

In vivo evaluation of wound healing properties of superoxidized solution (SOS) using an excision wound model

BIHE

Department of Biology

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Abstract

Superoxidized solution (SOS), which is made by electrolysis of sodium chloride, is a nontoxic disinfectant used to eradicate broad spectrum of microorganism and does not seem to damage host tissue or facilitate bacterial resistance. In addition, it is well tolerated, improves granulation of wound tissue, and reduces the need of debridement and less pain reported during the treatment. The purpose of this study was to assess wound healing effect of SOS and gel of SOS. Excision wounds were induced in Swiss Webster mice. Animals were assigned into five groups: negative control group received no treatment; positive control group received a wound ointment (Sebamed cream); SOS group; SOS gel group and blank gel group treated with gel base. Wounds were observed macroscopically until they were fully epithelialized, and they were sampled every three days to be examined microscopically. Efficiency of treatment for each animal in proportion to its three days earlier, revealed that repair of SOS and positive control group were accelerated significantly in day nine in comparison with day six. While it happened three days later for SOS gel group. According to microscopically observation, epithelialization occurred three days earlier in SOS and SOS gel group. It might be concluded that SOS and SOS gel group accelerate healing through inducing epithelialization. Result of investigation effect of gender on wound treatment revealed no significant differences between male and female, although the type of treatment can affect the wound healing activity for each gender.

Keywords: Superoxidized solution; Excision wound; Wound healing; Oxidative reductive potential; Epithelialization.

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List of Abbreviations:

8-OHdG: 8-hydroxy-2'-deoxyguanosine

AI: Antiirritant

AQP3: Aquaporin3

BMZ: Basement membrane zone

CMC: Carboxy methycellulose

HCECs: Corneal-epithelial-cells

DHEA: Dehydroepiandrosterone

ECM: Extra cellular matrix

EGF: Epidermal growth factor

ECAS: Electrochemically activated solutions

ESP: Exteracellular polysacharid matrix

FGF2: Fibroblast growth factor-2

HDFs: Human dermal fibroblasts

HP: Hydrogen peroxide

HCECs: Human corneal-epithelial-cells

HCLE: Human cornea–limbal epithelial

HK: Human keratonocytes

IgG: Immunoglobulin G

IL-1: Interleukin-1

LPS: Lipopolysaccharide

MIF: Migration inhibitory factor

MSCs: Melanocyte Stem Cells

MMP: Metalloproteinase

Ovx: Ovariectomized

Redox: Oxidation reduction

PDGF: Platelet-derived growth factor

PMN: Polymorphonuclear leukocytes

RBC: Red blood cell

ROS: Reactive oxygen species

SA- β -gal or SABG: Senescence-associated beta-galactosidase

SEM: Standard error of the mean

SOS: Superoxidized solution

TGF- α : Transforming growth factor α

TGF- β : Transforming growth factor β

TA: Transit amplifying cell

TEA: Triethanolamin

TNF: Tumor necrosis factor

VEGF: Vascular endothelial growth factor

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Chapter 1:

Introduction

Skin

Skin is the largest organ in the body. Over 5 kg of body weight in a 70kg individual is skin, and it covers almost 2 m^2 of a surface area (Burns *et al.*, 2010). Skin is composed of three layers, a stratified, cellular epidermis which is composed of viable keratinocyte and covered by a layer of keratin (stratum corneum). Underneath epidermis is dermis of connective tissues, principal component of dermis is fibrillar structural protein collagen. Beneath the dermis is subcutaneous fat (panniculus) which is composed of lobules of lipocytes and separated from other part of the body by vestigial layer of stratified muscle (Figure 1)(Burns *et al.*, 2010; James *et al.*, 2011).

Thickness of skin's layers are different in various part of the body (James *et al.*, 2011). The epidermis is thickest on the soles and palms, it is approximately 1.5 mm there, but it is very thin in eyelid where it is approximately less than 0.1. The thickest dermis is in the back where it is 30 to 40 times thicker than overlying epidermis. Large amount of subcutaneous fat is also on the abdomen and buttocks; still, very small amount of them could be seen on nose and sternum (James *et al.*, 2011).

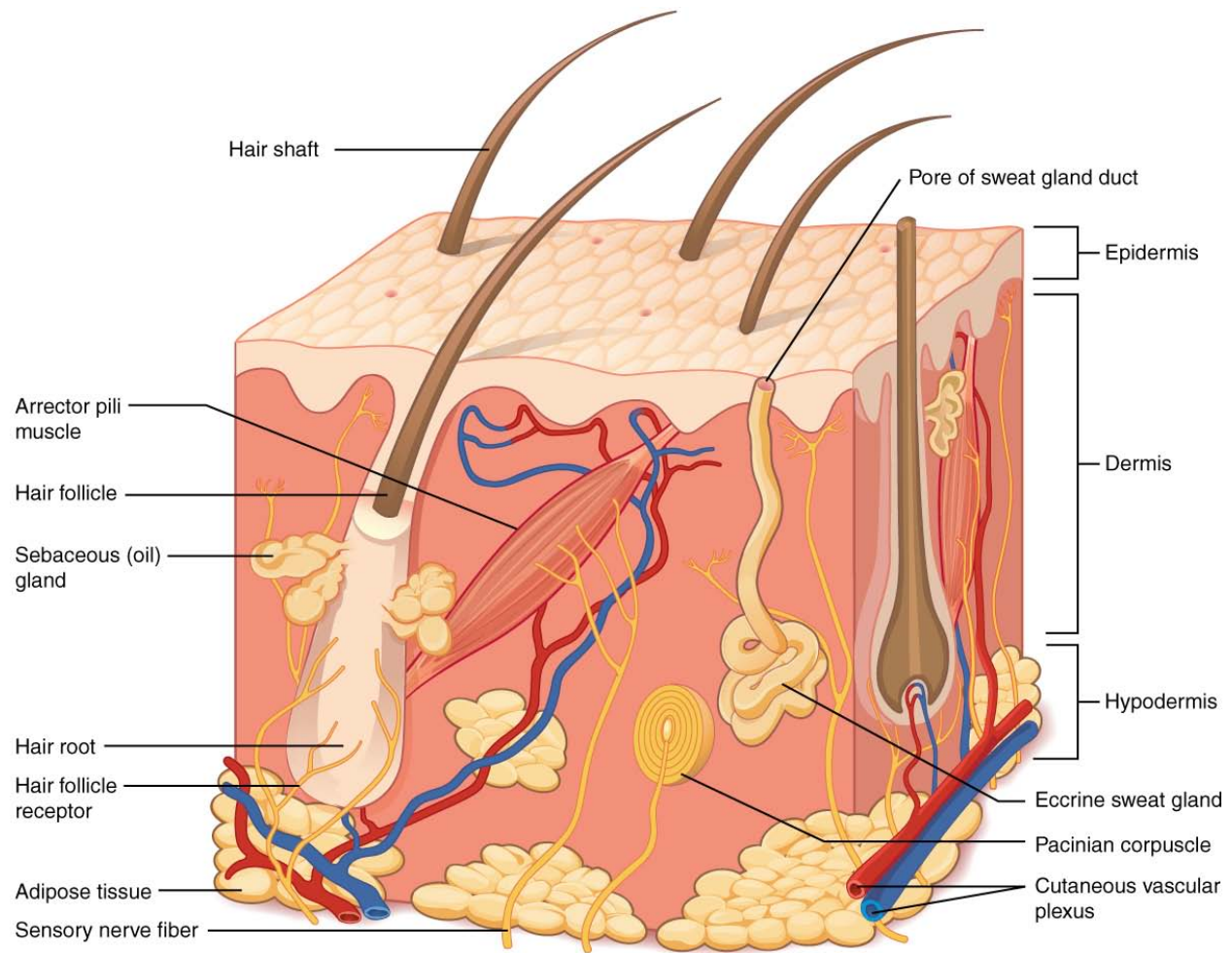


Figure 1. Schematic picture of skin.

(<http://philschatz.com/anatomy-book/contents/m46060.html>)

Epidermis

The fetus, during the first week, is covered by a single layer of cuboidal nonkeratinized cells called periderm (James *et al.*, 2011). Later, periderm is replaced by multilayered epidermis. Adnexia structures, eccrine sweat units and follicle downgrowth from developing epidermis during third month of fetal life. Later, sebaceous glands develop from midregion of follicular

epithelium and apocrine sweat units develop from upper portion of it. These structures develop first in cephalic portion of the fetus and later in the caudal portions (James *et al.*, 2011).

The normal epidermis is terminally differentiated stratified squamous epithelium which is typically 0.05-0.1 mm in thickness. Epidermis is mainly composed of keratinocytes (making up 95% of the total). Keratinocytes moving from base of epidermis to the surface of the skin and give rise to the four layers (Figure 2)(Burns *et al.*, 2010). Basal layer or stratum germinativum, prickly layer or stratum spinosum and granular layer or stratum granulosum and horny layer or stratum corneum (James *et al.*, 2011). The malpighian layer includes both basal and spinosum layer (Burns *et al.*, 2010). On palms and soles, another layer, stratum lucidum, is above the granulosum layer (James *et al.*, 2011). Keratinocytes progressively differentiate from basal cells to terminally, outermost layer, keratinized stratum corneum (Wolff *et al.*, 2008).

Basal layer (stratum germinativum), is a continuous one-cell-thick layer, but in glabrous skin it could have two or three layers (Burns *et al.*, 2010). Basal layer contain columnar and mitotically active basal keratinocytes which have large, dark stained nuclei with dense cytoplasm (Wolff *et al.*, 2008; Burns *et al.*, 2010). Basal keratinocytes attach to the basement membrane by keratin at hemidesmosome zones and attach to other cell through desmosome area. Basal keratinocytes enlarge and give rise to more superficial, differentiated epidermal layer, prickly layer or stratum spinosum (Wolff *et al.*, 2008; Burns *et al.*, 2010). Relatively undifferentiated stem cells of basal layer have the ability to reproduce for entire life span, they divide and develop two daughter cells, one of them stay in stem cells niche (termed niche) as a stem cell, and the other one, named transit amplifying (TA) cell leaves the niche. TA cells proliferate rapidly and undergo further

reproductive division needed for stable self-renewal and are most common cells in basal layer portion. Offspring of TA cells (post mitotic cells) expand cell population from initial stem cells mitosis and differentiate progressively and migrate to more superficial layers of epidermis (Wolff *et al.*, 2008).

Spinosum layer is named because of spine-like appearance of cell margins. Structure, shape and subcellular properties of cells depend on the positions of cell within epidermis. Cells which are above the basal layer have polyhedral shape with rounded nucleus, as cells move upward and differentiate; they become flatter and develop lamellated granules known as lamellar granules or bodies, membrane-coating granules or Odland bodies (Wolff *et al.*, 2008; James *et al.*, 2011). These lamellar granules are numerous in uppermost cells of the spinosum layer and as they enter granular layer they migrate toward periphery of the cells (Wolff *et al.*, 2008).

Granular layer or stratum granulosum named because of having prominent keratohyalin granules within the cells. Keratohyalin granules are composed of keratin filaments, profilaggrin and loricrin. As granular cells move upward toward stratum corneum, they discharge lamellar granule lipids into the extracellular space and result in the formation of the hydrophobic lipid envelope responsible for the skin's barrier properties, and cause intercellular cohesion within stratum corneum cells (Burns *et al.*, 2010). At the transition between stratum granulosum and stratum corneum, granulosum cells also release profilaggrin from keratohyalin granules which cause cleavage of the profilaggrin polymeric protein into filaggrin monomers by calcium dependant enzymes (Wolff *et al.*, 2008), and under filaggrin influence the cells become flattened and keratin filaments align into macrofibers and produce keratin filaments aggregations (Burns *et*

al., 2010). Stratum corneum complete differentiation of granular cells results in stacked layers of cornified flattened cells that lost their nucleus and other cell organelles and form stratum corneum. Stratum corneum functions are providing mechanical protection to the skin and prevent water loss and permeation of soluble substances from the environment (Wolff *et al.*, 2008).

The cellular progression from basal layer to stratum corneum layer takes about 30 days. During this trip, keratinocytes which are main cells of the epidermis (80 to 90% of epidermis) pass through synthetic and degradative phases (Burns *et al.*, 2010; James *et al.*, 2011). During keratinization journey, keratinocyte cells first have brick like shape which was the result of accumulation of cytoplasm intermediate filaments composed of fibrous proteins, keratin. As keratinocytes differentiate, they become more flattened. This process is done by the filament aggregating protein, filaggrin, a protein component of keratohyalin granules which cause keratin filaments aggregation (keratin and filaggrin comprise 80-90% of the mass of the epidermis). As cells become more flattened, during degradative phase, keratinocytes loose their nucleus and cytoplasmic organelles and they form the outermost layer of the epidermis, the stratum corneum, where cells now called corneocytes (Burns *et al.*, 2010; James *et al.*, 2011). The dead corneocytes have matrix protein, keratin filaments and protein shell called a cell envelope. The cell envelope is composed primarily of two proteins, loricrin and involucrin. These proteins contain extensive links between each other making the cell envelope which also surrounded by extracellular lipid layers and form the most insoluble structure of the corneocyte (Wolff *et al.*, 2008; James *et al.*, 2011).

The adult epidermis contains other type of cells, Melanocytes, Langerhans' and Merkel cells (James *et al.*, 2011).

Merkel cells are post mitotic cells which locate above basement membrane zone among keratinocytes (Figure 2). Merkel cells scattered throughout epidermis of vertebrates and constitute 0.25 to 5% of epidermis (Burns *et al.*, 2010; James *et al.*, 2011). Through transmission electron microscopy, Merkel cells are oval shape with long axis oriented parallel to the basement membrane, they have bilobed nucleus and clear cytoplasm. Merkel cells are found in hairy skin, tactile area of glabrous (Digit, lips), outer root sheath of hair follicles, taste buds, anal canal and eccrine sweat glands (Wolff *et al.*, 2008; Burns *et al.*, 2010). Merkel cells have neurosecretory granules in their cytoplasm and they have close contact with nerve fibers to develop merkel cell neurite complex. Still there is not any evidence about synaptic transmission; however, numerous vesicles could be seen in neurons opposed to Merkel cells. The Merkel cell neurite complexes act as mechanoreceptor and are responsible for touch sensation. Skin has extensive neural networks, some of these nerves contain Merkel cells and develop Merkel cell neurite complexes and some others are free nerve endings (Burns *et al.*, 2010).

Melanocytes are pigment producing cells of epidermis. During development, melanocytes could be found in eight week fetus. They derived from melanocyte precursors called melanoblasts. Melanoblasts emerge from neural crest cells and migrate to the skin (Burns *et al.*, 2010; James *et al.*, 2011). Through light microscope, melanocytes are recognized by ovoid nucleus, pale staining cytoplasm and color of melanosomes (Wolff *et al.*, 2008). In normal skin, melanocytes are inside basal layer and their population proportion is approximately 1 in every 10 keratinocytes (Figure 2). Areas like face, genitalia and shins have greater amount of them (James *et al.*, 2011). Melanocytes are dendritic cells that have packages of melanin in melanosomes and transfer their melanin to the keratinocytes and growing shaft in hair follicle through melanosomes (Burns *et al.*, 2010). Differences in skin color do not depend on amount of

melanocytes; however, the number, size and distribution of melanosome within keratinocytes determine the skin color. Exposure of sun light causes melanosome production stimulation in melanocytes to produce larger melanosomes and increase distribution of melanosomes within keratinocytes to protect skin (Burns *et al.*, 2010; James *et al.*, 2011).

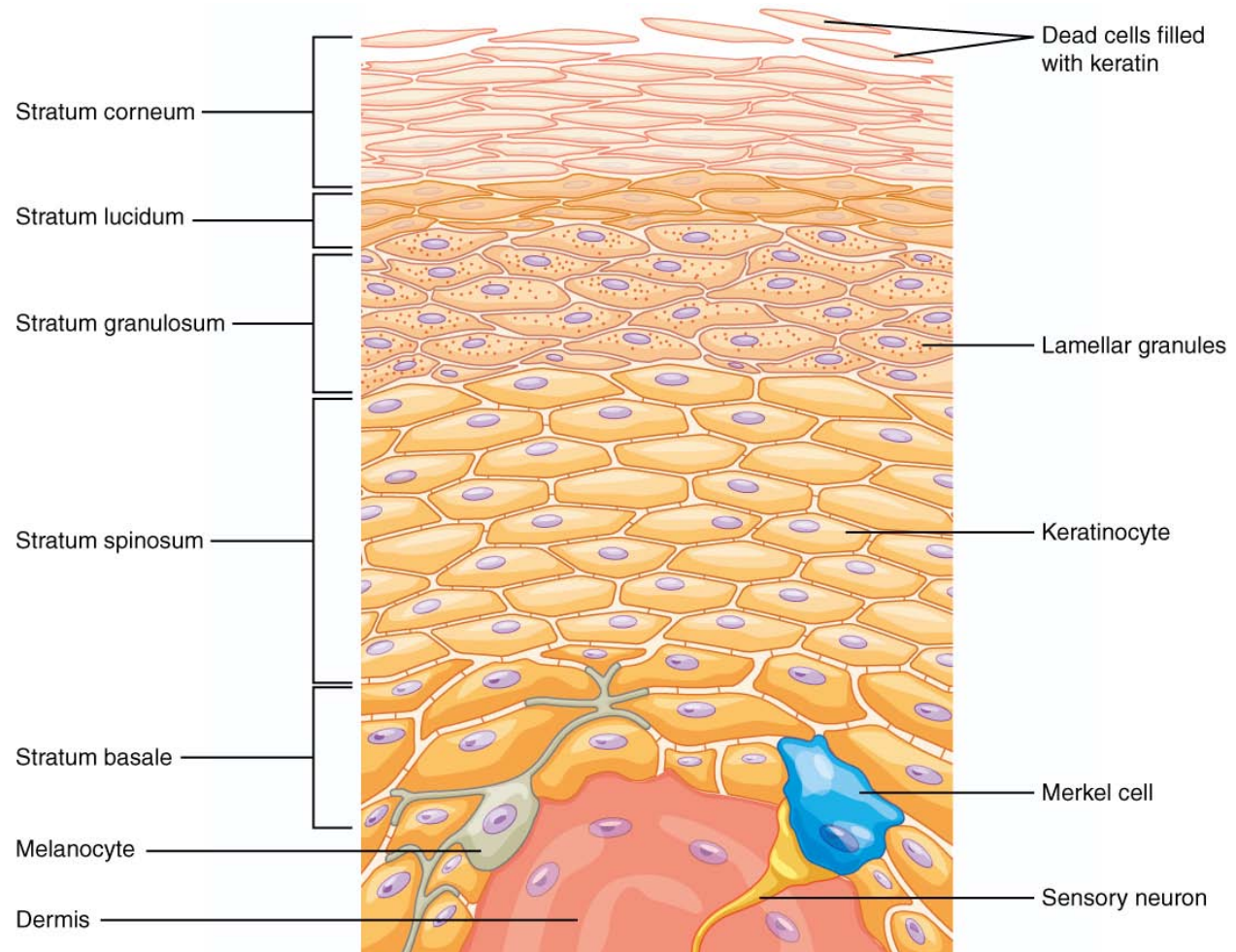


Figure 2. Epidermal layers.

(<http://philschatz.com/anatomy-book/contents/m46060.html>)

Langerhans cells are dendritic cells which are normally scattered among stratum spinosum's keratinocytes. They constitute 3 to 5% of spinosum layer (James *et al.*, 2011). They also distribute throughout the basal and granular layers (Wolff *et al.*, 2008). Langerhans cells are dendritic cells like melanocytes; still, they do not have pigments. Through electron microscopy, langerhans cells have folded and lobulated nucleus, clear cytoplasm, lysosome, golgi and endoplasmic reticulum. They also have distinct cytoplasmic organelle which is called birbeck's granules, these granules are rod shape and their function is under question. Langerhans cells originate in bone marrow and they are in the lineage of macrophage and monocyte (James *et al.*, 2011). Their roles are presenting and processing antigens of skins and bring them to lymph node, thus, they have key role in adaptive immune response in the skin. Langerhans cells work like macrophage among epidermal keratinocytes. They trap antigens by their dendrites and then migrate to lymph node via lymphatics. In lymph node, these cells process and present antigens to T lymphocytes that can then undergo clonal proliferation (Burns *et al.*, 2010). They also play an important role in regulating the balance between immunity and peripheral tolerance, under noninflammatory steady condition, they take skin specific antigens to lymph nodes to prevent immunization and induce peripheral tolerance against epidermal self antigens (Burns *et al.*, 2010).

Epidermal appendages: adnexia

Skin adnexia includes eccrine gland, apocrine gland and pilosebaceous units (hair follicle, sebaceous gland and arrector pili muscle) (Figure1). Embryologically, adnexia originate as downgrowth from epidermis, thus, they are originally from ectoderm. While different adnexia structures have specific functions, they all conserve skin from injuries. During injuries, keratinocytes of epithelium of these structures migrate to the surface of injury and cause

reepithelialization. So, in scalp and face, where pilosebaceous units are abundant, reepithelialization occurs rapidly than areas like back where adnexia structures are less. In area with large amount of adnexia, injury quickly is covered by epithelium and no more granulation tissue (connective tissue with tiny blood vessels that cover surface of the wound during healing process) will form, but in areas with few adnexia, granulation tissue will slowly fill the wound site until they are replaced by surrounding skin (James *et al.*, 2011).

Dermis

Dermis is a layer of the skin locating under the epidermis, and also known as corium or cutis (Burns *et al.*, 2010). It demarcated on the top by dermal-epidermal basement membrane and at the bottom by the subcutis. The dermis is the major component of human skin which its thickness varies from less than 0.5mm to more than 5 mm (Burns *et al.*, 2010).

The interface between epidermis and dermis comprises basement membrane zone (BMZ), which consist of different types of extracellular matrix macromolecules which are mostly glycoproteins. Ultrastructurally, this zone has two distinct layers. The upper layer, electron lucent region, is called lamina lucida and lower layer, electron dense region, is lamina densa. Some types of attachment structures also associated with the BMZ. These structures form a contiguous network containing hemidesmosomes, anchoring fibrils and anchoring filaments. Basal keratinocytes and dermal fibroblasts are the cellular components of the BMZ, and type IV collagen and a number of collagenous glycoproteins are the major biochemical components of the it (Burns *et al.*, 2010; James *et al.*, 2011).

