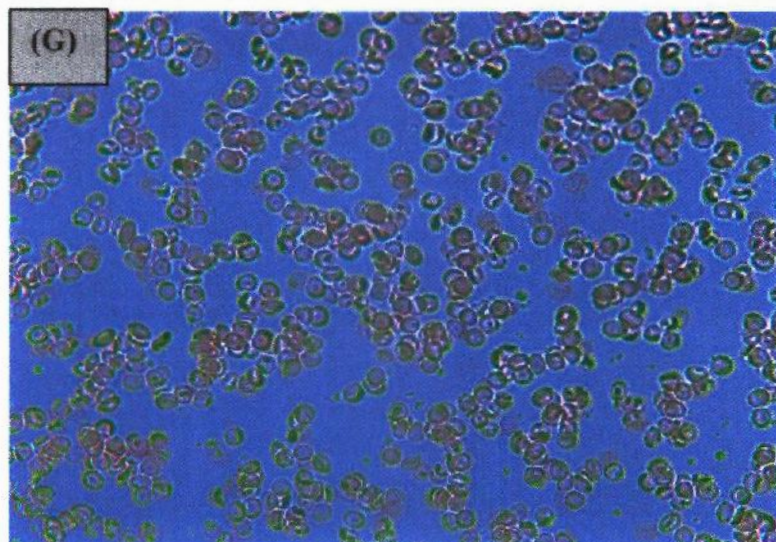


Figure 4.8: Microscopic view of blood incubated with gel samples at 20 $\times$ magnification; (G) Positive control.

4.5.4 Skin Irritation Studies

After topical application of all gels for a period of 1 day, it was found that the gels did not induce any edema or erythema (**Figure 4.9**). This result indicated the safety of gels to be applied topically.

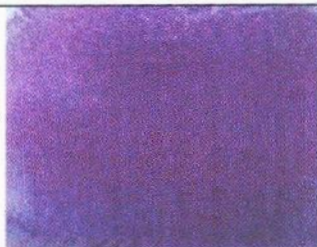
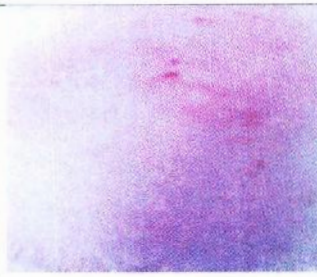
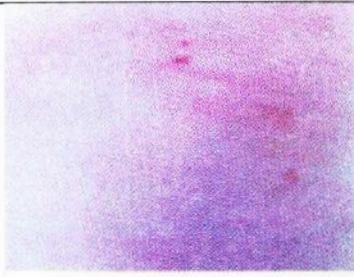
Gel	Shaved Skin of Rat	Applied Gel at Day-0	Skin Appearance at Day-01
AM			
AM-Collagen			
Collagen			
AM-Collagen-ES			

Figure 4.9: Skin irritation studies of final formulated gels.

4.6 Physiological Properties of Rats

According to the **Table 4.8** and **Figure 4.10**, the average body weight of negative control group and collagen treated rats increased slightly, while average body weight of combined group (AM-Collagen), AM group and positive control group treated rats, increased moderately. The rate of body weight increase was highest in the combination of (AM-Collagen) and epithelial sheath group. The protective barrier of epithelial sheath and the combination of wound healing properties may help the rats to be healthier and obtain somewhat enhanced body weight than rest

of the groups. This phenomenon is also implicated in case of food intake and water intake (Table 4.9, 4.10 and Figure 4.11, 4.12).