Dermis is a connective tissue which has mesodermal origin. It is mainly made up of collagen fibers and extracellular ground substance. The mesenchymal cells give rise to the fibroblasts and mast cells of the dermis. In the second month of fetus, the dermis and subcutis are not distinct from each other; however, by the end of the third month they are distinguishable by the collagen fibers which are present in the dermis stained deeply red (Burns *et al.*, 2010).

Dermis is supporting matrixes whose proteins and polysaccharides are linked and produce macromolecules that have the capacity for retaining water. Dermis gives structural integrity, pliability elasticity and tensile strength to the skin. It also has role in the thermal regulation and contains sensory and stimuli receptors (Wolff *et al.*, 2008; Burns *et al.*, 2010).

Dermis consists of extracellular matrix which includes various macromolecules. Proper quantity and appropriate interaction between these macromolecules are needed to maintain physiological homeostasis of this layer (Burns *et al.*, 2010).

Collagen

Collagen is the major component of the dermis which comprising 80-85% of the dry weight of it. This extracellular matrix protein serves as the main structural protein for the body. Twenty nine collagens have been identified in the vertebrates that twelve of them are expressed in the skin. Fibroblasts synthesize the collagen molecule. Triple helical collagen monomers polymerize into fibrils and fibers, and then stabilized by creating intra and intermolecular crosslink. Collagen fibers in the dermis are extremely tough and give tensile strength to the skin; which gives protective property to this organ. Collagen also is a stress-resistant material of the skin. Collagen has amino acids hydroxyproline, hydroxylysine and glycine. Presence of hydroxyproline and

hydroxylysine residues in the collagen is its characteristic feature. These two amino acids synthesized by hydroxylation of proline and lysine residues which catalyzed by enzymes in the rough endoplasmic reticulum. These enzymes used ascorbic acid, molecular oxygen and ferrous iron as cofactors. Hydroxylation of proline and hydroxylysine is essential for the stabilization of the triple-helical conformation and formation of stable covalent crosslinks, respectively. Thus, for example, low oxygen tension in wounds may impair collagen production and therefore resulting in inappropriate healing (Burns *et al.*, 2010; James *et al.*, 2011).

Fibrillar collagens are abundant group found in the skin. Type I collagen is the major component of dermis comprising about 80% of the total collagen. Collagen III accounts for approximately 10% of the total collagen in the dermis. Type I collagen associates with type III form bulk of extracellular fibers in the dermis (Burns *et al.*, 2010; James *et al.*, 2011).

Type IV collagen is the major component of the BMZ, which is a heterogeneous group of macromolecules includes six genetically distinct but structurally related α -chains that are present in diverse combinations of the basement membrane (Burns *et al.*, 2010; James *et al.*, 2011).

Type V collagens comprise less than 5% of the total collagen. It is located on the surface of collagen fibers in the dermis and regulates the lateral growth of these fibers.

Type VI collagen is a minor collagen in the dermis. It assembles into thin microfibrills and then this microfibrillar network stabilizes the assembly of collagen fibers by its anchoring function.

Collagen VII produces by keratinocytes and it is the major structural component of anchoring fibrils.

The proteolytic enzymes continuously degraded collagen fibers and then these degraded collagens have been replaced by newly synthesized fibers (Burns *et al.*, 2010; James *et al.*, 2011).

Elastic fiber

Elastic fibers account 2% to 4% of the extracellular matrix in the dermis, and they are synthesized by fibroblasts. Elastic fibers differ from collagens both structurally and chemically. They consist of two components, elastin and elastin associated microfibrils (Wolff *et al.*, 2008; Burns *et al.*, 2010; James *et al.*, 2011).

Elastin is synthesized as a precursor polypeptide called tropoelastin. Like collagen, in elastin approximately one-third of the total amino acid composition is glycine. It also contains valine, proline, lysine, alanine, desmosine and isodesmosine (Wolff *et al.*, 2008; Burns *et al.*, 2010; James *et al.*, 2011).

Elastic microfibrils are composed of several proteins including fibrillins, latent TGF-beta binding proteins (LTBPs) and fibulins which surround the elastin and associated with elastic fibers in the microfibrillar network, that can extended from the lamina densa of the BMZ throughout the dermis and into connective tissue of hypodermis (Wolff *et al.*, 2008; Burns *et al.*, 2010; James *et al.*, 2011).

Elastic fiber network has role in maintaining elasticity and provides resilience and elasticity to the skin (Wolff *et al.*, 2008; Burns *et al.*, 2010; James *et al.*, 2011). Some noncollagenous glycoproteins including fibronectins, fibulins and integrins also exist in the dermis which

facilitates cell adhesion and cell motility. Fibroblasts synthesize ground substance too. The ground substance is between the dermal collagen and elastic tissue (Burns *et al.*, 2010).

The laminin family

Laminins are other components of the cutaneous BMZ which are multidomain proteins. Approximately 16 different laminins have been identified that four of them are present in the skin. The members of the laminin family have important roles in the development and maintenance supramolecular organization of BMZ (Burns *et al.*, 2010).

Fibroblasts

Fibroblasts, macrophages and mast cells are other components of the dermis normally found surrounding vessels of the subpapillary plexus, around the papillary region and also between collagen fiber bundles.

The fibroblast is a mesenchymally derived cell and is responsible for synthesis and degradation of fibrous and nonfibrous connective tissue matrix proteins and some types of soluble factors. Fibroblasts are the most common cells of connective tissue which come in activated and inactivated form. The term fibroblast refers to an active cell, while the term fibrocyte refers to an inactive cell. Fibroblasts can be distinguished from fibrocytes by their abundance cytoplasm, well developed rough endoplasmic reticulum, ribosomes attached to the membrane surfaces and relatively larger size. Fibroblasts are essential for synthesizing collagen in the dermis. They also produce glycosaminoglycans, glycoprotein, polysaccharids for the ground substance and elastin.

Fibroblasts synthesize all of components of the extracellular matrix; therefore, the most important function of fibroblasts is maintaining the structural integrity of connective tissue by extracellular matrix. They also have critical role in wound healing. During the first days of wound healing, movement of fibroblasts through the ECM is important for wound contraction process. Fibroblasts are required for determining how well the wound will heal (Wolff *et al.*, 2008; Burns *et al.*, 2010).

Immunity

The monocytes, macrophages and dendritic cells constitute cutaneous immune surveillance in the dermis. This phagocytic system of cells restores tissue homeostasis by providing rapid and efficient immunological back up. This system is microbicidal, produces immunomodulatory molecules, processes and presents antigen to immunocompetent lymphoid cells and is involved in coagulation, tissue remodeling and wound healing (Wolff *et al.*, 2008).

Mast cells are secretory cells that reside in the papillary dermis near the BMZ, around blood vessels and nerves of subpapillary plexus, subcutaneous fat and also migrate through the basal lamina into the epidermis. Dermal mast cells are mononuclear or binuclear by a round or oval nucleus and their major feature is the presence of numerous cytoplasmic granules. Mast cells synthesize secretory granules of heparin, histamine, neutrophil chemotactic factor, eosinophilic chemotactic factor, tryptase, carboxypeptidase and chymase which are mediators in the type I hypersensitivity and production of subacute and chronic inflammatory disease in the skin. Activation of mast cells results in smooth muscle contraction, vascular permeability, fibroblast

deposition of collagen, induction of B cells, recruitment of neutrophils and eosinophils and promotion of T-cell (Wolff *et al.*, 2008; Burns *et al.*, 2010).

Stem cells

Stem cells reside in the epidermis, base of the sebaceous glands and the bulge area of hair follicles. These cells maintain skin homeostasis, hair regeneration and repair skin after injury by having the ability to self-renew and generating a proliferative progeny that go under differentiation (Burns *et al.*, 2010).

Vasculature

The dermal vasculature has role in providing nutrients and oxygen to the skin, regulating body temperature by controlling blood flow through the capillaries in the dermis, regulating blood pressure, wound repair and some immunologic events. There is a rich blood supply in the dermis; still, no blood vessels pass through the BMZ. The cutaneous vasculature is limited to the dermis, and epidermis has no vessels. (Burns *et al.*, 2010; James *et al.*, 2011).

Dermal lymphatics and nerves also associate with vascular network. The lymphatic network of the skin is important in transporting liquid materials and clearing the tissues of cells, bacteria, proteins and degraded substances. It is also regulates pressure of interstitial fluid (Wolff *et al.*, 2008; Burns *et al.*, 2010).

The neurovascular bundle in the dermis consists of nerve bundles that are found together with arterioles and venules. The ends of sensory nerves maybe free or may have expanded tips; some

of them also terminate in hair follicles. These sensory fibers function as receptors of pain, touch, temperature and mechanical stimuli. These receptors find more in hairless areas (Wolff *et al.*, 2008; Burns *et al.*, 2010).

Hypodermis

Fat is the major component of human body, 80% of it is distributed in subcutaneous region and others are around internal organs. Fat constitutes white and brown adipose tissue. In brown adipose tissue, heat production is increased and properties of mitochondria are different from white adipose tissue. Brown adipose tissue is more common in infant and children (Burns *et al.*, 2010).

Subcutaneous tissue (panniculus) lies beneath the dermis. In subcutaneous tissue cells with lipid droplets (Adipocytes) are located. Adipocytes organized into lobules which are separated from each other by septa. The septa is composed of fibrous connective, large blood vessels, nerves and lymphatics (Wolff *et al.*, 2008; James *et al.*, 2011). Although dermis and hypodermis are anatomically distinct from each other, they are structurally and functionally related to each other. They are integrated through vessels, nerves and epidermal appendages like hair follicle, eccrine and apocrien gland which may extend to the subcutaneous tissue (Wolff *et al.*, 2008). Like other layer of skin, hypodermis also varies in thickness in different part of the body (James *et al.*, 2011).

Fat storage and synthesis is a continuous process during lifelong. Increase in fat storage or fat production are the results of proliferation of existing adipocytes, developing new adipocyte cells from undifferentiated mesenchyme and accumulation of lipid within adipocytes (Wolff *et al.*, 2008). Fat function in subcutaneous tissue is providing buoyancy, insulation; allow mobility over underlying structure, protecting the skin, mechanical cushioning and energy storage (Wolff *et al.*, 2008; Burns *et al.*, 2010; James *et al.*, 2011). Fat also have endocrine function. Adipocytes regulate body weight by communicating with hypothalamus; they secrete leptin which regulate appetite by changing energy conversion in body (Burns *et al.*, 2010; James *et al.*, 2011). They also have signaling role in angiogenesis and osteogenesis. Multipotent stem cells are also found in fat tissues which are capable of giving rise to osteoblast, myoblast, adipocyte and chondroblast (Burns *et al.*, 2010).

Wound

Introduction

Wound is defined as a disruption or damage to normal body structure (Robson *et al.*, 2001). In skin it is characterized as any physical injury that creates an opening or break in the skin, and it could extend from epithelium to dermis, hypodermis and even deeper to tendon, muscle, nerve and bone (Alonso *et al.*, 1996; Kujath *et al.*, 2008). Skin is the largest organ in the body; it covers all over the body to protect it from environmental microorganism, dehydration and bleeding. Thus, any damage to the skin is dangerous for body (Burns *et al.*, 2010). In human and animals there is mechanism of wound healing to protect the skin integrity, in this mechanism reepithelialization occurs quickly over injured part and new matrix replace damaged dermis. In

adult, at the end of wound healing process, the skin does not retain its 100% normal structure and a scar remains on the skin surface (Burns *et al.*, 2010).

Wound term commonly is referred to acute wound. In acute wound, process of healing occurs quickly with few problem in most cases (Shai *et al.*, 2005; Burns *et al.*, 2010). In this form, wound undergoes complex interactive processes of different cell types and finally leads to complete mending (Schwartz *et al.*, 1999). Sometimes normal healing process does not occur and it fails during certain periods, which cause development of chronic wound. There is no clear definition for chronic wound; however, when wound does not heal during three to four months and trapped into constant inflammatory state, it could be named as chronic wound, although time estimation for healing depends on factors such as size of the wound, patient clinical situation, presence of microorganisms, blood supply, systemic diseases and drugs and nutrition that patients take (Lazarus *et al.*, 1994; Shai *et al.*, 2005; Burns *et al.*, 2010). In dermatology, chronic wound is called chronic cutaneous ulcer. Ulcer is a kind of lesion that epidermis and sometimes part of the dermis and even subcutaneous fat have been destroyed. The cutaneous ulcer does not appear *de novo* on the skin, it evolve from another initial pathologic lesion (Shai *et al.*, 2005). The most common form of chronic wounds is pressure sores, diabetic ulcers and venous ulcers. Factors include high blood pressure in diabetic ulcer and venous insufficiencies in venous ulcers develop a condition which wound healing process is impaired (Burns *et al.*, 2010).

General classification of cutaneous ulcer

Classification of skin ulcer is difficult for dermatologists. Accepted classification of skin ulcers is based on clinical appearance of them. In this classification, ulcers are categorized to yellow,

black and red one. The therapeutic methods mostly depend on these categories. In secreting yellow ulcer, presence of purulent secretions develops yellow appearance. Purulent secretion may be thick or thin. Black ulcer is a dry wound that is covered by black necrotic material. In addition to these three categories, sloughy ulcer should be included to the classification to make it complete. Sloughy ulcers' surface covered by soft material (ranging from liquid to semi solid) which is composed of devitalized fibrin and collagen and necrotic protein. These materials give yellow, green or gray color to the wound surface. Ideal ulcer or clean red ulcer is the one with best chance of healing. Therefore, dermatologists work other three mentioned types of sores to convert them to clean red ulcer, which bed is covered by granulation tissue that works as a substrate until all over the lesion bed covered by epithelial cells (Shai *et al.*, 2005).

Ulcers are also classified based on their depth and number of skin layers that are involved (Bolton *et al.*, 1991; Krasner *et al.*, 1993; Shai *et al.*, 2005). Injuries that affect just epidermis is superficial wound, if it extends to both epidermis and dermal layer (glands, blood vessels, sweat and hair follicle), it is called partial thickness, and the wounds which involves subcutaneous fat and underlying tissues are referred to full thickness ulcer (Boateng *et al.*, 2008). These classification help dermatologists and experts to use proper therapeutic agent for their treatment.

Process and phases of wound healing

Wounding and wound healing occurs in all tissues of the body. Even though all injured tissues undergo similar processes of repair, each of them has its own distinctive properties. Besides, regeneration period varies by tissue type. Some wounds may heal over a few days; while, others may need a year or more to regenerate completely (Flanagan, 2000; Komarcevic, 2000;

Richardson, 2004). In the skin, wounds heal by fibrosis and scar formation. There are different types of wound healing which includes:

- Primary healing:

This type of healing occurs when a wound is closed within 12-24 hours by using tissue glue, tapes, suture or other chemical material. Wounds that mend by this type of healing are created by incision which only few epithelial and underlying tissue cells are disrupted(Enoch *et al.*, 2007).

- Delayed primary healing:

In this type of healing, wound have been left open to prevent infection by host defense which use phagocytic and inflammatory cells to destroy bacteria and then it close after a few days(Enoch *et al.*, 2007).

- Secondary healing:

In wounds with loss soft tissue, this type of healing occurs. Inasmuch as epithelial cells alone cannot heal the wound completely, these wounds require ingrowths of granulation tissue and accumulation of extracellular matrix and then wound contraction and epithelialization. Myofibroblasts are important in this type of healing(Enoch *et al.*, 2007).

- Healing of superficial wounds:

In superficial wound only epithelium and sometimes superficial part of dermis are injured. Healing occurs by epithelialization that epithelial cells in hair follicles, sebaceous glands and dermal appendages replicate and cover exposed dermis (Enoch *et al.*, 2007).

Skin wound healing is a dynamic and complex process of cellular, humoral and molecular mechanisms which begins immediately after injury. Timing and interactions between the components in the wound healing process for acute and chronic wounds are different; however, the main phases are similar. Acute wounds normally heal through four distinct but overlapping phases: 1) Coagulation and hemostasis 2) Inflammation 3) Proliferation 4) Wound remodeling and scar formation (Degreef, 1998; Hunt *et al.*, 2000; Vanwijck, 2001; Attinger *et al.*, 2006; Broughton *et al.*, 2006b).

Coagulation and hemostasis

Coagulation and hemostasis are the initiating steps in the healing process which occur immediately after injury and will be completed after some hours. Coagulation cascade resulted in vasoconstriction, platelet aggregation and fibrin deposition. Fibrin mesh and aggregated platelets produced clot which is the end product of the hemostatic process (Lawrence, 1998b; Strodtbeck, 2001). The aim of this phase is preventing from exsanguinations, fluid and electrolyte loss and limiting contamination from outside. Providing a provisional matrix for invading cells which play role in the subsequent phases of healing is another aim of this stage (Pool, 1977; Jespersen, 1988; Lawrence, 1998b; Robson *et al.*, 2001).

The coagulation cascade is activated through extrinsic and intrinsic pathways. After wound formation, the injured blood vessels undergo vasoconstriction by the release of vasoactive amines triggered by platelets. Intrinsic coagulation cascade, which is activated by endothelial cells and adjacent platelets, initiates when blood is exposed to foreign surface. In extrinsic system different clotting cascades are then initiated by clotting factors from the injured skin. Platelets and blood components come in contact with exposed collagen and other extracellular matrix components which induce thrombin (a clotting enzyme) production that stimulates

vascular permeability and facilitate migration of inflammatory cells. Thrombin catalyzes the conversion of fibrinogen to fibrin (Stiernberg *et al.*, 1993). Fibrin is a key component of the provisional matrix and also is important for forming meshwork that stabilizes the platelet plug. Fibronectin is the other key component of the provisional matrix which is essential for cellular migration along the matrix and also trapping circulating cytokines for use in other stages of healing (Kurkinen *et al.*, 1980; Grinnell, 1984; Hynes, 1986).

Aggregation and attachment of platelets to exposed collagen surfaces activate them, which enables platelets to degranulate and release chemotactic and growth factors to attract inflammatory cells to the area, leading to the next phase in the healing process. Platelets contain α -granules in their cytoplasm. These α -granules are filled with cytokines and growth factors that initiate the inflammatory response. These factors are platelet derived growth factor (PDGF), transforming growth factor α and β (TGF- α and TGF- β), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), endothelial cell growth factor. PDGF and TGF- β are most important signals (Kim *et al.*, 1998; Lawrence, 1998b). The PDGF stimulates mitogenesis of smooth muscle cells and fibroblasts. It also initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts. TGF- β initiates the chemotaxis of fibroblasts and smooth muscle cells, and modulates collagen and collagenase expression. In addition, TGF- β is important for attracting macrophages and stimulates them to secrete further cytokines (Hart, 2002; Sylvia, 2003).

Inflammation

The inflammatory phase is activated during the late phase of coagulation and can be characterized by pain, heat, edema and erythema (Diegelmann *et al.*, 1981; Cohen *et al.*, 1999).

Mast cells have a role in representing the signs of inflammation by releasing granules filled with enzymes, histamine and other active amines (Artuc *et al.*, 1999). Based on the type of inflammatory cell involved and the duration of response, this phase can be divided into an early phase with neutrophil activity and a late phase with macrophage activity. The aim of this phase is creating an immune barrier against invading microorganisms by bringing inflammatory cells to the injured area(Hart, 2002).

The early inflammatory response activates the complement cascade and molecular events that lead to infiltration of the wound site by neutrophils (Broughton *et al.*, 2006a). The neutrophils attracted to the wound site within 24-48 hours of injury by chemoattractant agents including complement components (C3a and C5a), transforming growth factor β (TGF- β) and formylmethionyl peptides produced by bacteria. The chemotactic function of C3a and C5a is dependent on the release of TGF- β and formylmethionyl peptides (Wahl *et al.*, 1987; Glat *et al.*, 1997; Robson *et al.*, 2001; Enoch *et al.*, 2007). Neutrophils adhere to the endothelial cells in the capillaries in the wounded area through alterations in the regulation of intracellular adhesion molecules on the endothelial cell membranes that interact with integrins expressed on the surface of neutrophils, this process is called margination (Hynes, 1987; Lawrence, 1998b; Hart, 2002). Neutrophils moved forward by the blood flow and then in a process known as diapedesis they migrate through the vessel wall (Enoch *et al.*, 2007). Neutrophils phagocytose bacteria and other invading cells and destroy them by oxygen-derived free radicals and proteolytic enzymes (Skover, 1991; Flanagan, 2000; Richardson, 2004; Broughton *et al.*, 2006b).

Approximately 48-72 hours after injury, monocytes are attracted to the wound site by chemoattractive agents including complement components, clotting factors, breakdown products of elastin, collagen and cytokines. Monocytes undergo a phenotypic change and become

macrophages that have a longer lifespan than neutrophils (Hunt, 1988; Pierce *et al.*, 1991; Samuels *et al.*, 1999). Macrophages are key regulatory cells in the repair process. They perform phagocytosis of foreign debris, nonfunctional host cells, bacteria-filled neutrophils and damaged matrix. They release growth factors particularly platelet-derived growth factor (PDGF) and TGF- β that attracts fibroblasts and smooth muscle cells. The cytokines that produced by macrophages have a role in angiogenesis, collagen production and fibroblast migration. Macrophages also support cell proliferation and tissue restoration including synthesis of extracellular matrix (ECM)(DiPietro *et al.*, 1993).

Lymphocytes are attracted 72 hours after injury to the wound site by complement components, immunoglobulin G (IgG) breakdown products and interleukin-1 (IL-1) (Hunt *et al.*, 2000; Hart, 2002; Broughton *et al.*, 2006b).

Between the inflammatory cells, neutrophils are the first of them that become apoptotic and then phagocytosed by macrophages. Macrophages and lymphocytes remain approximately seven days in the wound area (Newman *et al.*, 1982; Cohen *et al.*, 1993).

Proliferative phase

The proliferative phase initiates on the third day after injury. Fibroblast migration, deposition of the ECM, restoring the vascular network and formation of granulation tissue are the main characteristics of this phase (Hunt, 1988; Arnold *et al.*, 1991; Glat *et al.*, 1997; Endrich *et al.*, 2000; Robson *et al.*, 2001; Strodbeck, 2001; Diegelmann *et al.*, 2004; Bauer *et al.*, 2005). Cytokines have important role in this stage by contributing to fibroplasia, epithelialization and angiogenesis (Fauci, 1996).

Fibroblast migration and collagen synthesis:

Following injury, native cells are damaged or lost in the wound area; therefore, fibroblasts are being attracted from the adjacent tissues to this site by factors including PDGF and TGF- β (Goldman, 2004). Cytokines may also influence on undifferentiated cells near the wound and transform them to fibroblasts. Fibroblasts appear in the wound on 2-4 days after injury. The proliferation of fibroblasts in the wound area is regulated by cytokines especially PDGF and TGF- β (Seppa *et al.*, 1982). After proliferation, fibroblasts produce matrix proteins including fibronectin, hyaluronan, proteoglycans and type 1 and type 3 procollagens (Witte *et al.*, 1997; Robson *et al.*, 2001; Ramasastry, 2005). These proteins further support cell migration and help to construct the new extracellular matrix. Collagens provide strength and integrity to tissues and have an important role in wound repair, especially in the proliferative and remodeling phases (Clark, 1993; Greenhalgh, 1998; Baum *et al.*, 2005). Collagen makes up 50% of protein in scar tissue. Wound granulation tissue that forms after injury expresses 30-40% type III collagen (Robson *et al.*, 2001). Fibroblasts turn into myofibroblasts that contain thick actin bundles and attach to fibronectin and collagen (Goldman, 2004; Ramasastry, 2005). Myofibroblasts appear at days 4-6 after injury and would be seen two to three weeks. These cells contract the edge of the wound. Wound contraction begins 4 to 5 days after injury and continues for two weeks. The rate of contraction is approximately 0.6 to 0.7 mm per day, although it varies between different locations. The shape of the wound also affects the rate of contraction; circular wounds contracting more slowly than square wounds (Gabbiani *et al.*, 1971).

Angiogenesis and Neovascularization:

There is an increasing demand for oxygen and nutrients at the injury site because of high metabolic activity which exists there. There is no vascular supply in the wound center. Factors in

the wound environment including decreased oxygen tension, low pH and high lactate levels initiate the release of factors that are needed for bringing new blood supply which is called angiogenesis (Knighton *et al.*, 1983; LaVan *et al.*, 1990).

Neovascularization is the de novo formation of new blood vessels; in contrast, in angiogenesis new capillaries are formed from preexisting blood vessels.

The angiogenesis process initiates from day 2 after injury and it continues during all phases of repairing. This process is stimulated by PDGF, TGF- β , TGF- α , angiogenin, vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Joseph-Silverstein *et al.*, 1987; Risau, 1990; Ribatti *et al.*, 1991; Battegay, 1995; Zhang *et al.*, 1997; Tonnesen *et al.*, 2000; Oike *et al.*, 2004). These growth factors bind to their receptors on the endothelial cells of existing vessels and then activate intravascular signaling cascades. In a process called “sprouting”, the activated endothelial cells secrete proteolytic enzymes that dissolve basal lamina, which help them proliferate and migrate into the wound area (Sorg *et al.*, 2007). The endothelial sprouts from the surrounding capillaries at the margins grow through migration and proliferation. The endothelial sprout comes in contact with a sprout from a different capillary, and then they interconnect and produce a new capillary. A microvascular network which is composed of many new capillaries would be formed within a few days. Then the new vessels differentiate into arteries and venules (Folkman *et al.*, 1987).

Epithelialization:

Reconstruction of epithelium is important for reestablishing barrier functions of the skin. Epithelial cells migrate from wound edges a few hours after wounding. Local keratinocytes at the wound margins and epithelial stem cells from hair follicles and sweat glands also contribute

to epithelialization process (Martin, 1997; Miller *et al.*, 1998; Roh *et al.*, 2006; Lau *et al.*, 2009). Epithelial cells migrate until they overlap with other epithelial cells moving from different directions. In this process, contact inhibition mechanism prevents further migration, which leads to formation of basement membrane (Niall *et al.*, 1982; Nanney *et al.*, 1984). This migration process is under control of various cytokines such as TGF- α , EGF, platelet derived EGF and keratinocyte growth factor (KGF) (Werner *et al.*, 1992; Werner *et al.*, 2003).

Granulation tissue formation:

Formation of the granulation tissue is the last step in the proliferation phase which is accompanied by remodeling stage. It is characterized by proliferation of fibroblasts, macrophages, granulocytes, loosely organized collagen bundles and loops of capillaries. It has a pink, soft and granular gross appearance. Granulation tissue is highly vascular. Owing to the fact that angiogenesis does not completely finish in this step, this tissue has red appearance and could be traumatized easily. Fibroblasts are dominant cells in this phase that do many functions such as production of ECM and collagen. ECM provides a scaffold for cell adhesion and regulates the growth, differentiation and movement of the cells within it (Enoch *et al.*, 2007; Eckes *et al.*, 2010; Barker, 2011). By accumulation of collagen in the granulation tissue, the density of blood vessels diminishes and the granulation tissue gradually matures to produce a scar (Enoch *et al.*, 2007).

Remodeling phase

Remodeling is the final phase of wound healing which initiates contemporarily with the development of granulation tissue and continues one or two years after wound formation. Remodeling phase equilibrates to a steady state about 21 days after injury. In this phase, regulatory mechanisms make balance between degradation and synthesis, which leads to normal

healing (Greenhalgh, 1998; Monaco *et al.*, 2003; Enoch *et al.*, 2007). The main feature of this stage is collagen degradation that achieved by specific metalloproteinases (MMPs) produced by neutrophils, macrophages and fibroblasts. The activity of MMPs should be regulated as long as they have the potential to degrade essential collagens and different extracellular matrixes (Madden *et al.*, 1968; Assoian *et al.*, 1986; Parks, 1999). Therefore, the synthesis and secretion of those enzymes are controlled by cytokines, growth factors and phagocytic stimuli (Monaco *et al.*, 2003; Enoch *et al.*, 2007). In this phase, collagen III is replaced by the stronger collagen (Gurtner *et al.*, 2000). In addition, density of fibroblasts and macrophages are reduced and also a reduction occurs in the angiogenic process and wound blood flow declines. The metabolic activity of the wound is diminished and finally stopped (Robson *et al.*, 2001). Moreover, myofibroblasts reduce the surface of the developing scar by their multiple attachments to collagen, which leads to wound contraction (Gurtner *et al.*, 2000; Profyris *et al.*, 2012; Tziotzios *et al.*, 2012). Granulation tissue formation stops through apoptosis. As scar matures, fine collagen fibers replace by thicker fibers, and fibronectin and hyaluronan are broken down. However, these collagen fibers never regain the strength of intact skin. The remodeling phase is important for creating a strong wound. Wound strength will be 3% of normal dermis one week after injury. After three weeks, by predominating of remodeling phase, wound will gain approximately 20% of normal dermis strength. It can achieve to a maximum of 80% strength of normal skin (Monaco *et al.*, 2003; Enoch *et al.*, 2007).

Infection

Various type and amount of microorganism are present in different area of body. Just presence of microorganisms does not indicate infection (Grice *et al.*, 2009; Grice *et al.*, 2011). A healthy

immune system defends the body against microorganisms like bacteria; however, if large amount of virulent bacteria is present or immune system becomes weak, bacteria can cause infection (Zulkowski, 2013).

Many processes can delay wound healing which cause chronic wound appearance. One of these factors is infection. All chronic wounds are colonized by bacteria. Although small amount of bacteria does not interfere healing process, their large amounts cause impairment in repairing (Edwards *et al.*, 2004).

Interaction of wound and bacteria ranges from contamination, colonization, local infection (critical colonization) and invasive infection which may cause cellulitis or septicaemia (Gardner *et al.*, 2001; Edwards *et al.*, 2004; Cutting *et al.*, 2005). In contamination situation, microorganisms are present on wound; however, they do not replicate. In colonization, microorganisms replicate; still, they do not cause host injury and any delay in wound healing; besides, their multiplication is without host reaction (Cutting, 2003). There is an intermediate stage between colonization and invasive infection named critical colonization. In this phase, wound granulation tissue is locally infected and wound has unhealthy appearance. In this situation, although high level of bacteria impairs healing, traditional clinical signs of infection like redness, heat and swelling do not appear, (Kingsley, 2001; Edwards *et al.*, 2004; Warriner *et al.*, 2005). However, absence of any reduction in wound size represents the delay in restoration of tissue (Sibbald *et al.*, 2006). Ultimately, bacteria can cause invasive infection defined as replication microorganism within the wound that induces host reaction and causes host injury (Dow *et al.*, 1999; Cutting, 2003). Generally, when bacteria numbers exceed 10^5 per gram of

tissue in open wound, infection occurs. Progression of critical colonization to the infection is not just determined by bacteria number. Factors such as virulence and pathogenicity of microorganism, immune response of host, microorganism interaction with surrounding microflora, bacteria species, number of different species (four or more species is related to nonhealing wound), and synergistic interaction of different microorganism species affect it. Furthermore, in some situations, bacteria in chronic wound live within biofilm which protect them from host defense and antibiotics (Trengove *et al.*, 1996; Bowler, 2003; E *et al.*, 2003; Shai *et al.*, 2005; Stashak, 2008).

Biofilm

In complete nutritional abundance and no physical or chemical stress, bacteria will live as free floating planktonic form. They will reversibly attach to the surface or adhere to each other and interact weakly via their component. In this situation they will proliferate at high rate in planktonic form. In contrast, under nutritional and environmental stress, planktonic bacteria aggregations convert to biofilm phenotype and begin extracellular matrix secretion to produce bacterial microcolonies (Costerton *et al.*, 2003; Hoffman *et al.*, 2005; E *et al.*, 2007). Chronic wound prepares suitable area for biofilm formation. Biofilm are found in 70 to 80% of chronic wounds. Collagen and fibronectin proteins of wound and damaged tissue prepare appropriate surface for biofilm attachment (James *et al.*, 2008).

Biofilm is composed of a community of aggregated bacteria in extracellular polysaccharide matrix (ESP). ESP is a bacteria self secretion. In mature biofilm, some complex structures are developed; for instance, water channel are developed to transfer nutrient and waste product (de

Beer *et al.*, 1994; Fuqua *et al.*, 1994). Bacteria also transfer intercellular signaling molecules to control growth pattern of other bacterial cells. These signaling molecules cause changes in gene expression of bacteria for environmental adoption. For example, in low nutrition and oxygen situation, this signaling causes slower metabolic rate or in more strict condition bacteria stop growing and become quiescent. Reduction in metabolic activity in quiescent bacteria and polysaccharide shield of biofilm increase antibiotic resistance. Polysaccharide shield and other products of bacteria also avoid phagocyte penetration into biofilm and protect bacteria from body immune system (Fuqua *et al.*, 1994; Ceri *et al.*, 1996; Nikolaev *et al.*, 2007; Xavier *et al.*, 2007).

Effects of bacteria on wound

In infection, bacteria communicate with each other. They use signaling molecules to coordinate their activity to increase their virulence capacity. They also express other virulence factor such as toxins and enzymes to manipulate their environment for higher multiplication. (Cutting, 2003; Stashak, 2008). In this situation, bacteria adhere to extracellular matrix and make their proteins unavailable for promoting tissue adherence. Bacteria also produce cytotoxic exotoxins which attack many types of cells and cause tissue damage and necrosis. Endotoxins, another production of bacteria, cause thrombosis in microvasculature. Thrombosis and vasoconstrictive metabolites of bacteria causes hypoxemia which increases tissue damage and bacteria proliferation. Endotoxin also causes immune dysfunction. They induce macrophage to release more inflammatory mediator such as interleukin-1 (IL-1) cytokines and tumor necrosis factor to prolong inflammatory phase of healing. Endotoxin also decreases growth factor production and collagen synthesis and cross linking. Besides, they produce proteolytic enzymes that digest collagens and reduce the number and proliferation of fibroblasts. Therefore, bacteria's

metabolites reduce tissue tensile strength by collagen degradation. They also cause exuberant granulation tissue formation leading to excessive scar production. Bacterial metabolites also inhibit epithelium migration. In addition, they mechanically separate wound edge from exudates accumulation. Bacteria with thick capsule are also resistant to phagocytic cells (C *et al.*, 2001; Konturek *et al.*, 2001; Stashak, 2008).

Number of organism and their virulence are two factors that determine severity of damage in infection. However, the most significant element is host resistance; in other words, its ability to prevent bacterial invasion and damage to the body. Some systemic and local elements can have adverse effect on host resistance. Systemic factors could be inadequate blood supply causing bacteria proliferation and tissue damage, poor nutrition, smoking, edema, excess alcohol, drugs that impair immunity, age, immunosuppression, or disease like diabetes mellitus and local ischemia. In addition, local factors include large wound size and presence of foreign body like gauze or suture have adverse impact on it (Krasner *et al.*, 2001; Burns *et al.*, 2010)

Clinical signs of infection

In infectious wound, first gram positive bacteria invade the wound and then gram negative and anaerobic bacteria species appear (F *et al.*, 1994; Gardner *et al.*, 2001; Schultz *et al.*, 2003). The first sign of infection in chronic wound is failure in decreasing wound size (Burns *et al.*, 2010). As infection progresses the amount of exudates will increase and a foul odor may accompany the exudates. The odor is the result of gram negative and anaerobe bacteria efforts to breakdown the tissues. Yellow or brown slough is another sign of infection, which shows cell death and tissue necrosis in wound. Exuberant friable granulation tissue in the wound bed with poor collagen

matrix quality and poor reepithelialization are another signs of infection (F *et al.*, 1994; Gardner *et al.*, 2001; Schultz *et al.*, 2003). Deeper infection and prolong inflammation also cause redness and heat at wound edge, swelling, pyrexia, excessive purulent, pain and wound breakdown (Gardner *et al.*, 2001).

Wound healing agents

Choosing appropriate dressing material for wound is a challenging issue for physicians. Different factors and wound characteristics affect on decision making for wound management. Wound etiology, patient medical condition, amount of blood flow to the wound area, presence of infection, wound bed, depth of damage, history of wound and place and anatomy of wound are some factors which should be known for optimal wound healing (Hunt *et al.*, 2000; Attinger *et al.*, 2001; Dissemond *et al.*, 2004; Sarabahi, 2012). These factors give efficient information about wound healing deficiency. As an illustration, poor nutrition and old age cause less fight against infection. Vitamin, mineral and protein deficiency also disturb collagen synthesis and inflammatory phase; in addition, disease like anemia and diabetes delay wound healing due to inappropriate blood circulation causing inadequate oxygen, nutrient and blood cells delivery. Thus, each wound type needs exclusive wound dressings in order to achieve rapid healing with minimum trouble for patient and low cost (Hemila *et al.*, 1999; Rojas *et al.*, 1999; Falanga, 2005; Patel, 2005; Lee *et al.*, 2006; Boateng *et al.*, 2008).

The aim of choosing different dressing for different wound types is to maximize rate of healing. Sometimes combination of different dressing increases their efficiency. Although, each dressing may have specific mechanism to facilitate repairing, they should be designed to have following

functions. Debridement meaning removal of nonviable and infected tissue is one of these tasks. As foreign bodies in necrotic tissue prolong inflammatory phase and prepare a place for bacteria growth, debridement accelerates healing. Moreover, preparing moist environment for wound is another function of dressings. Moisture prevents desiccation, cell death and also induces angiogenesis, epidermal migration and connective tissue synthesis. In addition of moisture maintenance, desirable dressings also control air and water vapor exchange. Low oxygen induces angiogenesis and high oxygen induces epithelialization and fibroblasts migration. They also control water vapor to manage wound exudates. Another function of dressing is removal of excess exudates and blood. When excessive amount of exudates aggregates in wound area, degrading enzymes block cell proliferation and degrade extracellular matrix and growth factor. Dressing should also provide thermal insulation since normal temperature of tissue induces blood flow which increase epidermal migration. Preventing bacterial infection is another duty of dressing agents. Bacteria prolong inflammatory phase, reduce collagen synthesis and epidermal migration and some other type of damages. Therefore, protecting wound from infection will increase rate of healing. Dressing should also be low adherent, because there is some problem like pain and dressing removal difficulty that would cause more damage to the wound (Eccleston, 2007; Boateng *et al.*, 2008).

Dressing could be classified in different ways. It can be classified as passive and interactive dressing. Passive dressings such as gauze have no direct effect on wound healing and just protect it from outside. In contrast, interactive dressings interact with wound to provide optimum environment for healing (Goodman *et al.*, 1956; Sarabahi, 2012). Dressing could also be classified based on their function as debridement, antibacterial, occlusive, adherent, absorbent

and so on. They also are classified based on type of material used to provide dressing such as hydrocolloid, collagen, and alginate. They also classified based on their physical form; for example, gel, foam, cream, film and ointment (Purna *et al.*, 2000; Queen *et al.*, 2004; Falabella, 2006). Classification of dressing as traditional and modern is usual. Traditional pharmaceutical formulations are liquid as solution, emulsion and suspension or semi solid like cream and ointment used extensively. Traditional dressing including gauze, cotton wool and synthetic bandage which are dry and do not provide moist environment for wound and mostly used as second dressing. Modern dressing distinguished characteristic is providing moist environment to accelerate wound healing and they are mainly classified based on the material they are made of. As a point in case, hydrocolloids, hydrogel, alginate which are produced in the form of gel, thin film and foam sheet (Boateng *et al.*, 2008). Another form of dressing is biological dressing. They play active role in wound healing process. They include tissue engineered products that could be combined with polymers like collagen which attracts macrophages and fibroblasts and also provides scaffold for tissue growth. Biologically active material and skin substitute are used as dressings to protect wound from infection, fluid loss and some of them are used as wound implant to replace extracellular matrix molecules or deliver growth factor to prepare bed of wound for permanent skin coverage (Barlett, 1981; Mian *et al.*, 1992; JAM *et al.*, 1995; Sarabahi, 2012). Some dressings are medicated to deliver drugs. They could be topical pharmaceutical agents in the form of ointment, cream and solution or they could be new generation of medicated dressings in form of hydrogel, alginate, hydrocolloid, foam, film and silicone gel. Their incorporated drugs may have direct or indirect role in wound healing. They may act as debridement, antibacterial and growth factor for tissue regeneration (Boateng *et al.*, 2008). Antibacterial agents (antiseptics and antibiotics) are used as first line of treatment for

colonized or infected wounds, they are also used contaminated wound and bacteria biofilm (Lawrence, 1998a; Schultz *et al.*, 2003; White *et al.*, 2006). While antibacterial agents treat infection, they necessarily do not take part in healing process physiologically. In contrast, growth factors work directly on healing process. Growth factors accelerate healing by inducing angiogenesis, cell proliferation, fibroblast activity production and degradation of extracellular matrix and cell inflammation. That is, they affect different phase of healing such as proliferation, migration and inflammatory phases. Growth factors are designed to target the special phase of healing, and different dressings administer some kind of growth factors on wound site (Chu *et al.*, 2006).

Another group of healing compounds are minerals and supplements such as vitamin A, C, E, zinc and copper. Topical use of zinc as ointment and liquid emulsion has been used as pharmaceutical formulation for wound healing. Dressings like ointment, cream, silicone gel, gauze, and liquid emulsion are also used to deliver vitamins to the wound site; nonetheless, they are mostly administered orally to patients. Vitamin A involves in synthesis of collagen and epithelial cell differentiation. Vitamin C improves immune function against infection and also involves in collagen synthesis and organic compounds of intracellular matrix of tissues such as skin and other connective tissues. Vitamin E induces angiogenesis, reduce scarring and also work as antioxidant and antiinflammatory compound (Delafuente *et al.*, 1986; Eaglstein *et al.*, 1988; Linda, 1991; Wallace, 1994; Osieki, 1995; G *et al.*, 1996; Ayello *et al.*, 1999; Porto da Rocha *et al.*, 2002).

Some natural products are also used in various part of the world for dressing wound. For example, honey, yoghurt, potato peeling, tea tree oil and so on. still, there is not enough evidence for some of them to approve their effects(Enoch *et al.*, 2006).

Antiseptics cytotoxicity

For good caring of chronic wound, topical prevention and management of bacterial contamination is necessary since infection has deleterious effects on wound healing. Ideal topical therapies contain removal of debris and reduction of bacterial contamination without adverse effect on tissues of wound and areas around it (Cooper *et al.*, 1991; Doughty, 1994; Maklebust, 1996; Robson *et al.*, 2001). Application of antiseptics on open wound is controversial. Both bactericidal activity and cytotoxicity of the antiseptics must be considered to choose appropriate antiseptics (Lineaweaver *et al.*, 1985; Doughty, 1994).

As mentioned the ultimate goal of wound management is to remove bacteria and develop healthy granulation tissue. In some situations debridement could alone remove bacteria and necrotic tissue of wound. However, debridement is not always enough and other factors, topical antiseptics and antibiotics, are needed to reduce bacterial load of wound (S *et al.*, 2003; Howell-Jones *et al.*, 2005; Thomas *et al.*, 2009). Antibiotics and antiseptics have different mode of actions, each class of antibiotics act against bacteria by unique mechanisms. Therefore, in most cases, their specificity limits their activity against bacteria and causes development of defense mechanism in bacteria (antibiotic-resistant bacteria). In contrast, antiseptics act nonspecifically and their toxicity is not selective and acts on any living tissue. Therefore, although antiseptics are less allergic and have lower resistant rate than antibiotics, their damage is not specific to pathogens but the host's cell as well (Shai *et al.*, 2005). Main antiseptics are oxidizing agents

like hydrogen peroxide and potassium permanganate, chlorine compounds like Eusol and Dakin's solution, iodine compounds like povidone iodine, silver compounds like silver nitrate and so on. Toxicity of each antiseptic depends on its compounds, concentration and ingredients in the preparation. Toxic antiseptics could damage tissue around wound bed and subsequently delay wound healing process. The damage potential of antiseptics was examined on animal model in past and now more accurate examination are done on fibroblasts and keratinocytes' cultures (Shai *et al.*, 2005). If wound healing process is divided to three phases, inflammatory phase is the first one that contains hemostasis to prevent blood loss and releasing growth factors, and causes inflammation to bring phagocyte cells and macrophages to break down necrotic tissue and invade bacteria. Therefore, infection and necrosis prolong this phase. Impact of antiseptics on reducing bacteria and necrotic tissue is beneficial for passing from inflammatory phase. However, their toxic effect on main cells of this stage, macrophages and leukocytes, should also be considered. Second phase, proliferating, contains granulation tissue formation, contraction and epithelialization, fibroblasts are main cells for connective tissue formation and endothelial cells and keratinocytes orderly act to develop angiogenesis and epithelialization; therefore, toxicity of antiseptics may have adverse effects on those mentioned cells. Last phase, maturation, contain remodeling of scar through collagen synthesis and break down, in this phase wound is closed and topical antiseptics are not needed (Shai *et al.*, 2005).