Table 4.8: Average body weight (g) of different groups on different observation days

Groups	AM-Collagen-ES	AM-Collagen	AM	Positive Control	Collagen	Negative Control
Day 0	128 ± 16.34	127.25 ± 21.84	122.75 ± 2.75	116.5 ± 11.73	104.5 ± 4.12	97.25 ± 2.06
Day 4	129.5 ± 7.55	128.75 ± 20.71	117.5 ± 15	116.75 ± 18.71	101.75 ± 2.22	91 ± 10.55
Day 8	136.5 ± 21.73	130.25 ± 5.38	122.75 ± 18.14	119.75 ± 13.60	104.75 ± 2.50	98.5 ± 10.63
Day 12	145.25 ± 22.35	136 ± 6.48	129.5 ± 14.75	126.75 ± 16.72	107.25 ± 3.30	102.5 ± 9.04
Day 16	148.5 ± 22.17	138.5 ± 6.24	132.25 ± 14.68	129 ± 16.57	109.25 ± 3.20	104.25 ± 9.29

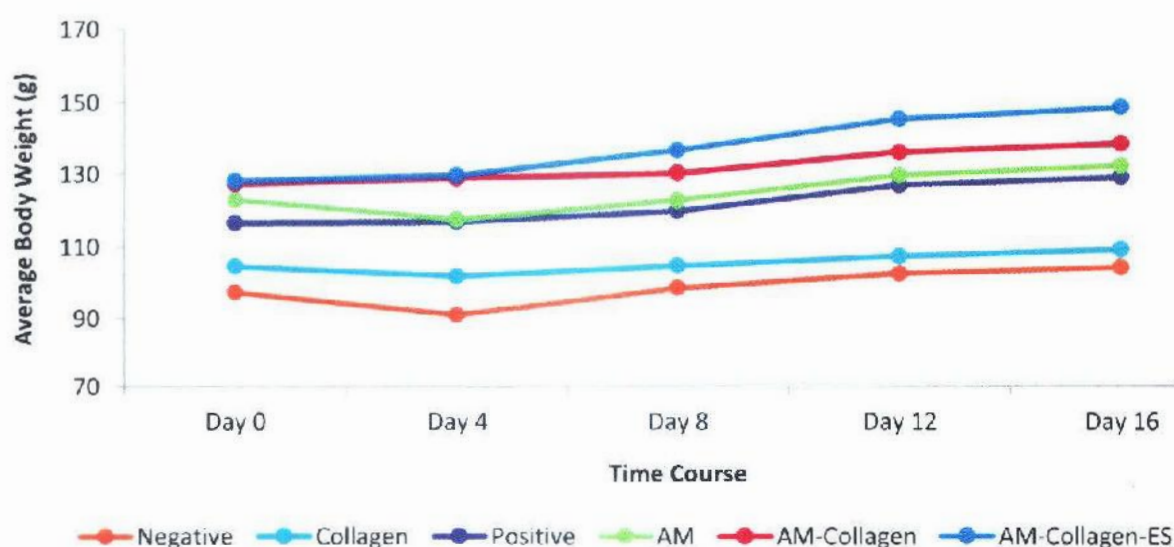


Figure 4.10: Average body weight (g) of different groups on different observation days.

Table 4.9: Average food intake (g) of different groups on different observation days

Groups	AM-Collagen-ES	AM-Collagen	AM	Positive Control	Collagen	Negative Control
Day 4	56	49.25	36	35.25	33.75	24.75
Day 8	78	71.75	63.5	45	44.5	44.5
Day 12	80.5	72	65	57.75	53.75	49.5
Day 16	86.9	76.25	69	62	58.5	52.75

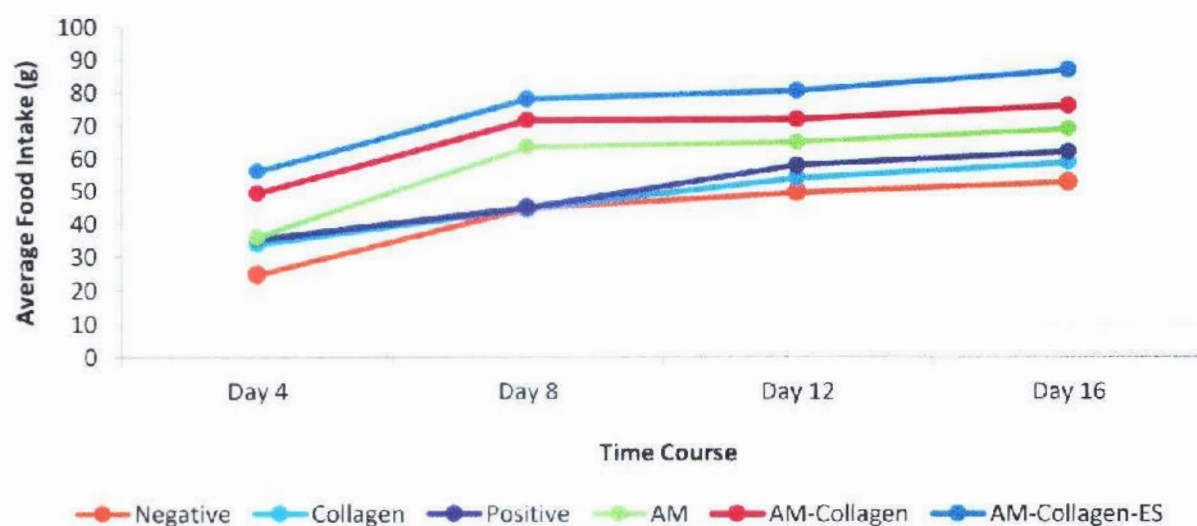


Figure 4.11: Average food intake (g) of different groups on different observation days.

Table 4.10: Average water intake (g) of different groups on different observation days

Groups	AM-Collagen-ES	AM-Collagen	AM	Positive Control	Collagen	Negative Control
Day 4	59	52.5	45	39.25	35.5	28.75
Day 8	64.25	57.25	45.5	46.25	42.75	34.75
Day 12	67.5	61.25	58.75	54.5	48.75	36.5
Day 16	73.5	69.25	63.75	60.75	57.25	45.25

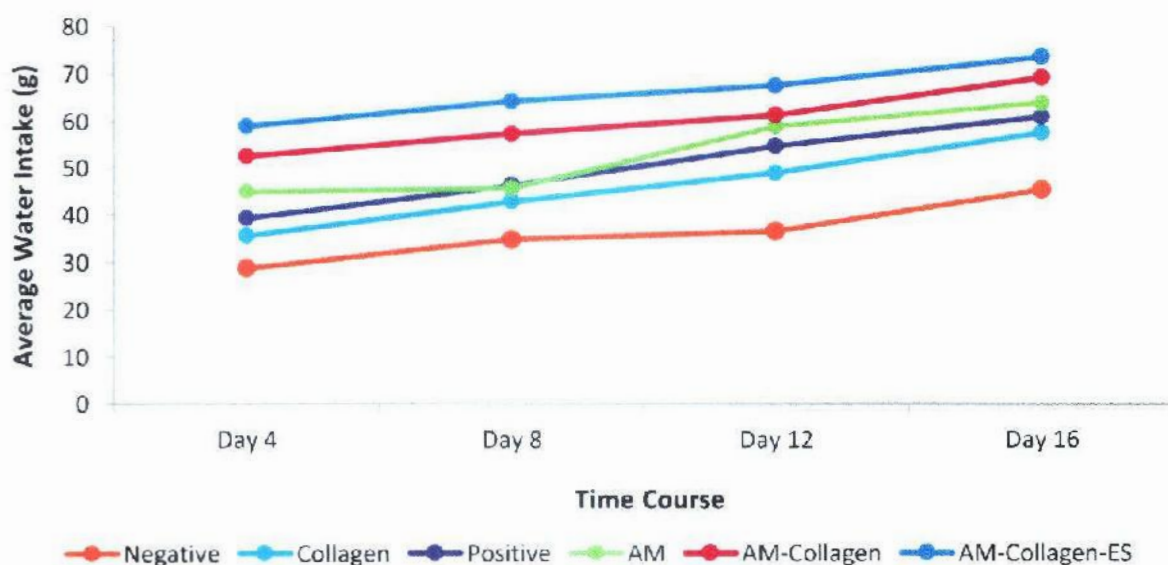


Figure 4.12: Average water intake (g) of different groups on different observation days.