In conclusion, effective antiseptic agent is needed to clean the wound and remove bacteria contaminants without toxicity and damaging healthy tissue (Martí'nez *et al.*, 2007).

Superoxidized Solution

Biocides are essential agents for preventing infection and drug-resistant bacteria in hospital or other health care system. As biocides use multiple sites of microorganisms as target, the risk of resistant development decreases in them compare to antibiotics, which usually have single target site. Therefore, biocides are highly effective as antimicrobial agents (Denyer, 1995; McDonnell *et al.*, 1999; Thorn *et al.*, 2012).

Electrolysis for disinfection has been used for one hundred years; however, the physiochemical properties of the electrochemically activated solutions (ECAS) were not examined until 1970 at Russian institute for medical engineering. It has been found that ECAS has biocide applications in different fields like potable water disinfection and food industry. Their high activity, ease of production and cheapness of their raw materials made the ECAS a functional application in clinical practices (Barratt *et al.*, 1990; Denyer, 1995; Prilutsky *et al.*, 1997; McDonnell *et al.*, 1999; Huang *et al.*, 2008; Kraft, 2008; Thorn *et al.*, 2012).

Superoxidized solution (SOS), which is made by electrolysis of sodium chloride, is a nontoxic disinfectant used to eradicate microorganisms such as bacteria, fungi, viruses and spores. As SOS is environmentally safe, there is no more need for costly disposal procedures. It has application in wound care, medical device sterilization, food sterilization, household, hospital and antibioterrorism (Alimi, 2005).

A broad application of SOS is in wound treatment. Different factors such as inadequate oxygenation, tissue necrosis and local infection have deleterious effects on wound healing. To prevent these factors an ideal antiseptic effect with high lethal potential to all forms of bacteria

and their spores and capable of keeping its antibacterial activity for long period of time with no harmful effect on wound healing tissue is needed. SOS may represent alternative to the conventional antiseptics for skin and wound. It is shown that SOS is safe and efficient that could lubricate, debride, moisten and reduce microbial load of various wound type (Tanaka *et al.*, 1996; Sekiya *et al.*, 1997; Kapur *et al.*, 2011). SOS is also well tolerated, improves granulation of wound tissue, and reduces the need of debridement and less pain reported during the treatment (Najafi *et al.*, 2008). SOS covers the broad-spectrum of microorganism and does not seem to damage host tissue or facilitate bacterial resistance. Besides, it might be active in the presence of biofilm (Gonzalez-Espinosa *et al.*, 2007).

The SOS has minimal impact on the environment due to the absence of hazardous chemicals in its product. It has been discovered that SOS accelerates wound healing; hence, it is good for skin dressing sterilization (Mori *et al.*, 1997; Yahagi *et al.*, 2000; Huang *et al.*, 2008; Ge *et al.*, 2012).

Preparation of SOS

Superoxidized solution (SOS), which is a physiologically-balanced and ionized solution, is produced by an oxidation-reduction process called electrolytic or redox reaction (Najafi *et al.*, 2008). In electrolysis, electric current passed through water or a solution and based on the solution and materials of the electrode, different chemical species and ions will be generated (Gutierrez, 2006). By electrolysis of water, hydrogen ions are reduced and provide hydrogen gas, and the hydroxide ions are oxidized and produce oxygen gas (Najafi *et al.*, 2008). The SOS is produced from the electrolysis of a solution which is a mixture of inorganic salts in physiologically-balanced proportions. The solution of inorganic salts and minerals is used

because it mimics the electrolyte concentration and mixture of body fluid in an isotonic state (Najafi *et al.*, 2002). During electrolysis process, in a chamber with positive and negative poles, reduction reaction occurs at the cathode and an oxidation reaction occurs at the anode; molecules are pulled apart and reactive species of molecules are formed. Different super oxidized solutions (SOS) have different properties (Sampson *et al.*, 2002; Alimi *et al.*, 2006; Gutierrez, 2006).

The electrolysis apparatus which used can be two-chamber or three-chamber. In the two-chamber type of electrolysis apparatus, the electrolysis vessel is partitioned into anode chamber and cathode chamber by a cation exchange membrane (Figure 3). This membrane acts as a diaphragm. There are anode electrode and cathode electrode in anode chamber and cathode chamber respectively. These electrodes can be in close contact with cation exchange membrane. Each chamber has an outlet and an inlet. Purified water is supplied to anode chamber and aqueous solution (for example saline solution) is supplied to cathode chamber to form anode-electrolyzed water in anode chamber (Yahagi *et al.*, 2002).

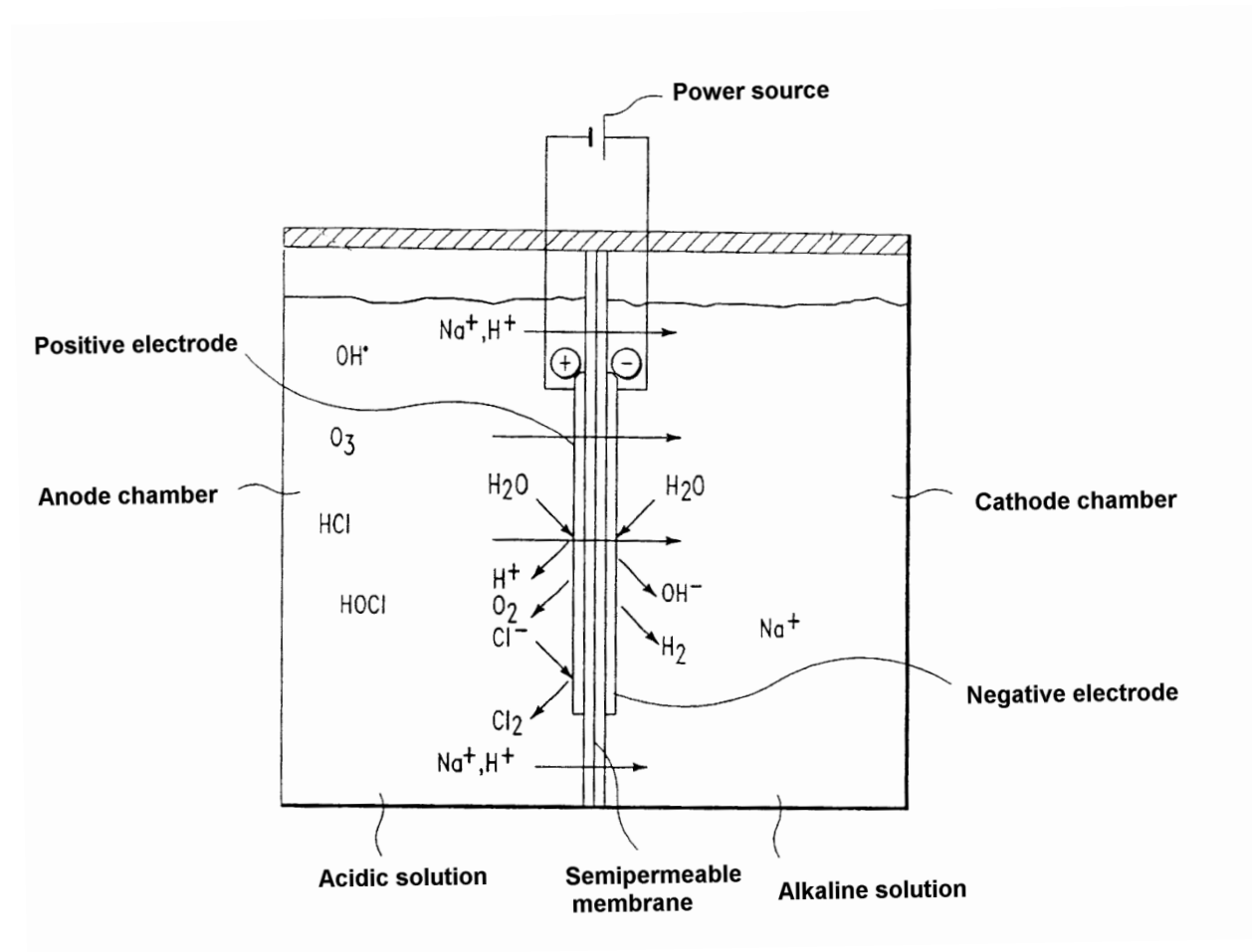


Figure 3. Schematic diagram of two-chambered electrolysis apparatus (Najafi *et al.*, 2008).

The three chamber type of electrolysis apparatus is partitioned into an anode chamber, a cathode chamber and a middle chamber or salt solution chamber that located between the anode and the cathode chambers (Figure 4). Both of anode chamber and cathode chamber have an inlet and an outlet which permit the water to flow through the chambers. The middle chamber has an inlet and outlet either. There are an anode electrode and an anion ion exchange membrane between the anode chamber and the middle chamber (Najafi *et al.*, 2002; Alimi, 2006). The cathode chamber also separated from the middle chamber by a cathode electrode and a cathode ion exchange membrane. Each of the electrodes may be positioned adjacent to its chamber and the membrane

located between the electrode and the middle chamber or membrane might be positioned adjacent to the chamber and the electrode located between the membrane and the middle chamber (Alimi, 2006).

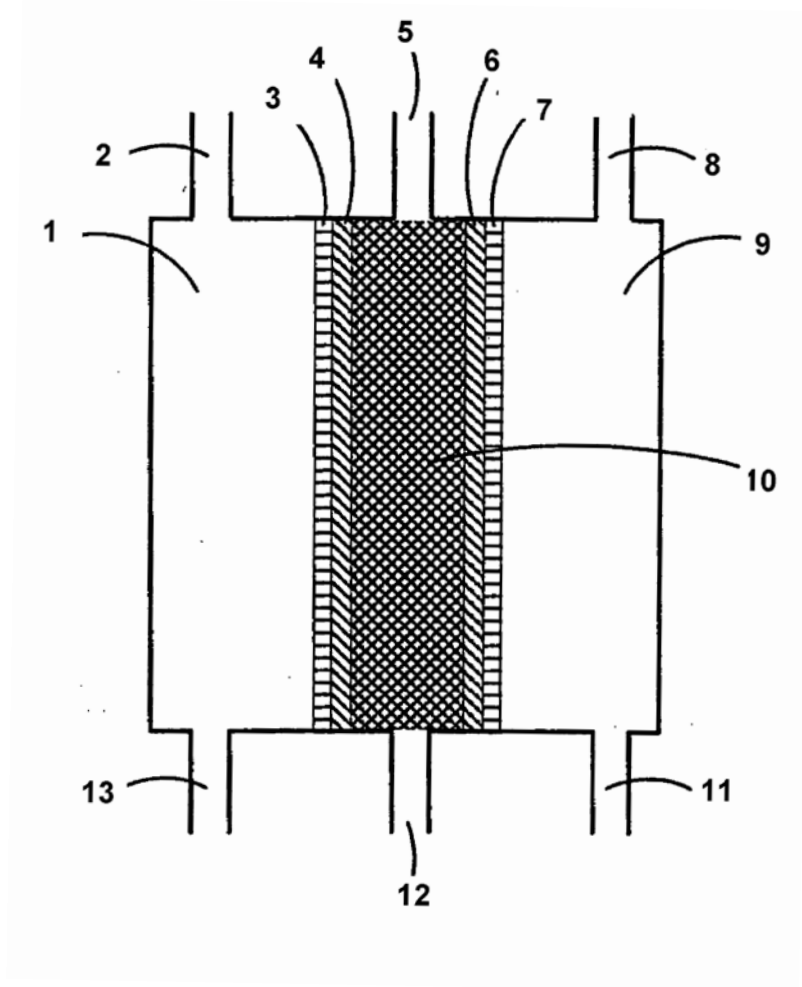


Figure 4. Schematic diagram of three-chambered electrolysis apparatus. (1) Anode chamber. (2) Outlet (3) Anode electrode. (4) Anion ion exchange membrane. (5) Outlet (6) Cathode ion exchange membrane. (7) Cathode electrode. (8) Outlet (9) Cathode chamber. (10) Salt solution chamber. (11) (12) (13) Inlet (Alimi *et al.*, 2006).

In electrolysis apparatus, the electrodes can be constructed from copper (Jnanadev *et al.*, 2011), platinum-plated titanium (Yahagi *et al.*, 2002), titanium coated with porous layers of a metal

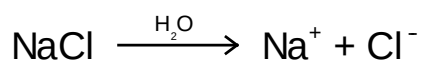
oxide catalyst (Cai, 2005) or any other types of suitable metals. The electrodes preferably are made of metal; therefore, the voltage potential can be applied between the anode chamber and the cathode chamber (Alimi, 2005). The electrodes are generally planar metals with similar dimensions and also have similar cross sectional area with the ion exchange membranes. Each of the electrodes exposed a significant portion of the surface of its ion exchange membrane to the water; therefore ionic species can migrate between the salt solution, cathode chamber and anode chamber (Alimi, 2005; Alimi *et al.*, 2006).

The ion exchange membranes are constructed from certain materials to permit the exchange of ions between the salt solution chamber and the anode/cathode chamber. The anode and cathode ion exchange membranes may be made of the same or different material, and also they may be constructed from a single layer or multiple layers. (Alimi, 2005).

In the electrolysis apparatus, purified water is supplied to the cathode chamber and anode chamber via their inlets and the salt solution is supplied to the salt solution chamber via its inlet. The source of the water for the cathode chamber and the anode chamber can be any suitable water supply. The water can be from municipal water or it is pretreated and is selected from softened water, distilled water, purified water or deionized water; however, preferably the ultrapure water, which is obtained from reverse osmosis and UV light purification equipment, is used (Alimi *et al.*, 2006).

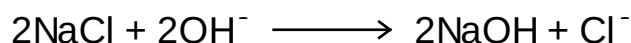
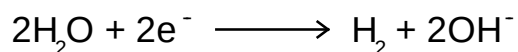
To produce SOS, the salt water solution in the middle chamber can be any aqueous salt solution with suitable ionic species. Salt solutions accelerate the process of electrolysis by allowing currents to pass between the electrodes. Generally a 1% or less saline solution is used. Chloride salts such as sodium chloride, ammonium chloride, potassium chloride and magnesium chloride

and also halogen salts such as potassium and bromine salts are suitable salt solutions. The salt solution is a mixture of salts which could be saturated or concentrated (Alimi, 2005; Alimi *et al.*, 2006). Preferably, the salt solution is a saturated sodium chloride which should be added to solution until it becomes isotonic with body fluid. Thus, solution also has similar ingredients, pH and osmotic pressure with body fluids (Yahagi *et al.*, 2002). The sodium chloride solution used in producing SOS is in ionized form and contains sodium ions (Na^+) and chloride ions (Cl^-) (Najafi *et al.*, 2002; Alimi *et al.*, 2006).

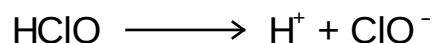
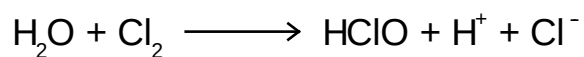
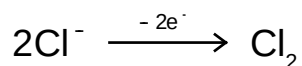


An electrical potential source is connected to the anode electrode and cathode electrode leading to an oxidation reaction in the anode chamber and a reduction reaction in the cathode chamber (Alimi, 2006).

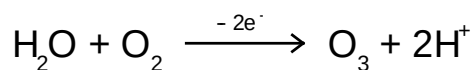
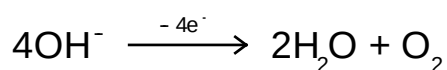
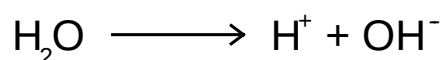
During electrolysis of saline, the sodium ions (Na^+) migrate from the salt solution to the cathode water or negatively charged electrodes and then counterbalance the hydroxide ions (Najafi *et al.*, 2002; Jnanadev *et al.*, 2011).



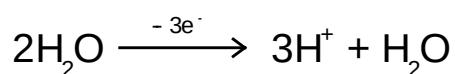
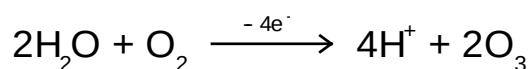
Chloride ions (Cl^-) migrate from the salt solution to the anode water or positive electrode and undergo an oxidative process producing small quantities of chloride gas that immediately will be consumed for generating hypochlorous acid (Najafi *et al.*, 2002; Jnanadev *et al.*, 2011).

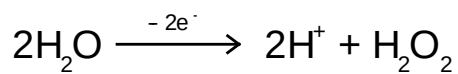


In anode chamber, the chlorine ions are in the form of HClO, ClO⁻, or Cl⁻; the pH of the solution affects the balance of these ions. HClO and ClO⁻ ions are effective sterilizing agents and actually HClO is ten times more effective than ClO⁻. Low levels of O₃ also are formed under electric current, which add antimicrobial property to the solution (Najafi *et al.*, 2002; Jnanadev *et al.*, 2011).



During electrolysis, sufficient chlorine ions are not supplied to the anode electrode therefore the below oxidative decomposing reactions also occur:





Based on these reactions, the electrolyzed water manufactured in the anode chamber contains ozone, active oxygen and hydrogen peroxide. The oxidative substances react with chlorine ions to form hypochlorous ions that are precursors for the hydroxyl radicals.

The water produced at an anode chamber is referred to as anode water and the water produced at a cathode chamber is referred to as cathode water. Anode water contains oxidized species including chlorine in various such as chlorine gas, chloride ions, hydrochloric acid or hypochlorous acid. Oxygen in various forms is also present. In contrast, cathode water contains reduced species including hydrogen gas, hydroxyl radicals or sodium ions (Alimi *et al.*, 2006). Highly oxidized water with controlled amount of free radical ions is the resultant output of this process.

Two three-chambered electrolysis cells can be used for producing SOS either. In this process water is pumped or dispensed to anode chamber and cathode chamber of the first electrolytic cell and also to anode chamber and cathode chamber of the second electrolytic cell. By using this process, about 1 liter/minute to about 50 liter/minute of SOS can be produced. through using additional electrolytic cells for instance three, four, five, six or more three-chambered electrolytic cells, the production capacity of SOS will be increased (Alimi, 2005).

After electrolysis products of anode chambers and a portion of the cathode water are collected in mixing tank and combined with each other. The remaining portion of cathode water that produced in the process will be discarded. Two gas separators may optionally added to the mixing tank which remove gases like hydrogen gas that formed in cathode water during the

process. In order to control pH of SOS, suitable devices optionally may be added to the mixing tank. In addition, for controlling the temperature of the SOS, the salt solution may flow through a heat exchanger in the mixing tank. The ions present in the salt solution are decreased over time; hence, an additional source of ions may be added to the mixing tank (Alimi, 2005).

The SOS contains various of ions including chlorine, ozone, and active oxygen which can be in different forms such as singlet oxygen ($^1\text{O}_2$) that formed from excitation of triplet oxygen, superoxide (O_2^-) formed by reduction of oxygen by a single electron, hydroxyl radical ($\text{HO}^\cdot$), hypochlorous ions (ClO^-), proxy radical ($\text{ROO}^\cdot$), alkoxy radical ($\text{RO}^\cdot$) and hydroperoxide (ROOH) (Yahagi *et al.*, 2002). Free chlorine species typically are hypochlorite ions (ClO^-), hypochlorous acid (HClO), sodium hypochlorite (NaOCl), chloride ion (Cl^-), chlorite ions (ClO_2^-), chlorine dioxide (ClO_2) and other species (Alimi, 2005). These active oxygen species that supplied from the SOS also activate the enzymes and the growth factors by the process similar to that of the bio-protective cells used by body. The disinfectant ability of the SOS for killing different microorganisms such as bacteria, fungi or viruses is achieved by the presence of one or more of these ions species (Yahagi *et al.*, 2002; Alimi, 2005; Alimi *et al.*, 2006).

ORP, pH and chlorine concentration

Each superoxidized solution has its own properties such as oxidation reduction potential (ORP), pH and concentration of chlorine. ORP indicates the degree to which a substance is capable of oxidizing or reducing another substance. ORP is measured in millivolts (mv) using an ORP meter. A positive ORP reading indicates that a substance is an oxidizing agent. A negative ORP reading indicates that a substance is a reducing agent. These factors determine SOS germicidal activity, shelf life and corrosion potential. Among them, neutral pH solutions have germicidal activity with long shelf life and more tissue compatibility (Nagamatsu *et al.*, 2002).

The superoxidized solution can be acidic, neutral and basic. SOS can have pH from 1 to 14 and in this range of pH; it could be safely applied in suitable quantities to surface without harming and damaging such as human skin. Preferably the pH of the SOS is about 3 to 8, more preferably 6.4 to 7.8 and still more preferably from 7.4 to 7.6 (Alimi *et al.*, 2007).

The ORP of the solution preferably has potential from -400 mV to +1300 mV. More preferably from 0 mV to 1250 mV; and still more preferably from +500 mV to 1250 mV. Even more preferably ORP should be from +800 mV to 1100 mV and the most preferably from +800 mV to +1000 mV (Alimi *et al.*, 2007).

Free chlorine of the SOS includes but not limited to, hypochlorite ions, hypochlorous acid, chloride ion, sodium hypochlorite, chlorite ions, chlorine dioxide, dissolved chlorine gas and other radicals of chlorine species. The ratio of hypochlorous acid to hypochlorite ion is dependent on pH and temperature of the solution (Alimi, 2005; Alimi *et al.*, 2007).

In terms of disinfection properties of SOS, it has been shown by different studies that ORP of the solution is more important than its free chlorine content. The ORP of the SOS is inversely proportional to its pH. Hence, pH reduction causes an increase in ORP and consequently antimicrobial properties of SOS even if the residual chlorine are kept constant. (Nakagawara *et al.*, 1998; Venkitanarayanan *et al.*, 1999; Kim *et al.*, 2000; Len *et al.*, 2000; Al-Haq *et al.*, 2002; Park *et al.*, 2004b; Liao *et al.*, 2007).