4.7 Macroscopic Evaluation of Wounds

After the burn induction, gross changes of burn wounds were evaluated at day 0 post burned and subsequently at days 4, 8, 12 and 16 post burned, and the image were captured using a digital camera (Canon IXUS 130) and the morphological evaluation such as appearance of the wound was recorded (**Figure 4.13**). Initially at day 0, a second-degree burn was created in all experimental rats that produced a painful injury, with no bubbles, white color and subsequently hyperemia occurred into the damaged tissue area. With time, in each group of rats, red blood cell undergoing extravasation due to white color burn turned into fully hyperemic zone. After 30 minutes of burn injury, respective gel was applied in each group of rats except negative control group. At day 4, negative control and collagen group showed wet crust where AM and AM-Collagen, AM-Collagen-ES and positive control group showed slight dry and thick dry crust respectively. At day 8, negative control and collagen group were pictured less detachment of crust edge rather than all treatment rat groups. On day 12, scar tissue and contraction became clearly visible in each group. At day 16, crusts were all disappeared and re-epithelialization was completed in almost all treatment groups. Finally, AM-Collagen-ES and AM-Collagen group showed best healing rate with no scar tissue while other groups showed less healing rate with less scar tissue.

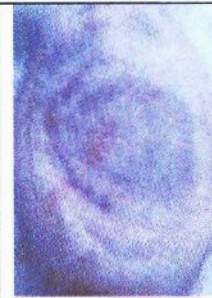
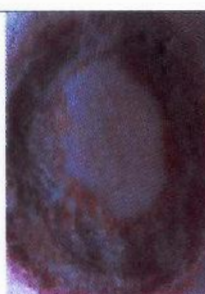
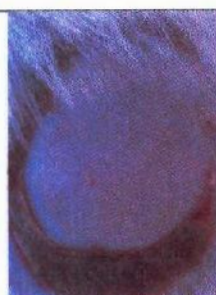
Day	(-) ve Control	(+) ve Control	Collagen	AM-Collagen-ES	AM	AM-Collagen
0						
4						
8						
12						
16						

Figure 4.13: Macroscopic wound images of different rat groups on different observation days.

4.8 Percent Wound Contraction

Wound contraction rate was observed in each group at day 4, 8, 12 and 16 of post burning. The rate of wound contraction were varied from group to group (Table 4.11 and Figure 4.14). Initially, at day 4, AM-Collagen-ES treated group was showed maximum healing rate, which was more than four times of control group and statistically most significant ($P<0.0002$), while AM-Collagen and AM gel treated groups were also statistically significant (AM-Collagen $P<0.0004$, AM $P<0.0006$). Positive control group and Collagen gel treated group were moderately significant than negative control group (Positive control $P<0.01$, Collagen $P<0.03$). After 8 days of post burning, negative control group had least healing rate of $9.33\pm2.31\%$ while AM and AM-Collagen treated group healed $25.33\pm2.31\%$ and $32\pm3.27\%$ of wound respectively and both were statistically significant (AM $P<0.0007$, AM-Collagen $P<0.00007$). AM-Collagen-ES treated group healed $34\pm2.31\%$ and statistically most significant ($P<0.000006$) than other groups. On 12th day of post burning, AM-Collagen-ES treated group reached maximum $52\pm3.27\%$ healing and was statistically most significant ($P<0.000003$). Healing rate of AM-Collagen and AM treated groups were $46\pm2.31\%$ and $40\pm3.27\%$ respectively. But, statistically AM-Collagen treated group was more significant ($P<0.000007$) than AM treated group ($P<0.00007$). On the day 16, AM-Collagen-ES treated group showed superiority ($72\pm3.27\%$) over other groups and it was statistically more significant ($P<0.0000009$) while negative control group healed only $22.67\pm2.31\%$. AM-Collagen, AM, positive control and collagen treated groups were healed $62.5\pm4.43\%$, $52\pm3.27\%$, $41\pm3.83\%$ and $36\pm4\%$ respectively (AM-Collagen $P<0.00001$, AM $P<0.00004$, positive control $P<0.0004$ and Collagen $P<0.001$).

Table 4.11: Percentage of wound contraction of different groups on different observation days

Groups	AM-Collagen-ES	AM-Collagen	AM	Positive Control	Collagen	Negative Control
Day 4	24.00 ± 3.27	20.00 ± 3.27	15.00 ± 2.00	12.00 ± 3.27	9.33 ± 2.31	5.33 ± 2.31
Day 8	34.00 ± 2.31	32.00 ± 3.27	25.33 ± 2.31	20.00 ± 3.27	16.00 ± 4.00	9.33 ± 2.31
Day 12	52.00 ± 3.27	46.00 ± 2.31	40.00 ± 3.27	30.00 ± 2.31	24.00 ± 4.00	18.67 ± 2.31
Day 16	72.00 ± 3.27	62.50 ± 4.43	52.00 ± 3.27	41.00 ± 3.83	36.00 ± 4.00	22.67 ± 2.31

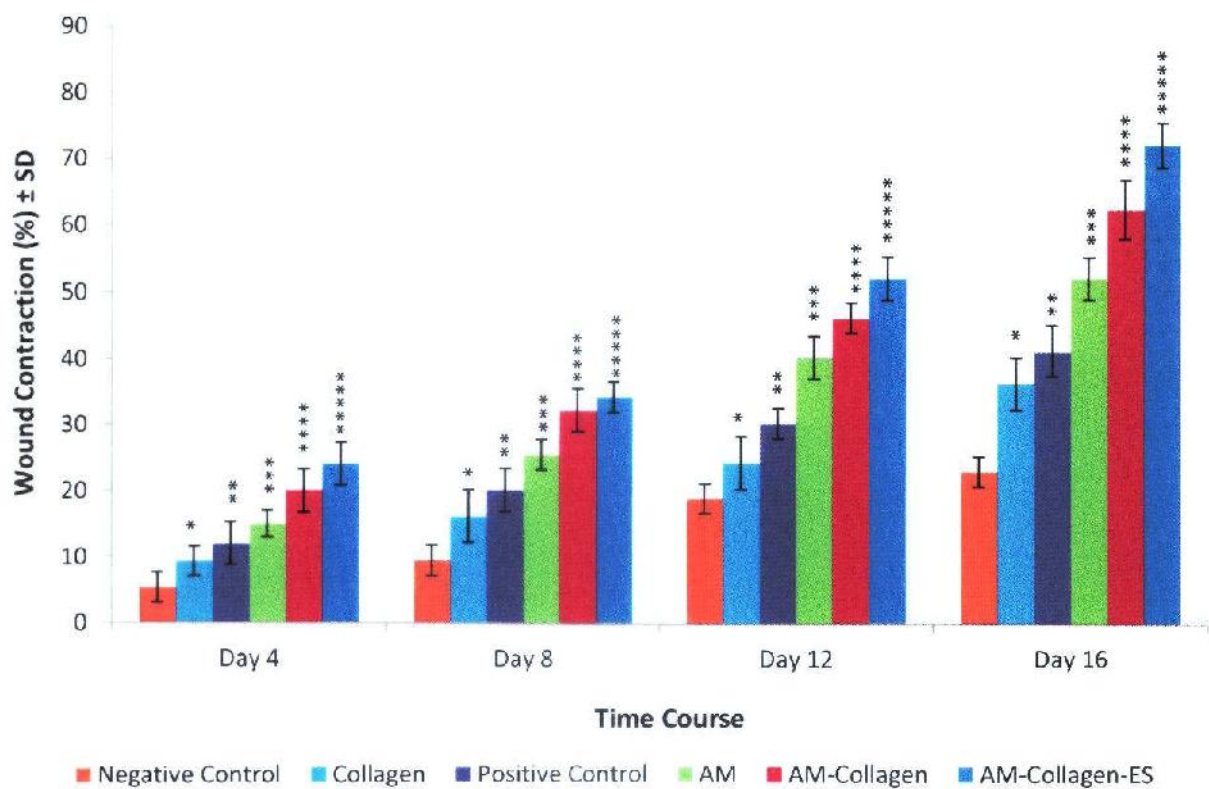


Figure 4.14: Percentage of wound contraction of different groups on different observation days. (N.B: * indicates level of significance against negative control group)

4.9 Epithelialization Period

Among the treated group, AM-Collagen-ES group required only 16.75 days while AM-Collagen, AM, positive control, collagen and negative control groups needed 18.75, 20.5, 22.25, 24.75 and 28 days respectively to achieve mean epithelialization (**Table 4.12**). Both AM-Collagen-ES ($P < 0.000002$) and AM-Collagen ($P < 0.000007$) treated groups were statistically more significant than AM treated group ($P < 0.0001$) and positive control group ($P < 0.0002$). Average re-epithelialization period in all groups were observed (**Figure 4.15**).