Mechanism of action against bacteria

Bactericidal effect of SOS against bacteria is based on the action of ORP, dissolved chlorine and concentration of hydrogen ion (Tanaka *et al.*, 1996). Various ions are present in SOS, it contains ozone and peroxides (e.g., hydrogen peroxide), and it also contains dissolved oxygen and chlorine (e.g., free and bound chlorine). The presence of one or more of these ions give disinfectant properties to the SOS (Alimi, 2005; Alimi *et al.*, 2007). The chlorine content of SOS, Cl_2 , is responsible for disinfection. Cl_2 affects depends on formation of hypochlorous acid (HClO) (Jnanadev *et al.*, 2011).

Mechanism of SOS disinfection includes two areas. First one focuses on the chemical and physical properties of the solution like high ORP, low pH and chlorine concentration and active oxygen. The other one is based on SOS disinfection target in cellular components such as outer membrane, cytoplasmic enzyme and so on (Len *et al.*, 2000; Koseki *et al.*, 2001; Park *et al.*, 2004a; Liao *et al.*, 2007).

The pH, ORP and chlorine content of SOS work as interrelated factors. Low pH, high ORP and hypochlorous acid work synergistically to exceed the stability limit of membrane potential of

organelles and inhibit respiration and metabolism of energy to kill microorganism (Jnanadev *et al.*, 2011). It has been demonstrated that level of ORP in SOS is inversely proportional to its pH; thus decreasing the pH of the solution increases the ORP and antimicrobial effect of the solution, even if the chlorine species of solution remain constant. At low pH, HOCl is dominant chlorine species that destroy microbial structures and proteins (McKenna *et al.*, 1988; Barrette *et al.*, 1989; Denyer, 1995; Nakagawara *et al.*, 1998; McDonnell *et al.*, 1999; Len *et al.*, 2000; Al-Haq *et al.*, 2002; Park *et al.*, 2004b).

Selective inhibition of bacterial cell division by HOCl is also observed in phagocytes. HOCl has small molecular size that help it to penetrate into the cell membrane components which increase cell wall permeability and cell membrane rupture (McKenna *et al.*, 1988). HOCl acts via attacking the surface and plasma membrane proteins in order to damage solute transport and salt balance of bacteria cells. However, HOCl has selectivity property which inhibits this molecule from attacking eukaryote cells (Selkon, 2007). In addition, presence of strong oxidizing agents such as hydroxyl radicals along with HOCl lead to high disinfection property of solution (Yonemori *et al.*, 1997; Cai, 2005; Martí'nez *et al.*, 2007; Xiong *et al.*, 2010).

High ORP of SOS causes bacteria destruction without the need for any toxic materials (Holton *et al.*, 1997). High ORP of SOS causes microorganism eradication. It alters integrity of bacteria cell wall (or viral capsids), and causes cell lysis via unbalancing the osmotic pressure between the cell and hypotonic SOS (Landa-Solis *et al.*, 2005; Martí'nez *et al.*, 2005). While microorganisms immerse into the solution, they are exposed to a powerful oxidant that sequesters electron from their structural components, which leads to rupturing biochemical bonds and subsequent losing

its integrity(Chittoria *et al.*, 2007; Thorn *et al.*, 2012). Oxidizing chemicals pull electrons from cell membrane and make it instable and leaky, which leads to rapid death (Suslow, 2004). As mentioned, high ORP creates imbalanced ions osmolarity (Chittoria *et al.*, 2007; Thorn *et al.*, 2012). During electrolysis reactive oxygen and chlorine are formed, these species cause unbalance osmolarity which cause damage to the integrity of the cell membrane and then reacts with protein and lipid of the microorganism and denature them. Unbalanced osmolarityonly affects single cell organisms, while multicellular organisms are not prone to osmolarity changes (Fuqua *et al.*, 1994; Kapur *et al.*, 2011). Damage in the membrane increases membrane porosity that induces oxidizing agent penetration into the cell cytoplasm and interacting with intracellular lipids, proteins and nucleic acids(Thorn *et al.*, 2012).

The lipid content of the cell membrane is the first target of SOS. Sequestering electron from the cell membrane cause penetration of more oxidants into the cell cytoplasm leading to inactivation of cell processes. It has been postulated that high ORP of SOS interferes with cellular redox signaling pathways causing cell permeabilization, oxidation of intracellular components, formation of disulfide bridges and consequently changes protein structure and function and cell lysis. Cellular redox signaling (also termed ‘redox cell signaling’) contributes to the control of cell development, differentiation, growth, death and adaptation through transferring electron (redox centers become reduced (gain of electrons) or oxidized (lose of electrons))(Zheng *et al.*, 1998; Levonen *et al.*, 2001; Pomposiello *et al.*, 2001; Liao *et al.*, 2007). In the cell cytoplasm, SOS also causes destruction of plasmid and chromosomal DNA, RNA and proteins (Zinkevich *et al.*, 2000; Cloete *et al.*, 2009; Zeng *et al.*, 2010). Oxidizing agents of the solution destroy covalent bonds in the nucleic acid chains and protein chains (Zinkevich *et al.*, 2000). One redox reaction in the cell is formation or breaking of protein disulfide bridges which is a good indicator

for bacteria redox environment. Oxidative stress also causes more oxidizing cell redox potential that results in the formation of disulfide bridges and at last changes the protein's structure and function. Therefore, ORP affects and damages redox state of disulfide bridges and penetrates outer and inner membrane of the bacteria and causes cell necrosis (Liao *et al.*, 2007). Inhibiting and damaging dehydrogenase enzyme is another mechanism of SOS to eradicate bacteria. Dehydrogenase is a key enzyme in respiration and anabolism. Low pH and high ORP have effect on dehydrogenase enzyme. Low pH influences the conformation of the enzyme's active site and high ORP causes electron sequestration from microorganism and interferes catalysis of enzyme in the cell. Small molecule HOCl also could enter the cell and influence on the metabolizing enzyme system. Therefore, SOS targets dehydrogenase and decreases its activity which result inhibition of respiration and anabolism of microorganism (McPherson, 1993; Wang *et al.*, 2002; Zhao *et al.*, 2004; Qu, 2005).

SOS also enhances pathogen permeability which leads to leakage of more elements from the cytoplasm of the bacterial cell. The HOCl in the solution binds the pathogens and penetrates into the cell wall. HOCl is able to inhibit protein synthesis by its reactions with amino acids; it also reacts on polysaccharides and lipids that lead to cell wall loosening and cell membrane destruction. Increase in membrane permeability of microorganism leading to unbalanced permeate pressure between intracellular and extracellular ion concentrations causes a large amount of K^+ leak from inside the cell. Since K^+ is important for intracellular physiologic function, great loss of K^+ causes bacterial mortality. Longer disinfection causes increase in protein leakage. Longer SOS contact with microorganism causes more damage on the cell wall thus more leakage of protein occurs. However, after long period of SOS disinfection, proteins

leakage slow down as they denature and also react with reactive oxygen, hypochlorous acid. The DNA leakage is another result of SOS treatment. DNA is in the nucleus; hence, its leakage suggests that cell wall and cell membrane of microorganism were both damaged and SOS reaches inside the cell and nucleus. In later period, leakage of DNA, protein and K^+ of the bacteria slow down (Xue, 1985; Marklund *et al.*, 2001; Zeng *et al.*, 2010).

Strong acidic superoxidized solutions have another mechanism to kill the bacteria. Most microorganisms cannot survive at very acidic pH (CKJ, 2000; Nagamatsu *et al.*, 2001). The required environment for mitochondria is pH 2 to 12 and ORP -400 to 850. Superoxidized solutions which have pH below 2.7 and ORP around 1100 mV fall out of the mitochondrial range which is one reason of their germicide effect (Shimizu *et al.*, 1996; Nakae *et al.*, 2000a).

Different studies have been done to demonstrate the effect of SOS on different organisms.

SOS can be used to prevent infection by pathogens such as viruses, bacteria and fungi. The virus can include adenovirus, herpes virus, coxsackie virus, HIV, rhinovirus, flu virus, hepatitis, coronavirus, rotavirus, avian flu virus, respiratory syncytial virus, herpes simplex virus, varicella zoster virus, rubella virus, pox virus and papilloma virus (Alimi, 2005; Alimi *et al.*, 2006; Alimi *et al.*, 2007).

Bacteria that have been shown to be sensitive to SOS are: *Escherichia coli*, *pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* (Alimi, 2005; Alimi *et al.*, 2007). *Enterococcus hirae*, *Acinetobacter baumannii*, *Acinetobacter* species, *Bacteroid fragilis*, *Enterobacter aerogenes*, *Enterococcus faecalis*, Vancomycin resistant – *Enterococcus faecium*, *Klebsiella pneumonia*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Micrococcus luteus*,

Staphylococcus epidermidis, *Proteus mirabilis*, *Serratia marcescens*, *Staphylococcus haemolyticus*, *Staphylococcus Pneumoniae*, *Staphylococcus pyogenes*, *Shigella dysenteriae*, *Salmonella choleraesuis*(Alimi et al., 2006). *Streptococcus pneumoniae*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Lactobacillus*(Najafi et al., 2008). *Bacillus subtilis* and *Bacillus atrophaeus* (Alimi, 2005; Alimi et al., 2007).

The fungi include *Candida albicans*, *Trichophyton mentagrophytes*, *Candida tropicalis*, *Histoplasma capsulatum*, *Aspergillus* species and dermatophytes (Alimi, 2005; Alimi et al., 2006; Alimi et al., 2007). *Cryptococcus neoformans*, *Microsporum canis*(Najafi et al., 2002).

Tissue repairing activities

When skin is damaged, bioprotective cells such as macrophages and neutrophils which gathered at the wound site, produce active oxygen that functions as biosignals and activates the enzymes and growth factors for promoting the healing mechanism (Yahagi et al., 2002).

The active oxygen is not similar to the molecular oxygen based on biochemical activities. The molecular oxygen has a role in the metabolism and has not any role in the growth of the cells. In contrast, the active oxygen species function as bio-signals for the cell growth and do not have any role in metabolism. Through using SOS, greater amount of active oxygen is supplied to the wound site; thus, greater amount of bio-signals are secreted and it results in more promotion of the growth of cells, which leads to tissue reconstruction. The active oxygen concentration should be between 1ppm to 500ppm, if it is less than 1ppm, it does not function as bio-signal, and if it

is 500ppm or more, it produces undesired effects on the cells because of its toxicity (Yahagi *et al.*, 2002).

In the SOS preferably the active oxygen is in the form of hydroxyl radical ($\text{HO}\cdot$) because in the body the same active oxygen is produced. The hydroxyl radical needs a precursor to be synthesized. This precursor forms hydroxyl radical in the presence of an iron or copper ingredient as a catalyst. The hypochlorous ions in the body react with proteins or amino acids and form hydroxyl radicals; then, they promote the growth of tissue cells by bio-signaling (Yahagi *et al.*, 2002). At the wound site, the hypochlorous acid (HOCl) attacks to the surface and plasma membrane proteins of bacterial cells and impairs transport of solutes and the salt balance of them. The combination of hypochlorous acid and ozone also give antimicrobial properties to the solution (Najafi *et al.*, 2002; Selkon, 2007). In addition, oxygen is important in collagen synthesis. Based on in vitro studies, there is a proportion between the amounts of collagen synthesis by fibroblasts and the extracellular oxygen concentration. By increasing the oxygen concentration, the collagen fibers crosslinking also increases, which raise tensile strength of collagens. Furthermore, In a vivo studies, leg ulcers exposed to excess oxygen had more fibroblast proliferation (Najafi *et al.*, 2002).

Hydrogen peroxide, chlorine, hypochlorite and hypochlorous acid have deodorant properties in the wound site. These radicals react with putrefaction substances from necrotic tissues and anaerobes and cause odour reduction (Thomson, 2000).

In conclusion, active oxygen species have promoted the growth of tissue cells and also function as sterilizer for the wound sites during wound healing.

Toxicity of SOS

Most disinfectants used for wound care can cause damage to the skin and granulation tissue. Common disinfectants like hypochlorite, povidone iodine and hydrogen peroxide can cause cell damage and interfere with wound healing. Furthermore, although saline solution and water are not cytotoxic, they cannot remove adequate contamination from the wound and do not have antiseptic properties. Therefore, nontoxic disinfectants with powerful antiseptic properties are needed to clean the wound and induce healing. Different studies have revealed that superoxidized solution is nontoxic with power to kill different microorganisms so as to treat infectious skin condition (Fowler *et al.*, 1999; Nakae *et al.*, 2000a; Sen *et al.*, 2002).

In a clinical study (Martínez *et al.*, 2007), a group of patients with diabetic foot infection were treated with conventional chemical antiseptics and another group was treated by SOS. The study revealed that SOS as compared with the other antiseptic had higher disinfection properties while minimizing skin and tissue damage. The SOS revealed greater odor and cellulitis reduction and less tissue toxicity in comparison with conventional chemical antiseptics. The bactericidal effect and minimal cytotoxicity of the SOS also cause better control of exudates volume and sudden deterioration in the quality or quantity of granulation tissue, which increased rate of patients whose wound advances to granulation phase (Martínez *et al.*, 2007). However, just a clinical study is not adequate and more in vivo and in vitro studies are needed to reveal effects of SOS on wound and different cell types and cell components such as RNA and DNA.

Different studies have revealed SOS effects on wound (Alimi, 2005; Alimi, 2006; Alimi *et al.*, 2007). In a study, local and systemic toxicities of a neutral SOS (Microcyne) were examined in a

deep wound on rats. No abnormalities or significant differences in hematic cytology and parameters of blood chemistry were observed. The autopsies were also normal. The irritation of skin, histopathology of wound and tissue around the wound revealed no differences between groups who were treated by SOS and saline solution (Alimi *et al.*, 2007).

Another study examined affects of SOS (Dermacyn) on histopathology of wound bed in full thickness dermal wound healing. The expression of collagen type 2, connective tissue proliferation, inflammation and dermal ulceration were investigated. The findings indicate that SOS is well tolerated in rats. It left no histopathologic lesion in skin and there were no histopathologic differences and collagen type 2 expressions between SOS and saline treatment. These evidences indicate that SOS does not have adverse effects on fibroblast and collagen formation during wound healing (Alimi, 2006; Alimi *et al.*, 2007).

In another study, local and systemic toxicities of topically applied SOS (Microcyne) on deep wound was evaluated in rats. In this one also no differences were observed between SOS and saline treated group in parameters of blood chemistry or hematic cytology. The irritation grading, skin histopathology and tissue around the wound also were not different in both groups. Collagen II also was assessed and no alteration was observed during the wound healing process in SOS group (Alimi *et al.*, 2006).

Different studies have also been done to examine effect of SOS on skin irritation and sensitization. Two studies were carried out to demonstrate the effect of SOS (Microcyne) on rabbit skin to evaluate irritation and dermal toxicity. No clinical sign, dermal irritation and abnormalities in the skin autopsy were found in group of animal who were exposed to SOS (Alimi *et al.*, 2006).

Another study was conducted to evaluate skin irritation. In that study two intact and two abraded skin sites were prepared on the back of six animals. The SOS was applied to the intact and abraded skin sites for four hours a day for five days. The control group (distilled water) also included another intact and abraded skin sites under the same condition. No skin irritation was observed in SOS group compared to the control group (Najafi *et al.*, 2002; Najafi *et al.*, 2008).

Skin potential for sensitization was evaluated by using SOS (Microcyne) on guinea pig. No irritation was observed in control group and group that were treated by SOS. Therefore, SOS does not induce sensitizing reactions (Alimi, 2006).

Other studies have also been done to show the effect of SOS on skin cells like fibroblasts and keratinocytes. One study compared toxicity of SOS and hydrogen peroxide (HP) on viability of human dermal fibroblasts (HDFs). In vitro, HDF were exposed to SOS and HP for five minutes. The cell viability of SOS group was 75% and 55% respectively. The viability of cells decreased to 70% and 5% respectively after 30 minutes. SOS induces cell death through necrosis and apoptosis does not seem to be the underlying mechanism of action as just 3% of the cells reveal cellular surface marker for apoptosis. In contrast, HP induced necrosis in 20 % and 75% of treated cells and apoptosis in 15% and 20% after 5 and 30 min of exposure. Overall, these results show that SOS is far less toxic for HDF than HP (Alimi *et al.*, 2007).

Another study examined toxicity of SOS on fibroblast cells. In this study a filter disc with 0.1 ml of SOS was placed on monolayer of mouse fibroblast cells and the sample were studied after 24 hr incubation at 37°C. The SOS sample did not reveal any cell lysis or toxicity, which indicate that SOS does not have cytotoxic effect on murine fibroblasts (Alimi, 2006; Alimi *et al.*, 2007).

In vitro effect of SOS (acidic solution) application on human skin cells were also assessed. The application of SOS on keratinocytes, fibroblasts and melanocytes was well tolerated and their cytotoxicity was minimal and parallel to sterile saline solution. The SOS was also applied in vivo to rabbit eyes. The rabbits received randomly saline solution and SOS. The results showed that the cytotoxicity index was zero for both groups and no irritation response existed. Therefore, the SOS is revealed to be nontoxic to biological tissue in comparison with saline solution (Najafi *et al.*, 2008).

In another study, impact of SOS on human keratinocytes (HK) proliferation was investigated. Keratinocyte cultures in the presence of SOS showed cell proliferation enhancement and no toxicity was seen in the presence of SOS (Selkon, 2007).

Research also has been conducted to reveal the effect of SOS on cell components like DNA and RNA. In a study, cell viability and oxidative stress of SOS were examined on human dermal fibroblast culture (HDF). In this study 8-hydroxy-2'-deoxyguanosine (8-OHdG) adducts, nucleic acid stability and ageing process were evaluated. HDFs were exposed to different concentration of SOS (neutral pH solution). The experiments were conducted with primary HDF cultures that contained more heterogeneous cell population than established cell lines to have more cell population with different fibroblast phenotype which closely mimics fibroblasts of the wound. In the study, senescence-associated beta-galactosidase (SA- β -gal or SABG) and 8-OHdG were observed as markers of oxidative stress since elevation of their levels correlates with carcinogenesis, mutagenesis and cellular ageing. Under the experimental conditions just 30% of the fibroblasts died after 30 minutes of exposure to the SOS, while 90% of them died after exposure to HP. The study revealed that SOS is significantly less cytotoxic than commonly used antiseptic, HP. Besides, SOS does not induce DNA oxidation and cell ageing. The analysis also

revealed that HP induced both apoptosis and necrosis, while SOS only induced necrosis. The expression of SABG (a factor associated with cellular aging) has also been examined after one month of exposure of fibroblast cultures to SOS and HP. There was increased expression of SABG in HDFs just in HP group. Therefore, exposure of HDF to SOS is significantly less cytotoxic than HP and it does not induce DNA oxidation, cell ageing. Moreover, its safety is supported by the lack of irritation, genotoxicity and sensitization (Severino *et al.*, 2000; Wolf *et al.*, 2002; Naderi *et al.*, 2003; Tanaka *et al.*, 2004; Arrigo *et al.*, 2005; Wilson *et al.*, 2005).

Another study also demonstrated the effect of SOS relative to hydrogen peroxide on oxidative DNA damage and formation of the DNA adducts (8-OHdG) in HDF. 8-OHdG production is a marker for oxidative damage of DNA and high level of 8-OHdG is correlated with mutagenesis, carcinogenesis and cell aging. HDF cultures were exposed to SOS and HP for 30 minutes. The SOS-exposed group did not reveal increase in 8-OHdG adduct, in contrast to the cells treated with HP showed an increase in 8-OHdG adduct by about 25 fold relative to the control and SOS treated cells. The result demonstrates that SOS does not induce significant DNA oxidative damage, mutagenesis and carcinogenesis in vitro or in vivo (Alimi *et al.*, 2006).

SOS administration also does not cause significant RNA degradation. In a study, RNA which was extracted from human cell culture was exposed to SOS for 30 minutes. Gel electrophoresis analysis revealed no significant RNA degradation and also showed two discrete bands corresponding to ribosomal RNA of eukaryotic cells. These evidences reveal that SOS leaves the RNA substantially intact (Alimi *et al.*, 2007).

In conclusion, SOS is toxicity free for normal mammalian cells and tissue. Administration of SOS does not cause a significant decrease in eukaryotic cell viability. Furthermore, it does not

raise apoptosis, cell ageing and oxidative DNA damage and RNA degradation in mammalian cells (Alimi, 2006; Alimi *et al.*, 2007). Histopathological and immunohistochemical analyses also demonstrated that SOS does not interfere with wound healing and also in vitro studies have shown no cytotoxicity induction in fibroblasts (Gutierrez, 2006). SOS is nonirritating, nonhazardous and nonsensitizing to skin and also nonirritating to eye. In addition, it shows no mutagenic activity (Selkon, 2007). Inasmuch as superoxidized solution's disinfection power is roughly equivalent to the hydrogen peroxide with significant less toxicity, it is a proper and safe option for use in mammals including human (Alimi, 2006; Alimi *et al.*, 2007).

Wound type

Recent studies also revealed how SOS is useful in acceleration of wound healing. SOS can treat both human and nonhuman mammals. The skin ulcers which are treated by SOS could be located on different parts of the body such as head, neck, upper extremity, hand, finger, trunk, genitalia, lower extremity, foot, toes, paws, hooves or combination of them (Alimi *et al.*, 2006).

The SOS also can treat skin ulcers of any size, shape and depth such as ulcers on superficial epidermis, ones that preserve the epidermal basal layer, ulcers that penetrate epidermis, ulcers that involve dermis and penetrate into deep tissues such as fat, muscle and bone. Their shape also could be any kind such as linear, round, oval or irregular (Alimi *et al.*, 2006).

SOS could be utilized in different mode. For instance, the damaged tissue can be treated by irrigating, steaming or spraying with SOS. In addition, aerosolization, nebulization or atomization are also used to administer the effective amount of it (Alimi, 2005; Alimi *et al.*, 2007).

SOS is also proper candidate for irrigation, debridement and moistening and microbial load reduction of acute and chronic wounds. It is also used to remove foreign material and debridement from acute and chronic dermal lesions such as diabetic ulcer, pressure ulcer, postsurgical wounds, first and second degree burns and minor skin irritation (Gutierrez, 2006).

In addition, the SOS could be used for treating nonsurgical and surgical wounds. It is used for treating tissues which have been impaired by incisions and surgeries such as oral surgery, graft surgery, transplant surgery, implant surgery, amputation, radiation, chemotherapy and a combination of them. It is also used as surgical open wound cleansing agent, wound disinfection, wound and burn healing promotion, and it is employed as treatment agent for dermal abrasion, pressure ulcer, laser resurfacing, donor sites or grafts, exuding wound and superficial injuries like laceration, cut, abrasion, minor skin irritation, scaps, rashes, punctures wounds and a combination of them (Alimi, 2005; Alimi, 2006; Alimi *et al.*, 2007).