Table 4.12: Epithelialization period in different groups on different observation days (Values $\pm$ SD)

Groups	Epithelialization Period
AM-Collagen-ES	16.75 ± 0.96
AM-Collagen	18.75 ± 0.50
AM	20.50 ± 1.29
Positive Control	22.25 ± 0.96
Collagen	24.75 ± 0.96
Negative Control	28.00 ± 0.82

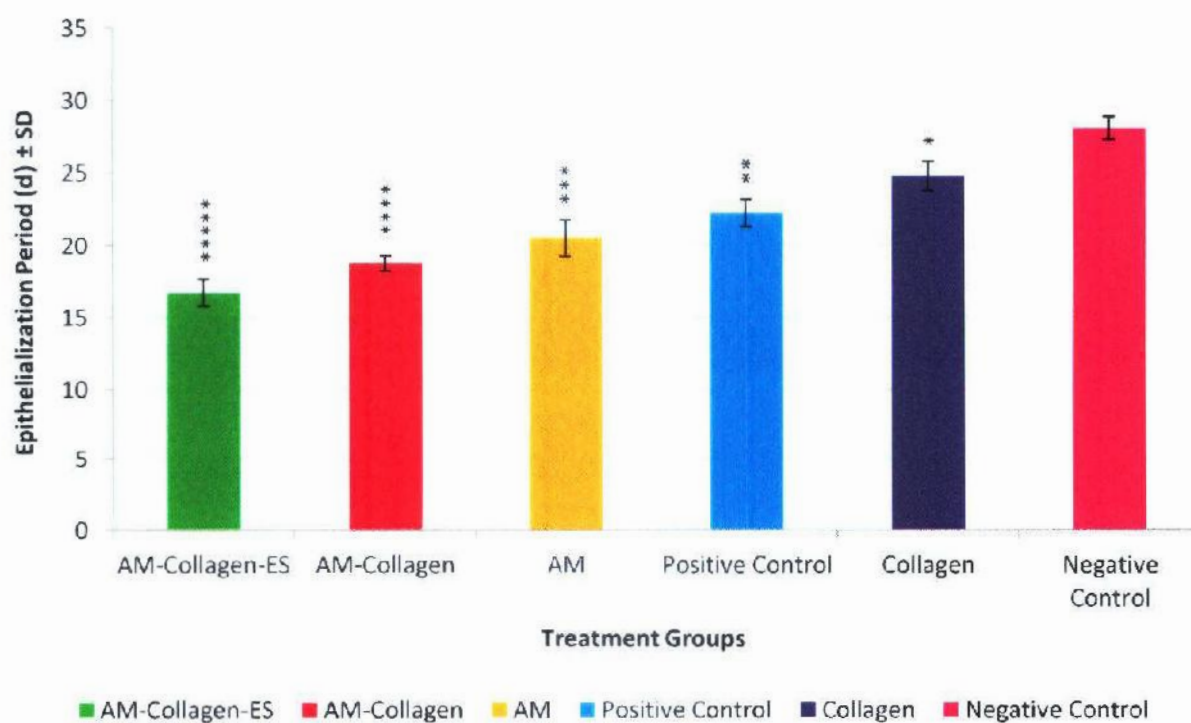


Figure 4.15: Epithelialization period in different treatment groups. (N.B: * indicates level of significance against negative control group)

CHAPTER FIVE

DISCUSSION



Burn injury is one of the most common and devastating phenomena worldwide. Since the ancient period; humans have known the tragedy of severe burn injuries. Globally, 265000 deaths every year are caused by burn. The majority of these occur in low- and middle-income countries and almost half occur in the South-East Asia Region (Sabale and Bhimani, 2012). It is the most common traumas in developing countries like Bangladesh. According to World Health Organization (WHO), nearly 173,000 Bangladeshi children are moderately or severely burnt every year (http://www.who.int/violence_injury_prevention). Most recent examples of burn related casualties in Bangladesh include Chawkbazar tragedy in Old Dhaka and FR Tower tragedy in Banani which claimed a lot of lives and a lot of people got injured in the process of escaping from fire burn. In addition, road accident has also become a common phenomenon in Bangladesh which incurs considerable number of skin injuries. Most skin injuries consumes large amount of medical resources. But it is hardly possible to bear the enormous economic and human cost for the people with lower socio-economic class. So, it is crucial to find an appropriate material for dressing of burn and skin wounds that improves healing and is readily available, easily applicable, and economical and also be protective from infection and desiccation, and facilitates healing (Mohammadi et al., 2009).

Both amniotic membrane and collagen fulfill the above criteria. Amniotic membrane is being widely used as burn wound healing material worldwide and known as an ideal biological burn wound dressing material (Mostaque and Rahman, 2011; Mohammadi et al., 2009). On the other hand, collagen is one of the body's key natural resources and is thus used as a natural wound dressing and has properties that artificial wound dressings do not have. When collagen is used as a burn dressing, healthy granulation tissue is able to form very quickly over the burn, helping it to heal rapidly (Singh et al., 2011). Moreover, Epithelial sheath is considerably a recent and newer concept of covering the wound area along with other wound healing agents which itself may be composed of biomaterials. It may serve the synergistic function of wound healing and protecting the wound area from external environment.

In our study, we maintained several physicochemical factors of gel such as, pH, physical appearance, homogeneity, viscosity etc. We also used many active ingredients, such as glycerine (used as a moisturizing agent), CMC-Na (thickening agent and maintain viscosity), methyl and propyl paraben (act as an anti-fungal agent), triethanolamine (used as a pH adjunct). We

analysed various physical properties like swelling ratio, water absorption (%), equilibrium water content (%), gel fraction (%) and spreadability of gel formulations to find out the best concentration of CMC-Na which better contains the optimum physical properties to be considered for final wound healing applications. 6% CMC-Na concentration was found to be best for final application. The same process was adopted for epithelial sheaths as well and we found epithelial sheath containing 10 mg/ml concentration of both collagen and chitosan to be optimum for final animal experiment. Outer surface of our body acts as a defense barrier due to acidic pH level. As pH level increase from epidermis to dermis and remains neutral state (pH-7) in internal body environment and it is required to formulate a gel having pH range 6.5 to 7.3 which are desirable to skin. So, pH of all final formulations was found to be in between 6.95 to 7.04, which denotes that gels are suitable to apply in skin. The final gel and sheath samples also passed the *in vitro* cytotoxicity test and red blood cell compatibility test which indicates the gel and sheath samples to be biocompatible.

After applying AM-Collagen-ES, AM-Collagen, AM and Collagen on different rat groups, rats showed no edema or erythema and it was obvious the gels were safe to use (**Figure 4.9**). According to the scatter plot, it was deducible that the combined (AM-Collagen-ES) and (AM-Collagen) rats group average body weight, food intake and water intake were significantly greater than any other rats group (**Figure 4.10, 4.11 and 4.12**). It also showed that the gel had no negative effects on rats' physical properties.

Wound contraction is an integral part of wound healing process, which closes wounds to the external environment and it is accountable for the shrinkage of the wounded area and healing primarily depends on the repairing ability of tissues in addition to type and degree of damage and general health status of the tissue.