Infected tissue, which is the result of one or more pathogens, could also be treated by SOS. It manages various infections such as eye, ear, lung, nasal and sinus infection (Alimi, 2005; Alimi, 2006; Alimi *et al.*, 2007).

The SOS can treat ulcers which are appeared by different factors for example, arterial insufficiency, venous insufficiency, lymphatic insufficiency, neuropathy, pressure, trauma or combination of them (Alimi *et al.*, 2006).

SOS also provides method for treating skin ulcer in patients with metabolic disorders (such as diabetes), inflammatory conditions (such as lupus and hypersensitivity reaction), infectious diseases (such as herpes and sepsis), hereditary diseases (such as sickle cell anemia),

degenerative diseases (such as scleroderma), trauma (such as abrasion and radiation) or a combination of them (Alimi *et al.*, 2006).

SOS is also sufficient to treat other various skin ulcers such as diabetic foot ulcer, gangrenous ulcer, ischemic ulcer, venous stasis ulcer, decubitus ulcer or traumatic ulcer, bed sores, post operative infective wounds, cellulites and abscesses. SOS also provides a method for decreasing likelihood of amputation and dehiscence (Alimi *et al.*, 2006; Kapur *et al.*, 2011). Therefore, SOS is a proper solution to treat different types of wound varying from superficial wounds like abrasion to deep wound which penetrate to muscle and bone.

Review of SOS histological effect on wound

Previous research showed that superoxidized solution is nontoxic and nonirritating and it possesses antimicrobial and antiseptic properties. The acidic ORP solution was tested against different species of bacteria, fungi and viruses that showed reduction in microorganism activity in most of them (Najafi *et al.*, 2002). To study irritating effect of electrolyzed acidic water, male rabbits were used. In this study certain volume of electrolyzed acidic solution was instilled into each eye of rabbits. There were no signs of irritation in any of the treated eyes (Najafi *et al.*, 2002). In another in vitro study that compared disinfection capability of electrolyzed acid water with 2% glutaraldehyde, the disinfection potential of 2% glutaraldehyde and electrolyzed acid water were similar when the disinfection time was 5 minute (Jnanadev *et al.*, 2011).

Recent studies also revealed that superoxidized solution promotes healing mechanism, and is effective in wound healing. The effectiveness of electrolyzed strong acid aqueous solution with high ORP and high concentrations of chloride and oxygen was examined rats with three-degree

burns. Three groups of rats were used which received normal saline, electrolyzed solution and no treatment, respectively. The wound area was observed macroscopically until they were fully healed. After healing, rats were sacrificed and the healed wound area excised for microscopy by using hematoxylin-eosin staining method. In the group treated with electrolyzed aqueous solution, epithelialization occurred significantly earlier. Histological studies showed that proliferation of lymphocytes and macrophages associated with collagen deposition were more extensive in the normal saline group and electrolyzed aqueous solution treated groups compared with no treatment group. This study also revealed that electrolyzed aqueous solution did not cause any tissue damage and it may promote tissue growth in wounds (Nakae *et al.*, 2000b).

Previous research compared the efficacy of superoxide solution with other medicines and also evaluated the outcomes of wound treatment with this agent. Pandey *et al* reported that in a group of patients with different wounds that received treatment with SOS compared to a group receiving povidone iodine solution (betadine), allergic reactions, infection occurrence and persisting of it was lesser and the reduction in wound area was greater in this group, too. Their research also showed that in SOS group elimination of bacterial strains and granulation tissue formation occurred earlier (Pandey *et al.*, 2011).

In another study by Kapur *et al* which have been done on 200 patients suffering from different wounds, patients were equally divided into two groups (SOS-treated vs. betadine treated-group). The average length of hospital stays in SOS treated patients were less than betadine group. The granulation tissue formation and epithelialization were earlier in the SOS group compared to other group. Furthermore, this study showed a higher reduction in wound size and less scar formation in SOS group. Minimum toxicity and moistening effects of SOS that was found in this study made it a good choice for using in wound care management (Kapur *et al.*, 2011).

In a study conducted by Alimi on rats, he attempted to illustrate the activity, stability and the lack of toxicity of SOS solution and also to evaluate the local tolerability and histopathology of wound beds (Alimi, 2006). The SOS solution used in this study is known as Microcyn which is a superoxidized solution with neutral pH and nontoxic, germicidal and sterilizing activities. This study used Masson's staining and collagen type II staining for Microcyn and saline-treated sections. Comparisons of histological samples of these groups showed that there were no significant differences in collagen II expression and Microcyn did not have any adverse effects on collagen and fibroblasts production. In general, there were no histopathological differences between these two groups and the results of this study showed that Microcyn treatment was well tolerated (Alimi, 2006).

Another study compared the effects of superoxidized water with betadine as a topical treatment for chronic wound within 30 patients. It demonstrated that superoxidized water is well tolerated in comparison with betadine with less local adverse effects. Besides, it induces wound healing through fibroblast migration and proliferation; hence, tissue regeneration (Abhyankar *et al.*, 2009).

Electrochemically activated solutions (ECAS) were also reported to have histological effect on wound repair via reducing the bacterial load, enhancing local blood supply, accelerating neovascularisation and reducing inflammation (Bongiovanni, 2006).

Pervious researches showed that superoxidized solution has antimicrobial and antiseptic properties without any tissue damage. Histological studies showed that epithelialization and granulation tissue formation occurred earlier in wounds treated by superoxidized solution. It also

accelerates neovascularization and does not have any adverse effect on fibroblasts and collagen production.

Objectives of the present study

Even though a great number of clinical studies were done to reveal antiseptic effect of SOS and its influence on different wound types, just few studies did evaluate its histological effect. Therefore, **the first** objective of the present study was to investigate the mechanism of wound healing properties of SOS histologically.

Despite the fact that SOS is an effective therapy for wound healing, its fluidity makes its application difficult. Besides, it has been claimed that application of SOS that quickly loses contact with tissue does not maximize the effectiveness of treatment. Hence, to increase the healing effect a SOS dosage form is required to be stable on wound for longer period of time (Alimi *et al.*, 2006). Thus, **the second** objective of the present study was to prepare a topical dosage form containing SOS and compare its healing effect with that of pure SOS.

Moisture prevents desiccation, cell death and also induces angiogenesis, epidermal migration and connective tissue synthesis (Eccleston, 2007; Boateng *et al.*, 2008). Modern dressings distinguished characteristic is providing moist environment to accelerate wound healing and they are mainly classified based on their materials such as hydrocolloids, hydrogels, alginates, which are in the form of gel, thin films and foam sheets(Boateng *et al.*, 2008).

Overall, the aim of this study was to understand better the mechanism of wound healing properties of SOS and assess whether this agent could be incorporated in a topical formulation

without altering its therapeutic activity. An excision wound model in mice has been used in this study.

Chapter 2:

Materials& Methods

Materials

Table 1. Used chemicals.

Chemicals	Company	Place of purchase
Absolute ethanol 99.6% (V/V)	JATA Co.	Tehran, Iran
Absolute ethanol 96% (V/V)	JATA Co.	Tehran, Iran
Ethanol, absolute	Bidestan Co.	Tehran, Iran
Ethanol 96%	Taghtir Khorasan Co.	Tehran, Iran
Paraffin non caking for histology Solidification point about 56-58 C °	Merck	Tehran, Iran
Paraffin 56-58 C °	Merck	Tehran, Iran
Canada balsam	Merck	Tehran, Iran
Toluene	Merck	Tehran, Iran
Picric acid	Merck	Tehran, Iran
Toluene	Ghatran Shimi Co.	Tehran, Iran
Acetic acid (glacial) 100%	Merck	Tehran, Iran

anhydrous		
Formaldehyde solution min.37%	Merck	Tehran, Iran
Glycerol	Farabi Pharmaceutical Co.	Tehran, Iran
Hydrochloric acid	Ghatran Shimi Co.	Tehran, Iran
Acetic acid (Extra pure)	Dr Mojallali Chemical laboratory	Tehran, Iran
Benzalkonium chloride Vegetable disinfectant	Man Co.	Tehran, Iran
Super oxidized solution	ORYN S.O.S	Johannesburg, South Africa
Carboxymethylcellulose	Pars Chemie	Tehran, Iran
Diethyl ether		Tehran, Iran
Sebamed Baby Healing Cream	Sebamed	Tehran, Iran
Light green yellowish for microscopy	Merck	Tehran, Iran

Ponceau 2R	Sigma Chemical Co.	Tehran, Iran
Molybdatophosphoric acid	Titran. Co.	Tehran, Iran
Acid Fuc sine	Evans Sons Lescher & webb Ltd.	Tehran, Iran
Iron (III) chloride hexahydrate 99+%, extra pure	ACROS organics	Tehran, Iran
Hematoxylin	Fisher Scientific Co.	Tehran, Iran
Hematoxylin	BDH Chemical Ltd.	Tehran, Iran
Depilatory Cream	CineRe	Tehran, Iran
L-Ascorbic acid	Titranchem. Co.	Tehran, Iran
Hydroxypropyl cellulose	Titranchem. Co.	Tehran, Iran
Food for Laboratory mice	Behparvar Co.	Karaj, Iran
Protein%	19.5 - 30.5	
Lipid%	3.5 – 4.5	
Fiber%	4 – 4.5	
Ash%	Max 10	

Calcium%	0.95 – 1		
Phosphor%	0.65 – 0.7		
Salt	0.5 – 0.55		
Humidity%	Max 10		
Lysine%	1.15		
Methionine%	0.33		
Methionine and cysteine%	0.63		
Threonine%	0.72		
Tryptophan%	0.25		
Energy%	16.16 - 17		

Preparation of the gel

Different formulations of gels were examined to reach the best one based on OPR and pH evaluation.

First carbopol polymer was used as thickening agent. carbopol and SOS (Figure 5) were mixed together by continuous stirring. Then, since mixture of SOS and carbopol was completely liquid,

the required amount of neutralizing agent, triethanolamine (TEA), was added to increase its viscosity. Mixing was continued until a transparent gel was formed. At last, certain amount of glycerin was also added to the gel to make it more compatible with skin. Gel was prepared by a pharmacist's help (Table 2).



Figure 5. Super Oxidized Solution (SOS).

Table 2. Quantities of ingredients of carbopol-based gel.

Ingredients	Amount
SOS	22 ml
Carbopol	0.2 g
Triethanolamine 100%	1.7 ml
Glycerin 100%	5 ml

Carboxy methylcellulose (CMC) was another thickening agent which was used for gel preparation. Two gel formulations of CMC and SOS were prepared. These gel formulations are detailed in table 3. To prepare the gel required amount of CMC was mixed with SOS; then, continuous stirring was started until a transparent gel was prepared. At last, glycerin was also added to the formulation to make it more compatible with skin.

Table 3. Quantities of ingredients of CMC-based gel.

Ingredients	Gel 1	Gel 2
SOS	25 ml	25 ml
Carboxymethylcellulose	0.8 g	0.5 g
Glycerin	5 ml	5 ml

As ascorbic acid is an antioxidant, it was also added to one of the gels (Table 4) to examine ORP changes in presence and absence of this agent.

Table 4. Gel formulation containing ascorbic acid.

Ingredients	Gel 1
SOS	25 ml
Carboxymethylcellulose	0.8 g
Ascorbic acid	0.05 g

To make the blank gel (negative control), SOS was replaced by water; still, the same amount of CMC and glycerin was added (Table 5).

Table 5. Blank gel formulation used as a negative control.

Ingredients	Blank Gel 1	Blank Gel 2
Distilled water	25 ml	25 ml
Carboxymethylcellulose	0.8 g	0.5 g
Glycerin	5 ml	5 ml

Hydroxypropyl cellulose was also used (Table 6) as a thickening agent in gel preparation in order to assess its effect on SOS properties.

Table 6. Formulation of gel with hydroxylpropyl cellulose.

Ingredients	Gel
SOS	25 ml
Hydroxypropyl cellulose	1.5 g

ORP and pH measurement

As high ORP is the most important characteristic of SOS, it is essential to do our best to maintain this characteristic during gel production. Therefore, the ORP of gels were measured in different intervals. This measurement was done by Ezdo ORP meter, which was water proof and could be inserted into any solution. For the purpose of ORP measurement, the ORP meter was fixed on a holder to avoid any movement, and then its sensor was immersed (Figure 6) into the solution or gel. The ORP meter remained into the solution/gel until it showed a fixed number (mV), then the number was recorded as ORP of the solution/gel under study.



Figure 6. ORP Meter.

ORP level and pH of SOS and the prepared formulations were measured during experiments. pH was measured by pH indicator strips. Mentioned actions were done to find a suitable formulation (Table 7).

Table 7. Prepared gel to find a suitable formulation.

	Ingredients	Amount
Formulation 1	Carbopol	0.2 g
	TEA	1.7 ml
	Glycerin	5 ml
	SOS	22 ml
Formulation 2	CMC	0.8 g
	Glycerin	5 ml
	SOS	25 ml
Formulation 3	CMC	0.8
	SOS	25 ml
	Ascorbic acid	0.05 g
Formulation 4	CMC	0.5 g
	SOS	25 ml
	Glycerin	5 ml

ORP of below formulations were evaluated to find out whether presence of glycerin in formulation had any effect on ORP level or not. Their pH was also measured at the day of

production. In addition, ORP level of the formulation containing glycerin was measured three and eight days later (Table 8).

Table 8. Formulations with presence and absence of glycerin.

	Ingredients	Amount
Formulation 1	CMC	0.5 g
	SOS	25 ml
Formulation 2	CMC	0.5 g
	SOS	25 ml
	Glycerin	5 ml

ORP of below formulation was recorded at the day of production and three and five days following their preparation. The pH also was measured at the day of production (Table 9).

Table 9. Formulation used to measure ORP three and five days following their preparation.

Ingredients	Amount
CMC	0.5 g
SOS	25 ml
Glycerin	5 ml

The reason for ORP measurement of the same formulation in different days was to evaluate ORP changes following gel preparation. Since in our first and second experiments, we prepared again both SOS-containing gel (SOS gel) and blank gel (gel without SOS) on the days 7 and 5 of experiments, respectively, the ORP level was checked on the days 7 and 5 following gel preparation.

Another ORP meter device (Jeneway 3010) was also used to measure the ORP level in the below formulations (Table 10).

Table 10. Formulation used to measure ORP with another device.

	Ingredients	Amount
Formulation 1	CMC	0.5 g
	Glycerin	5 ml
	SOS	25 ml
Formulation 2	CMC	0.5 g
	Glycerin	5 ml
	Distilled water	25 ml
Solution	Superoxidized solution	

Animals

Swiss Webster outbred mice were maintained under standard experimental conditions; fed on normal pellet diet and water ad libitum. Vegetable including lettuce was also prepared once a week to supply their water soluble vitamins. Lettuce was washed and disinfected before use.

Prior to experiments, animals were housed in the groups of three or four in each cage.

The experiment started from 15 Swiss Webster mice including nine females and six males. These mice were used for breeding to provide first experiment animals. Therefore, they divided between four cages (Table 11).

Table 11. Parents.

Cage number	Number of male	Number of female
1	1	2
2	2	2
3	2	3
4	1	2

Nearly after two months new born mice were seen in each cage. The condition of each cage after breeding was as follows (Table 12).

Table 12. Number of Filial in each cage.

Cage number	F₁ (Filial 1)
1	23
2	11
3	9
4	17

Nearly after two weeks the females and males of F₁ generation separated from each other and were housed in the groups of 3 or 4 in each single-sex cage. Approximately after 3.5 and 4 months F₁ mice were used for the first experiment.

For the second experiment, some mice were selected from the F₁ group and they were used as parents for obtaining F₂ mice. The mating cages for the second experiment were as follows (Table 13).

Table 13. Number of male and female in each cage for mating.

Cage number	Male	Female
1	1	2
2	1	2
3	1	2
4	1	2

128 newborn mice were obtained as F₂ generation that nearly 60 of them (3-3.5 months old) were selected for second experiment.

Throughout the experimentation period, animals were kept single in each cage and they were maintained at 25 °C ± 2 and 12 h light/dark cycle. On the initial day of dosing, animals were 3 to 4 months old.

Excision wound model in mice

Between 72 hours to 24 hours before the beginning of wound generation, hairs of the interscapular region (between two shoulders) of mice were shaved by depilatory cream. Required amount of cream were added to the interscapular region for few minutes and then the hairs were removed by depilatory blade. After shaving, remaining cream was cleaned with wet cotton, and mice were returned back to their cages.

The day of wounding in animals was designated as day 0. The mice were lightly anesthetized using diethyl ether. Required amount of diethyl ether was added in a glass container with wide opening in order to drop animals easily into it. To avoid ether evaporation, the bottom of container was covered completely by cotton wool.

The mice should be anesthetized in transparent container to check its anesthetic situation regularly. If mice remain more than required time in the ether container, they will die and if they remain less than enough, they will become conscious during wound generation.

After anesthesia, animal skin was swabbed with 70% ethanol for disinfection.

Then, the wound area was outlined on the back of the animals with fine tipped pen using a quadrangular pattern plastic stencil. A full thickness of the excision wound of square area 8mm × 8mm was created along the markings using forceps and pointed scissors. Then post-surgery animals were housed individually in cages and their wounds were left undressed. In these procedures, animals were treated gently and all possible measures were taken to reduce pain in animals. The initial wound size was measured on day 0.

Animal grouping

After excision wound establishment, animals were divided into five groups consisting of 5 female and 5 male mice in each of them.

- Group I was treated with superoxidized solution.
- Group II was treated with SOS gel.
- Group III was treated with blank gel.
- Group IV served as positive control and was treated with Sebamed® Baby Healing Cream containing allantoin and panthenol which show healing property.
- Group V served as negative control with no treatment.

Animal treatment

The wounding day was considered as day 0. In this day just after making excision wound, animals in each group were treated with corresponding test product once. However, in the following days the wounds were treated with topical application of the test products twice a day every twelve hours for 15 consecutive days. For sampling one male and one female of each

group were sacrificed every three days. In day 15 all remaining animals were sacrificed and no application was done.

The test product was applied on the wound with clean cotton swab; each swab was used just once. As SOS was provided as a spray, we sprayed on the wound twice every application time to make sure that wound surface is fully covered. All wounds were left undressed to the open environment.

Weight and wound size analysis

In this study two animals in each group, a male and a female, were randomly chosen to survive until the end of experiment for wound size measurement and photography. Wound sizes of specified mice were measured on days 1st, 3rd, 6th, 9th, 12th and 15th under light diethyl ether anesthesia. This process was done placing a transparent paper over the wound and tracing it out by permanent marker to analyze wound size reduction.

Epithelialization occurs when the scab falls off and no residual of wound remains (Nayak *et al.*, 2010).

The wound contraction percentage was calculated using following formula:

$$\% \text{ Relative improvement} = \frac{\text{Wound area on day 0} - \text{Wound area on day } n}{\text{Wound Area on day 0}} \times 100$$

n = number of days (3rd, 6th, 9th, 12th and 15th day) (Kumar *et al.*, 2008)

While mice were still under light diethyl ether anesthesia, the progressive changes in wound area were also monitored by a digital camera (Canon 12.1 megapixel SD 780). During photography, the distance between mouse and camera was fixed and the camera lens was at the right angle to the wound surface.

Sampling of wound tissue

Tissue sampling was done to study cells, tissues and their structures.

Sampling from wound area should be done accurately by precision tools to avoid tissue damage or crush. It must also be perpendicular to the skin's wound surface to represent all layers of skin (Bahadori, 1991).

In these experiments, on days 3rd, 6th, 9th, 12th and 15th one male and one female mouse in each group were selected randomly to go through tissue sampling procedure. Each animal was sacrificed by diethyl ether. The animal was laid on a smooth surface and wound sampling was performed using fine forceps and sharp scissors.

After tissue removal, samples must be labeled to be identified easily. Since labels must be remained until staining phase, the type of paper and ink of marker are important (Bahadori, 1991). In this study, after sampling, specimens were put on a fine net. Then, their numbers were written by pencil on small filter paper and were placed into the same net. Finally, they were fixed by a thread and situated into fixative solution for required period of time.

Sample fixation

The aim of fixation is to preserve cells and other components of tissues in condition identical to that existing during life. Nonetheless, as materials are removed from body for histological examination, this aim could not be fulfilled completely. In practice the purpose of fixation is: (1) Prevent or arrest autolysis. Autolysis means self destruction and it is caused by intracellular enzymes after cell death. Normal behavior of intracellular enzymes change and they start to breakdown proteins and eventually promote cells' liquefaction. Autolysis accelerated at 37 °C but almost inhibited by heating the tissue to 57 °C. Autolysis affects the highly specialized cells of complex organ like kidney and brain more rapidly than collagens and elastic fibers. (2) Prevent bacterial decomposition. Bacterial decomposition causes changes in the tissues very similar to the autolysis by the presence of multiplying bacteria at time of death or by bacteria which normally exist in body such as nonpathogenic organisms. (3) Fortify tissue against deleterious effects of the different phases in the preparation of sections. (4) Leave tissue in a condition that facilitates differential staining with dyes and other reagents (Drury *et al.*, 1967).

Fixation has different effects; one of the most important of them is coagulation of tissue proteins and constituents to prevent their loss or diffusion during tissue processing. Another effect of fixation is to improve optical differentiation of tissues. Since the refractive index of some elements of cells is very close to the surrounding structures, they are invisible in living state when examined by ordinary microscope. Fixation causes optical differentiation of cells and tissues by changing their refractive indices in varying degrees which results in differentiation of structures often even in the absence of staining. Fixation also has a significant effect on the subsequent tissue staining as fixative agents make connections between tissue constituents and dyes (Drury *et al.*, 1967).

There are large varieties of fixative agents; in addition, it should be noted that single substance or known combinations of substances cannot preserve all tissue components. Hence, some fixations have limited application and most fixative solutions are a mixture of different reagents. Since it is crucial to fix the specimen immediately after sampling, fixative agents should already be prepared. The fixative solution has to be 25 to 50 times greater than sample volumes and the specimen should be thin enough for fixative agents penetration. Fixation time could be changed based on tissue thickness (Drury *et al.*, 1967; Bahadori, 1991).