Wound contraction rate was observed in each group at day 4, 8, 12 and 16 of post burning. The rate of wound contraction were varied from group to group (**Table 4.11, Figure 4.14**). Initially, at day 4, AM-Collagen-ES gel treated group stood out among other groups in wound contraction which was more than four times than control group and statistically most significant ($P < 0.0002$), while groups other than AM-Collagen-ES and AM-Collagen were relatively significant [AM-Collagen ($P < 0.0004$), AM ($P < 0.0006$), Collagen ($P < 0.03$) and Positive Control ($P < 0.01$)]. On the day 16, AM-Collagen-ES group showed superiority ($72 \pm 3.27\%$) over other groups and it was

statistically more significant ($P < 0.0000009$). While negative control group healed only $22.67 \pm 2.31\%$. AM-Collagen group, AM group, positive control group and collagen group healed $62.5 \pm 4.43\%$, $52 \pm 3.27\%$, $41 \pm 3.83\%$ and $36 \pm 4\%$ respectively [AM-Collagen ($P < 0.00001$), AM ($P < 0.00004$), Positive Control ($P < 0.0004$) and Collagen ($P < 0.001$)]. Among different treated rat groups, AM-Collagen-ES treated group was showed faster epithelialization (16.75 ± 0.96 days) in comparison with other rat groups [AM-Collagen (18.75 ± 0.50 days), AM (20.5 ± 1.29 days), Positive Control (22.25 ± 0.96 days), Collagen (24.75 ± 0.96 days) and Negative Control (28 ± 0.82 days)]. The results indicated that the combination of amniotic membrane and collagen imparted exclusive wound healing properties of both the biomaterials to the AM-collagen group. In addition, epithelial sheath served as an excellent wound covering material which protected the wound area from external environment as well as added extra wound healing properties to the existing AM-Collagen combined gel as the epithelial sheath itself was composed of wound healing biomaterials like collagen and chitosan.

CHAPTER SIX
CONCLUSION

In this study, we have developed a novel, biocompatible wound healing gel with a combination of amniotic membrane and collagen; and characterized their properties. Amniotic membrane is a globally accepted biological biomaterial for second and third degree burn. Collagen has been reported to provide an optimal structure for cellular ingrowth and modulation of the chronic wound microenvironment to facilitate burn healing. From the analysis report, it was revealed that, the formulated gels had neutral pH, acceptable swelling ratio, good spreadability and acceptable gel fraction rate. The gels were also found biocompatible at brine shrimp lethality and red blood cells compatibility test. From the *in vivo* study, no erythema and edema were observed in skin irritation experiments confirming the applicability of the gels. Our results also showed that AM-Collagen-ES gel treated group was demonstrated superiority in wound contraction over other treatment groups and it was statistically more significant. However, further investigation is required to unfold the underlying molecular mechanisms of wound healing.

CHAPTER SEVEN

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APPENDIX-I

1. Normal Saline (0.9%) Solutions

Chemicals	Amount
NaCl	0.9g
DW	Up to 100ml

2. 70% Alcohol Solutions

Chemicals	Amount
Absolute Ethanol	70 ml
DW	Up to 100ml

3. 0.5 M Acetic Acid Solutions

Chemicals	Amount
Concentrated Acetic Acid	30 ml
DW	Up to 1000ml

4. 2.6 M NaCl Solutions

Chemicals	Amount
NaCl	76 g
DW	Up to 500ml

5. 0.5 M NaOH Solutions

Chemicals	Amount
NaOH	2 g
DW	Up to 100ml

APPENDIX II

Glassware and other accessories:

- A) Beaker (50 ml, 100 ml, 250 ml, 500 ml)
- B) Conical flask (100 ml, 250 ml, 500 ml)
- C) Measuring cylinder (10 ml, 50 ml, 250 ml, 500 ml, 1000 ml)
- D) Spoon
- E) Glass pipettes (1 ml, 2 ml, 5 ml, 10 ml), Micro pipettes (1000 μ l, 200 μ l, 10 μ l)
- F) Tips (1000 μ l, 200 μ l, 10 μ l)
- G) Pipette holder
- H) Petri dishes
- I) Scissors
- J) Forceps
- K) Sterile cotton
- L) Needle holder
- M) Surgical blade
- N) Surgical suture
- O) Scalpel
- P) Sterile aluminium foil
- Q) Filter paper
- R) Glass rod
- S) Glass pipette
- T) Spirit lamp
- U) Sterile inoculation box
- V) Sterile cotton
- W) Tissue paper
- X) Sterile container (tissue container)
- Y) Falcon tube