In these experiments, Bouin's solution was used as fixative agent. It was composed of:

Saturated aqueous picric acid 100%	75 ml.
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Formaldehyde 100%	25 ml.
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Acetic acid glacial 100%	5 ml.
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Mixture of saturated aqueous picric acid and formaldehyde could be stored for long period of time; however, as glacial acetic acid is added to the solution, it must be used immediately.

Bouin's solution is a proper fixative for cytology; it is also used for microanatomy activities and small biopsies. However, it is not suitable for big blocks as its penetration degree is low and it lyses red blood cells (Bahadori, 1991).

In this study, after removing wound samples, they were placed into the net and then transferred to the Bouins' solution for 24 hours.

It is critical to make sure that all tissue samples are floating in the fixative solution completely.

After 24 hours tissue samples were removed from Bouin's solution and transferred to the 70 percent ethanol. Samples could remain intact in this solution for a long time. Since only one sample was available for each animal, to avoid losing it, the sample was cut by scalpel from the middle, and both halves were marked and wrapped into the net separately and transferred into two different jars of 70% ethanol. One half of each sample was kept in 70% ethanol and the other half went through the next phase of tissue processing, dehydration. In case anything goes wrong during sample processing, the other half could be reused.

Preparation of tissue for microtomy: Tissue processing

To examine and observe tissue with the light microscope, the slice of tissue must be thin enough to transmit light. Preparation of such thin slice is called section cutting or microtomy. Before microtomy, tissues should undergo preparatory treatment entailing impregnation of the specimen with an embedding medium to provide support and consistency for sectioning. These preparatory treatments are known as tissue processing which include dehydration, clearing, impregnation and blocking or embedding (Drury *et al.*, 1967).

The most commonly used embedding medium is paraffin wax which solidifies by cooling. In addition, cutting quality of paraffin blocks is appropriate; beside, they have high durability (Drury *et al.*, 1967).

Dehydration

Inasmuch as paraffin wax cannot penetrate the tissues in the presence of water, dehydration is done to solve this problem. The percentage of initial alcoholic solution in dehydration phase depends on the fixative agent, size and texture of tissue. Dehydration phase usually starts with immersion of tissue samples in 50% to 70% ethanol. Immersion of sample in a more concentrated alcohol causes quick shrinkage of tissue as its water is pulled out rapidly. On the other hand, dehydration, clearing and wax impregnation phases cause inevitably shrinkage and hardening of tissue no matter what technique is used, therefore, by careful choice of processing procedure shrinkage may be kept to minimum (Drury *et al.*, 1967; Bahadori, 1991).

In this study, the tissue samples were immersed respectively into 70%, 80%, 85%, 90%, 95% ethanol, and absolute ethanol twice for 15 minutes. The samples remained in each of them for 15 minutes. The volume of each alcohol solution must be 50 to 100 times greater than samples (Bahadori, 1991).

Clearing

Since alcohol is hardly miscible with paraffin wax, after dehydration, it is essential to treat tissues with reagent that mixes with both substances and which will be eliminated in the process of wax impregnation. This phase is called clearing as most reagents are used to raise refractive index of samples and make them more or less transparent (Drury *et al.*, 1967).

Different reagents are used in this phase and each has their own advantages and disadvantages. In this study toluene was used as a clearing agent. Toluene acts rapidly and clears small tissue blocks in 30 to 60 minutes and prevent the samples from hardening (Bahadori, 1991).

In this study, immediately after dehydration phase tissue blocks were transferred to equal mixture of absolute ethanol and toluene, then toluene twice for 15 minutes. Tissues were immersed in each solution for 15 minutes.

Impregnation

The function and aim of Paraffin wax impregnation is to remove clearing agent from tissue and to permeate paraffin wax into tissue which subsequently solidifies to produce a block for section cutting or microtomy (Drury *et al.*, 1967).

Ideally the consistency of solidified embedding medium should be exactly the same as the specimen, but it is rarely achieved since specimen consistency is in a wide range and it is impractical to use large variety of solidifying media with different consistency. The hardness of paraffin wax is determined by its melting point, waxes with 54-56 °C melting points are harder than waxes with 45-50 °C melting point. The wax will be chosen based on the average temperature in working area, nature of the specimen and the thickness of the sections (Drury *et al.*, 1967).

The duration of wax impregnation and number of changes of wax depend on the size of specimen and its consistency. Tissues generally are transferred from clearing agent to paraffin wax; however, working with fragile tissues need one more step. With fragile specimen it is recommended to transfer specimens from clearing agent to equal mixture of clearing agent and wax as an intermediate step and then transfer them to pure paraffin wax (Drury *et al.*, 1967).

In this study, paraffin wax with melting point 56–58 °C was used. Specimens were immersed first in an equal mixture of toluene and paraffin wax for 15 minutes in 57 °C in an incubator; they

then were transferred to paraffin wax I, paraffin wax II and paraffin wax III each one for 15 minutes in 57°C in the incubator.

Note: the incubator's temperature should be watched carefully; its temperature must be between 56 and 57°C as higher temperature causes tissue hardening and shrinkage.

Embedding

As specimens were completely impregnated with paraffin wax, it is necessary to obtain a solid block containing the tissues. Block production is done by filling a mould of suitable size with paraffin wax and putting the specimen in the mould in correct orientation to be sure that it is being cut in the correct plane and at last cooling the paraffin mould to promote solidification (Drury *et al.*, 1967).

In this study, paper moulds were used which were made by appropriate paper folding, the moulds were cubes with 1.5cm × 1.5cm × 1.5 cm in dimension. At the end of impregnation phase, the container of paraffin wax III was removed from incubator and was put on leg with metal net above the alcoholic lamp. The glassy jar of paraffin wax III should be kept at that situation until the end of embedding to avoid solidification of paraffin.

The paraffin container must not be in direct contact with alcohol lamp's flame, because it may increase the paraffin temperature which causes tissue shrinkage.

Then the specimens were removed from Paraffin wax III container individually and were placed into moulds to make paraffin blocks. In this process; first, specimen's number was recorded on paper mould. Second, the mould for each specimen was filled half with paraffin wax from

paraffin wax III by pipette and waited for few seconds until the paraffin in the mould solidified a little, then specimen was transferred from the paraffin jar into mould in right angle by fine forceps. The specimen should retain perpendicularly into the paraffin wax mould (Figure 7).

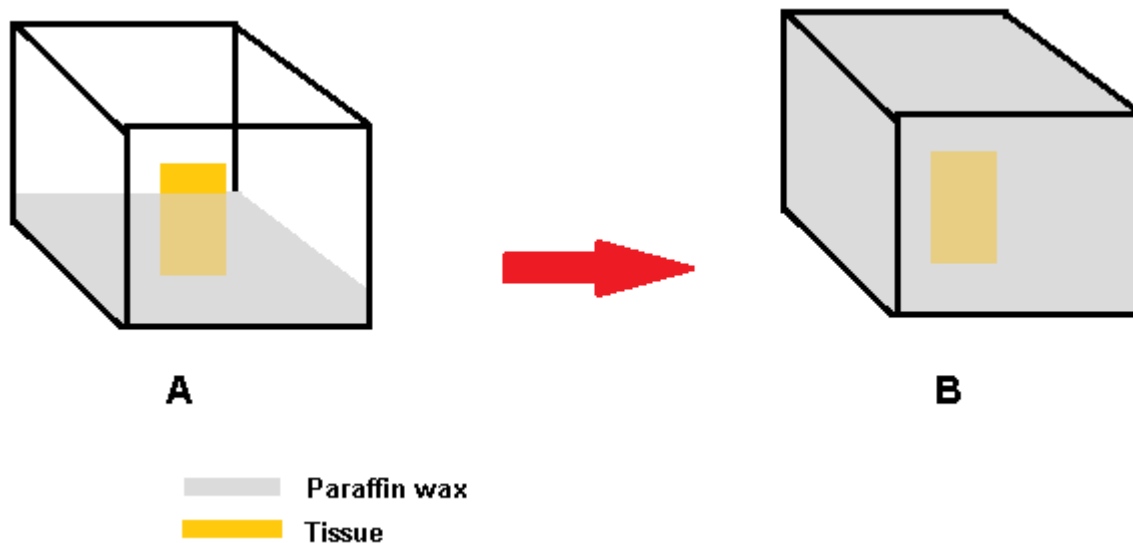


Figure 7. Orientation of specimen in paraffin block. The specimen should be retained perpendicularly into the paraffin wax mould. (A) make the specimen constant into the half filled paraffin wax. (B) fill the mould completely with paraffin wax.

After the specimen stayed firmly on the paraffin wax in the mould, the mould was filled completely with paraffin wax until all part of specimen was immersed into it. At last, the moulds were incubated at room temperature for 24 hours for complete solidifying.

There must not be long pause between first and second paraffin filling in the mould, because long pause causes complete solidifying of first layer of paraffin, and when second portions of paraffin is poured, the first and second paraffin cannot fuse to each other and the mould will contain two distinct layer of paraffin which will cause block shrinkage in the next steps.

When the blocks were completely cooled, they were removed from the mould and stored in a cool place and protected from dust.

There is no doubt that if tissues are intended for future microscopic examination, paraffin blocks preserve them better than any fixative or preserving reagent(Drury *et al.*, 1967).

Trimming the blocks

When everything has gone well in fixing, dehydrating, impregnation and embedding, the result is a paraffin block containing the specimen surrounded by generally a too generous amount of paraffin wax. The surplus paraffin needs to be removed and the paraffin block will be trimmed in its final form prior to sectioning.

How thick the paraffin layer surrounding the specimen should be is dictated by personal preference of the microtommist. As a general rule, it's a good practice to make the paraffin layer surrounding the specimen not too thick, the larger the surface of the paraffin block to be sectioned, the higher the risk of problems in section cutting and it serves no purpose to cut blank paraffin. On the other hand, there should be enough paraffin left to handle the sections in a comfortable manner. Two opposite side of blocks may be cut close to the tissue as just 2-3 mm is left around the tissue. The upper and lower surface of blocks should be parallel and flat, square or rectangular. The depth of the block depends on the thickness of the tissue plus 2-4 mm of underlying embedding medium. Shallow block is more stable than deeper block when it is in the microtome (Drury *et al.*, 1967).

When several serial sections should be mounted under a common cover slip the surrounding paraffin should be kept to a minimum, 1 or 2 mm. The thinner the surrounding paraffin coat, the more sections can be mounted under a single cover slip. When only individual sections are to be mounted onto slides the surrounding paraffin coat can be kept larger (Drury *et al.*, 1967).

Trimming should be done with straight edge sharp scalpel, and paraffin in narrow layer should be cut from the block since cutting too thick layer of paraffin might cause the block (and the specimen) crack. If the block cracks, it should be melt and mould again. As mentioned the upper and lower sides of the trimmed block should be parallel, if not this will result in curved ribbons of sections. This causes problem when slides containing several serial sections (Figure 8).

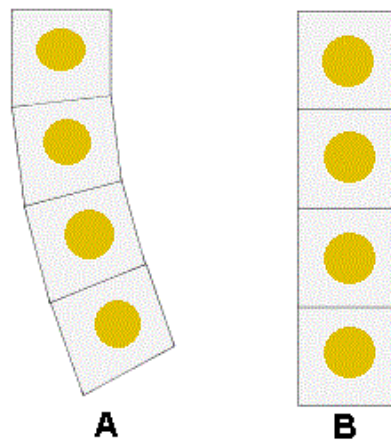


Figure 8. Curved and straight section ribbon. (A) Curved ribbon due to inadequate trimming, upper and lower side of the block is not parallel. (B) Block trimmed properly.

In this study, blocks were taken on a clean table, and then they individually were trimmed by sharp scalpel until just 2 to 3mm of paraffin covered each side of the tissue. The layers of

paraffin were removed slowly and delicately to avoid block shrinkage. The top and bottom surfaces of blocks also were flattened to near the tissue. The trimming were done delicately and tried to make blocks with right angles to avoid curved ribbon in sectioning.

Mounting the trimmed block on paraffin table

Wooden blocks of suitable size to fit microtome chuck were used as object holder. The blocks were made from hard wood with plane surface. After the wooden blocks were labeled by a permanent marker or sticky paper on both sides to show the tissue's number, in order to fix the paraffin wax block on holder (wooden block), a flat metal blade was heated on Bunsen burner. Then melted the lower side of the paraffin block with the hot blade for a second and then press the paraffin block on the wooden block slightly. In addition, in order to complete block adhesion, the hot blade was also applied to the four side of wax block at the junction with wooden blocks (Drury *et al.*, 1967). At last paraffin blocks were left in room temperature for cooling (Figure 9).



Figure 9. Wooden blocks.

Orientation of paraffin blocks should be considered in the experiment. The specimen should be set perpendicularly to the holder surface, as all layers of skin must be sectioned through microtome.

After mounting the trimmed block, two opposite sides of paraffin block were covered by very thin layer of paraffin 44 °C. A heated scalpel was used to melt the paraffin 44 °C and cover two opposite sides of paraffin blocks. As melting point of paraffin 44 °C is lower than paraffin 56 – 58 °C which was used for embedding, during microtomy paraffin 44 °C melts sooner and provides attachment between serial sections. In addition, to achieve this purpose, the sides of paraffin blocks covered by paraffin 44 °C should be parallel to the microtome knife (Figure 10).

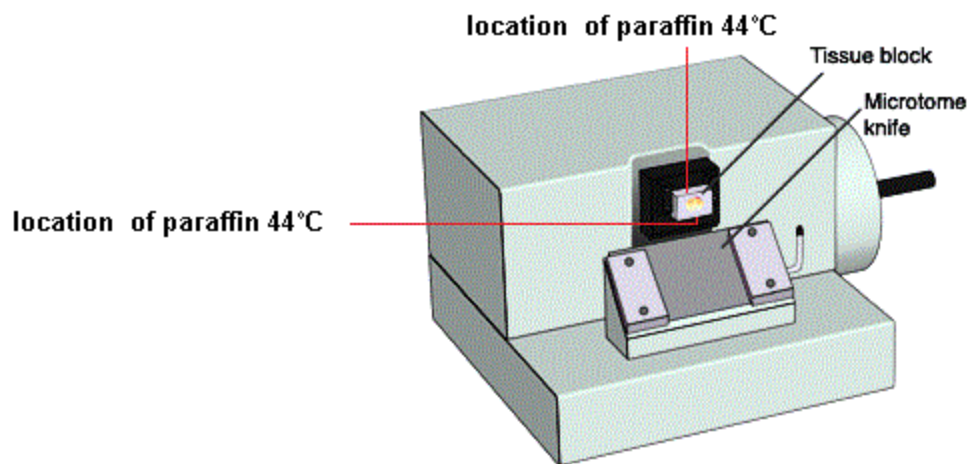


Figure 10. Correct location of paraffin block in microtome. The microtome's knife should be parallel to the surface covered by paraffin 44 °C.

(<https://www.nationaldiagnostics.com/histology/article/sectioning>)

Microtomy

Microtomy is a significant phase of tissue sectioning. The production of good quality sections sometimes is wasted by poor technique and unsatisfactory equipments at the section cutting stage. The well preserved tissue during tissue processing may be damaged during attempts to cut sections, which causes serious difficulty in microscopy and interpretation.

Sections are prepared on an instrument known as microtome. Various types of microtomes are available. Their all common features are separate and firm support for knife and tissue block and a mechanism which predetermine the number of micron on each cutting stroke. The thickness of sections may vary from one micron to several hundred microns. The most common range is 5-10 μ , but the thickness of sections depends on the tissue consistency and embedding medium. The tissue with looser texture such as adipose tissue the thicker can section be made. The thick sectioning (15-40 μ) in such tissues are not only easier but they are often more informative. On the other hand a dense cellular tissue like spleen is easier to observe with the microscope in thin section (5 μ) (Drury *et al.*, 1967).

The rotary microtome was used in this study. The rotary microtome works by turning a wheel at one side of the machine. Its knife is fixed and the object move against the knife. According to the selected thickness of cutting the object rising and falling vertically (Drury *et al.*, 1967).

Cutting technique

For cutting the paraffin blocks the following steps should be done:

1. Fix the object in the clamp.

2. Tighten securely and check that paraffin block is correctly oriented in relation to the knife edge.
3. Secure the knife in knife's clamp and select appropriate angle of tilt.
4. Moving the block upside down or moving the knife back and forward to reach each other and make sure that block's surface and knife are located in against each other evenly.
5. Select the required thickness setting.
6. After microtome adjustment, the paraffin block is cut until a complete section is obtained.
7. In serial sectioning, the ribbons in almost 30 cm length could be provided. The free end of the ribbon is held by needle or fine forceps, and guided away from the microtome.
8. When sufficient sections have been cut, the ribbon is detached from the knife by inserting the scalpel blade between ribbon and knife edge.
9. Then lay the ribbons on a piece of dark paper prior to mounting on glass (Drury *et al.*, 1967)

In this study all above mentioned steps were done to provide intact serial sections of tissue and 100 sections were obtained from each single tissue.

Mounting tissue on slides

After providing ribbons of tissue, since it is extremely difficult to manipulate those thin sections without damaging them, it is necessary to attach the sections onto glass slides. For this purpose, first the slides were prepared. Second, the slides were covered by glue. Finally, the tissues were attached to them properly.

Glass slides are available in various sizes, the most common size being 75 × 25 mm with the thickness of 1-1.2 mm were used. Prior to section mounting, the glass slides should be labeled. The number of tissue and the number of slide were written on one side of slides by diamond blade. The slides were not clean enough for use when received from the suppliers; hence, after labeling they were subsequently polished by the polishing solution which was the mixture of 100 ml of 70 percent ethanol and 3 to 4 drops of hydrochloric acid. The slides remained for seconds in the solution and then cleaned with dry fluff free cloth.

The sections should be tightly attached to the glass surface of the slide to prevent them from loosening during preparation process. Egg albumin was used for tissue attachment. For albumin glue preparation, albumin of the egg was separated and added to the graduated cylinder; subsequently, the equal volume of glycerin and distilled water were also added to the container. Then, all those materials were placed into refrigerator to pass through filter paper. The filtered solution was the final product. Albumin glue's shelf life is about a month; however, it's better to prepare a fresh solution prior to use. To avoid mold growth in albumin glue a piece of thymol was located into its container. The glue smeared thinly on slide before mounting the sections.

For mounting sections; first, the glue was smeared thinly on the slide; then, one to two drops of distilled water were added to it to ease sections movement and arrangement on slide. Then, for production of each slide, the first ten sections were removed and cut by fine forceps and scalpel delicately and located on top of the glass slide. As each slide contained 20 sections, second ribbon containing ten subsequent sections was placed on the same slide exactly under the first serial sections. For easier observation under microscope the sections must be in the same direction and exactly in a column under each other (Figure 11). After sections arrangement, the extra amount of water on slides was dried by fluff free cloth. Then, to avoid tissue shriveling, the slides were put on the water bath or Bain marie in 70°C until the paraffin around the tissue was melted completely. Finally, the slides were placed on a clean table for three to four days to dry.



Figure 11. Mounted sections.

Tissue staining

Section preparation

Paraffin sections are almost stained on the slide after attachment. Before attaining, the sections must go through some preparatory treatments which are removal of paraffin wax, removal of toluene with absolute alcohol, treatment with decreasing concentrations of alcohol and immersion into water (Drury *et al.*, 1967).

As paraffin wax is poorly permeable in stains it should be removed by a solvent. In this study, toluene was used as a solvent. For this purpose, the slides were placed into a jar and immersed into toluene twice and then equal mixture of toluene and absolute alcohol for 15 minutes each. Then, since toluene is not miscible with aqueous solution and low concentration alcohol, it must be removed by absolute alcohol (Drury *et al.*, 1967). Subsequently, toluene was removed by immersion of slides into absolute alcohol twice for 15 minutes. Then, to avoid the possibility of detachment of sections the absolute alcohol treatment was followed by 95%, 90%, 85%, 80% and at last 70% ethanol (Drury *et al.*, 1967). Then, they were immersed into distilled water for few minutes before initiation of staining phase.

Masson Trichrome stain

This staining technique is ideal for selective demonstration of collagen fibers, muscle, fibrin, and erythrocytes. In this technique all used dyes are in an acidic solution. They include hematoxylin nuclear stain followed by staining of cytoplasm with red dye, fixing and differentiation of red dye with phosphomolybdic acid and finally light green to stain fibers (Drury *et al.*, 1967).

To start staining, the tissue sections first went through post fixative phase. Slides were immersed into Bouin's solution and were incubated for 60 minutes at 60 °C. After that, the following steps (Table 14) were done for staining(Bancroft *et al.*, 2002) .

Table 14. Protocol for Masson Trichrome staining

Wash slides in running water	10 minutes
Stain nuclei with Weigert's Iron Haematoxylin :	2 minutes
Solution A	
Haematoxylin	5 gm
Absolute ethanol	500ml
Solution B	
30% aqueous ferric chloride	20 ml
Distilled water	500 ml
Concentrated hydrochloric acid	5 ml

Mix equal parts of solutions A and B immediately before use. Solution A and B could remain in refrigerator separately for several days. However, after mixturing they must be used immediately. For 30% aqueous ferric chloride preparation the following formula was used: $\frac{30}{x} = \frac{100}{20ml} = \frac{600}{100} = 6 \text{ g ferric chloride in 20 ml distilled water}$	
Wash in distilled water: The slides were washed twice with distilled water for further cleaning.	
Stain samples with red cytoplasmic stain: 1 %Ponceau 2R in 1 % acetic acid. 2 parts 1 % Acid Fuchsin in 1 % acetic acid 1 part These two solutions could remain in refrigerator for several days separately. However, mixture of them should be prepared immediately before use. For preparation of 1% acetic acid, 1ml acetic acid should be added to 100 ml distilled	5 minutes

Staining fibers by mixture of 2% Light Green in 1 % Acetic Acid:	10 minutes
Light Green 2 g	
1% Acetic Acid 100ml	
Light green solution could remain for 6 months in the refrigerator.	
Wash well in 1% acetic acid	1 minute

After staining the slides went through dehydration, clearing and mounting phases and the results were:

Nuclei-blue/black

Cytoplasm, muscle and red blood cell (RBC)-red

Collagen-blue/green

(Bancroft *et al.*, 2002).

Dehydration

Toluene would not clear and render sections transparent unless the section become completely dehydrated(Drury *et al.*, 1967). Therefore, the slides went through dehydration by passing through 70%, 80%, 85%, 90% and 95% ethanol, and absolute alcohol twice for 10 minutes.

Clearing

Clearing is done to remove alcohol and cause transparency of sections before mounting. In this process, the slides were immersed in equal mixture of absolute alcohol and toluene, toluene I and toluene II each for 10 minutes.

At last, the slides were transferred from jar on the clean table to dry out.

Mounting coverslip

A coverslip is applied to protect and preserve the sections. Two type of material are used for coverslip mounting, aqueous media and resinous media. In this study, Canada balsam was used to mount coverslips on slides which falls to resinous media category.

In this process, coverslips should have been cleaned in cleaning solution comprising 100 ml 70% ethanol and 2 to 3 drops of hydrochloric acid. They remained in the solution for a few seconds, and then they were dried with fluff free cloth. Cleaned coverslips were ready to mount. The largest available coverslip in market (24 mm× 50 mm) was used to cover all sections.

The required amount of Canada balsam, three to four droplets, was added on coverslips for its attachment. To avoid bubble formation, one side of coverslip was mounted on the sections first, and then the other side was mounted slowly. Then, the cover slip went under pressure to spread the Canada balsam all around it smoothly. If any bubble remains under the coverslip, it may crack under microscope and also cause problem during microscopy. Therefore, the Canada balsam spreading should have done delicately and accurately. After mounting, surplus of Canada

balsam was cleaned by toluene soaked cloth. Finally, slides transferred to a clean table to dry out for 10 days.

Microscopy

Tissue samples were examined for the following histological criteria under light microscope:

The extent of reepithelization

The maturation of the epidermal cells

The thickness of the epidermal layer

The degree of granulated tissue formation:

- Fibroblast formation
- Amount of collagen
- Organization of collagen fibers

The presence of disorganized collagen fibers with prominent vascularity considered as an indication for immature granulation tissue. And the presence of well formed and horizontally-oriented collagen fibers, as evident by Masson Trichrome stain, along with inconspicuous blood vessels were considered as an indication for the maturity of the scar (Khalil *et al.*, 2007).

The presence of angiogenesis

The presence of glands

The presence of hair follicles

For microscopy, at first one slide for each animal was observed and if more examination was needed, two more slides were observed. Again, if more observation was needed, to more slides assessed until proper result obtained.

Photography of sections

To show what was observed under microscope, the slides were photographed by 1.3 megapixel CCD camera. The camera was mounted on the microscope and connected to the laptop through USB cable. It was recognized in laptop by AMcap program.

The photos were taken under $4 \times$ magnifications to show perspective of the sections and $10\times$ and $40\times$ magnifications to reveal details of sections.

Measurement of wound size

In order to increase the accuracy of wound size measurement, a software analysis was implemented. First, in wound pictures, which were taken under the same condition, boundaries of wounds were pointed. Then coordinates of those points and a point in the center of wound were uploaded to the software called image area. This software calculates the wound area through dividing it to as many as triangles it could with the help of those points.

Statistical analysis

Obtained results from five groups were calculated by applying Anova and T-test. In addition, relative improvement was used to calculate wound areas' changes in every three days from day 0 to day 15. The relative improvement is calculated as follows:

$$\text{rel.imp3} = \frac{\text{day 0} - \text{day 3}}{\text{day 0}}, \text{rel.imp6} = \frac{\text{day 0} - \text{day 6}}{\text{day 0}}, \text{rel.imp9} = \frac{\text{day 0} - \text{day 9}}{\text{day 0}},$$

$$\text{rel.imp12} = \frac{\text{day 0} - \text{day 12}}{\text{day 0}}, \text{rel.imp15} = \frac{\text{day 0} - \text{day 15}}{\text{day 0}}$$

Differences were considered significant at $P < 0.05$.

Chapter3:

Results

Preparation of gel

The carbopol-based formulation containing 22 ml SOS and 1.7 ml TEA showed a gel-like texture. In contrast, both CMC-based formulations formed a viscous liquid instead of gel. Besides, since CMC works as a thickening agent, first formulation with 0.8 g CMC had a higher viscosity than the second one with 0.5 g CMC (Table 3).

Adding 0.05 g ascorbic acid to 0.8g CMC and 25 ml SOS did not cause any changes in the viscosity of formulation.

Another formulation containing 1.5 g of hydroxypropyl cellulose and 25 ml SOS did not turn into gel. It maintained its liquid form, although it was stirred a lot and kept for 24 hours at room temperature. Thus, it was not suitable for purpose of present study.

Notwithstanding the fact that CMC and carbopol-based formulations had different textures, both had sufficient viscosity to remain on the wound area.

ORP and pH measurement

The ORP and pH of all formulae and also SOS were measured to obtain a suitable formulation for SOS gel production.

To compare ORP and pH level of prepared formulations to SOS, first the SO properties were measured for three times (Table 15).

Table 15. ORP and pH of SOS

Name	ORP (mV)	pH
SOS	509	2<pH<3
SOS	525	2<pH<3
SOS	510	pH=3

Also, ORP and pH of below formulations were measured (Table 16).

Table 16. ORP and pH of formulations.

Formulation	ORP (mV)	pH
Carbopol 0.2 g TEA 1.7 ml Glycerin 5 ml SOS 22 ml	(-)120	9<pH<10
CMC 0.8 g Glycerin 5 ml SOS 25 ml	(+)135	6<pH<7

In above formulations (Table 16) CMC-based formulation had higher level of ORP than carbopol-based one. In addition, as skin surface pH is between 4 and 6.5 (Yosipovitch *et al.*,

1998), the pH of CMC-based SOS gel was more compatible with skin, and its protective natural acid mantle.

To find out which ingredient in carbopol-based SOS gel caused the huge decrease in ORP, the pH and ORP level of formulations with and without TEA were measured (Table 17).

The results of measurement demonstrated that TEA factor working as neutralizing agent (Alimi, 2011) increased pH of the formulations by converting the acidic Carbopol polymer to a salt to achieve maximum thickening. Hence, inasmuch as pH is inversely proportional to ORP level of SOS (Thorn *et al.*, 2012), it might be concluded that low ORP level is due to this factor. Therefore, as without TEA the mixture of carbopol and SOS remained liquid and could not turn into gel, the carbopol-based gel was omitted from the test.

Table 17. ORP and pH of carbopol base gel with and without TEA

Formulation		ORP (mV)	pH
Carbopol	0.15 g	(-)125	10<pH<11
TEA	3 ml		
SOS	25 ml		
Carbopol	0.15 g	(+)330	2<pH<3
SOS	12.5 ml		

Since CMC-based formulation had significantly a higher ORP value among other ones, this formulation was investigated further (Table 16).

As ascorbic acid shows antioxidant activity(Cort, 1974), pH and ORP level of formulation containing ascorbic acid was also examined in order to see whether it has positive or negative impact on ORP level of formulation (Table 18).

As ascorbic acid decreased the ORP level of formulation, it was not utilized any more during this study.

Table 18. pH and ORP of CMC-based SOS gel with and without ascorbic acid

Formulation		ORP (mV)	pH
CMC	0.8 g	(+)199	5<pH<6
SOS	25 ml		
Glycerin	5 ml		
CMC	0.8 g	(-)38	4.5<pH<5
SOS	25 ml		
Ascorbic acid	0.05 g		

Due to the fact that ascorbic acid did not increase ORP level of the mentioned formulation (Table 18), more efforts were done to increase the ORP value.

Thus, to increase SOS level in formulation, CMC was reduced from 0.8 g to 0.5 g (0.3 g reduction). Changes made caused an increase in the level of ORP (Table 19). Since, gel B containing 0.5 g of CMC showed a higher ORP value and its viscosity was acceptable, we decided to use this formulation in our experiments.

Table 19. Comparison of ORP level in formulations containing 0.8 and 0.5 g of CMC.

	Gel A		Gel B	
Formulation	CMC	0.8 g	CMC	0.5 g
	SOS	25 ml $\approx$ 25 g	SOS	25 ml $\approx$ 25 g
	Glycerin	5 ml $\approx$ 6 g	Glycerin	5 ml $\approx$ 6 g
ORP (mv)	(+)130		(+)220	

To examine influence of glycerin on ORP level of CMC-based formulations, the ORP of formulations with and without glycerin were measured (Table 21).

The glycerin did not have considerable effect on formulation's ORP level. In addition, since glycerin make the formulation more compatible with the skin, in the rest of study below formulation was utilized as ultimate product (Table 20).

Table 20. Ultimate product formulation.

Ingredients	Amount
CMC	0.5 g
SOS	25 ml
Glycerin	5 ml

Table 21. ORP of formulations with and without glycerin

Formulation		ORP (mV)	pH
CMC	0.5 g	(+)216	5<pH<6
SOS	25 ml		
CMC	0.5 g	(+)231	5<pH<6
SOS	25 ml		
Glycerin	5 ml		

As approved formulation was remade in two main experiments after 7 days in test one and 5 days in test two, to make sure that formulation retains its ORP level during the experiment, its ORP was measured after 3 and 7days in test one and after 3 and 5 days in test two (Table22 and 23).

Table 22. Measuring ORP of CMC-based formulation for test one.

Formulation		Day 0	Day 3	Day 7
CMC	0.5 g	ORP = (+)231	ORP= (+)223	ORP= (+)227
SOS	25 ml			
Glycerin	5 ml			

Table 23. Measuring ORP of CMC-based formulation for test two.

Formulation		Day 0	Day 3	Day 5
CMC	0.5 g	ORP = (+)192	ORP= (+)188	ORP= (+)196
SOS	25 ml			
Glycerin	5 ml			

To be sure about the accuracy of ORP measurement, the ORPs of SOS, SOS gel and blank gel were measured twice by another device (Table 24).

Table 24. Measurement of ORP of SOS, SOS gel and blank gel by another device (Jeneway 3010).

Name		ORP (mV)
SOS gel		1. (+)231 2. (+)203
CMC	0.5 g	
SOS	25 ml	

Glycerin 5 ml	
Blank gel	1. (-) 120 2. (-)101
CMC 0.5 g	
Distilled water 25 ml	
Glycerin 5 ml	
Super oxidized solution	1. (+)606 2. (+)560

Results of wound closure analysis

Comparing the results of test one and two, SOS, blank gel and negative control group in first experiment had better healing than second experiment. In contrast, healing of SOS gel group was better in second test, and positive control group's healing was approximately the same in both of them. However, the statistical comparisons between results of first and second experiments of each group in different days show no significant difference.

The summary of wound closure results for the experiments is shown in Figure 12, 13, 14, 15 and 16. Groups were compared with each other based on their rel.imp values in days 3, 6,9,12 and 15.

Even though there were some differences between values of rel.imp of each group, no significant differences were seen statistically in day 3 (Figure 12).

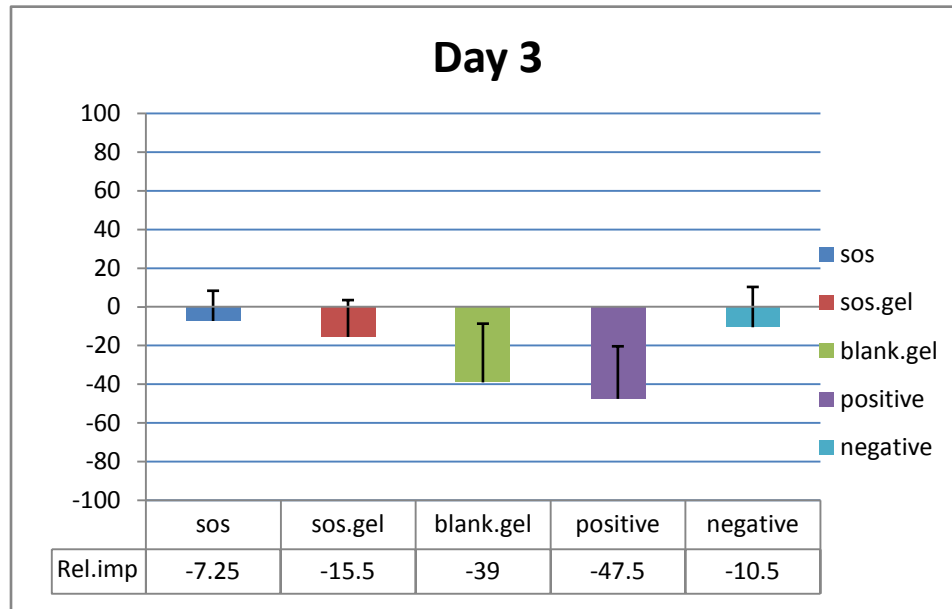


Figure 12. Comparing rel.imp of all groups on day 3. The results are expressed as means $\pm$ SEM of two independent experiments.

Notwithstanding the fact that wound area of all groups underwent some changes, still no significant differences were observed between their rel.imp on day 6 (Figure 13)

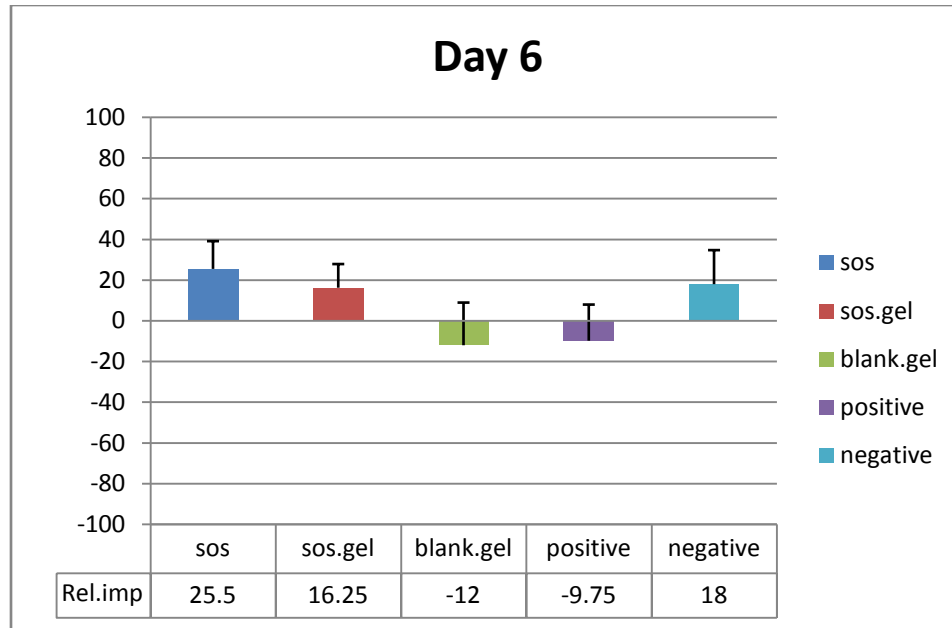


Figure 13. Comparing rel.imp of all groups on day 6. The results are expressed as means $\pm$ SEM of two independent experiments.

In day 9 all groups show an increasing trend in their wound closure, however, no statistically difference was observed among them (Figure 14).

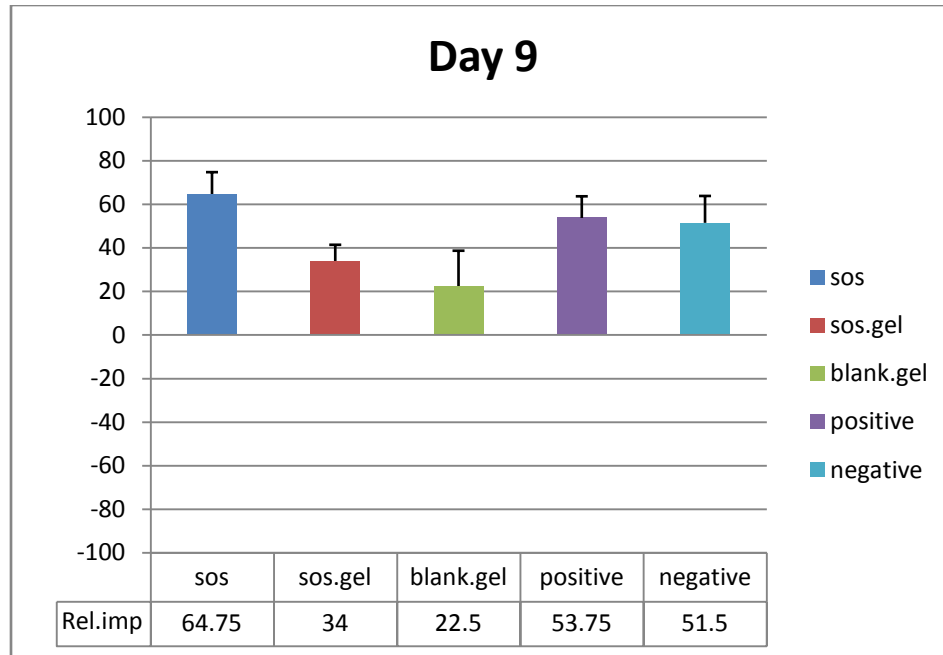


Figure 14. Comparing rel.imp of all groups on day 9. The results are expressed as means $\pm$ SEM of two independent experiments.

Contrary to previous days, in day 12, mice treated with blank gel exhibited substantially less wound closure than other groups which was also statistically significant. Furthermore, SOS was the only group displaying complete epithelialization (Figure 15).

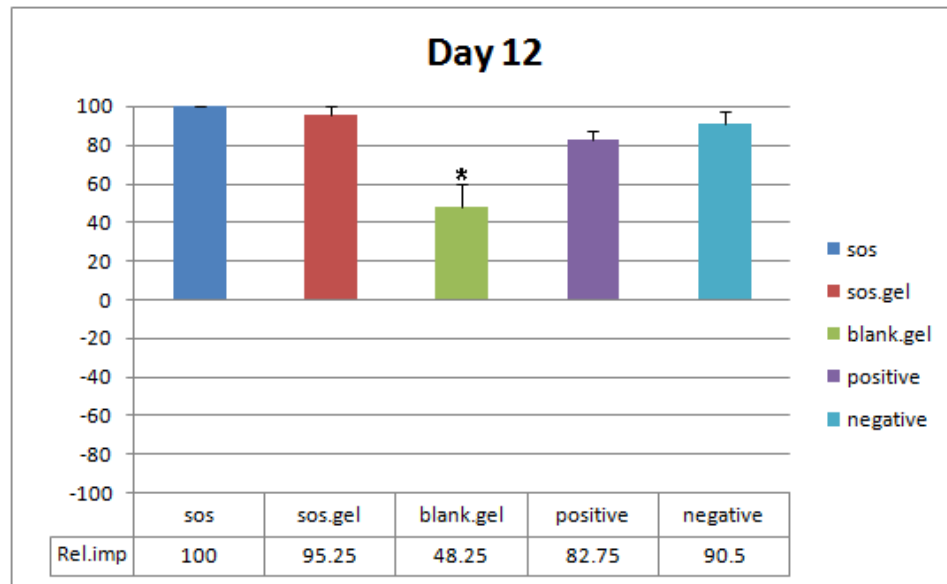


Figure 15. Comparing rel.imp of groups on day 12. Star indicates significant difference for rel.imp of blank gel group in day 12 in comparison with others ($*P < 0.05$). The results are expressed as means $\pm$ SEM of two independent experiments.

In day 15 except blank gel four other groups reveal complete recovery; hence, there was a significant difference between values of rel.imp of blank gel in comparison with other groups (Figure 16).

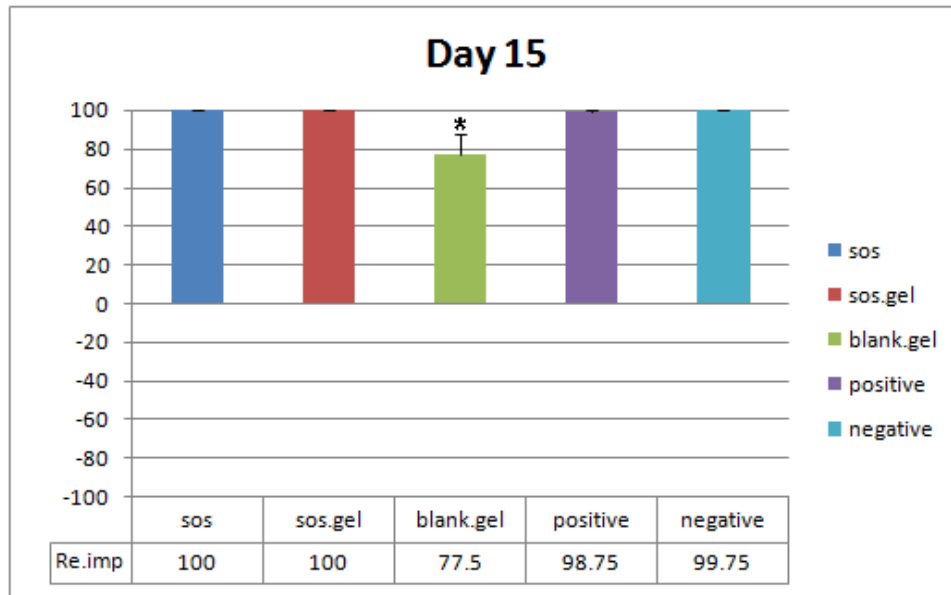


Figure 16. Comparing rel.imp of groups in day 15. Star indicates significant difference for rel.imp of blank gel group on day 15 in comparison with others ($*P < 0.05$). The results are expressed as means $\pm$ SEM of two independent experiments.

What was explicit in Figures 12 to 16 was that generally wound closure in SOS was more pronounced than the other groups in all days. However, SOS gel just displayed its healing effect in day 12 onwards, which are also shown in Figure 18.

Next graphs compare relative improvement of days 3, 6, 9, 12 and 15 for each individual group. In their statistical analysis relative improvement of each day was compared with three days earlier. For SOS group, a sharp decrease in the wound area was observed in the first 12 days, and healing was completed on day 12. Statistical analysis also reveals significant increase in value at day 9 (Figure 17).



Figure 17. Comparison of relative improvement of SOS group. Star indicates significant difference between rel.imp of each day in comparison with three days earlier ($*P < 0.05$). The results are expressed as means $\pm$ SEM of two independent experiments.

In SOS gel group, a noticeable decline in the wound area was observed in the first 12 days, while a gradual reduction in the wound area was recorded between day 12 and 15 when wound was fully healed. Statistical analysis revealed a significant increase in value of rel.imp of day12 comparing to day 9 (Figure 18).

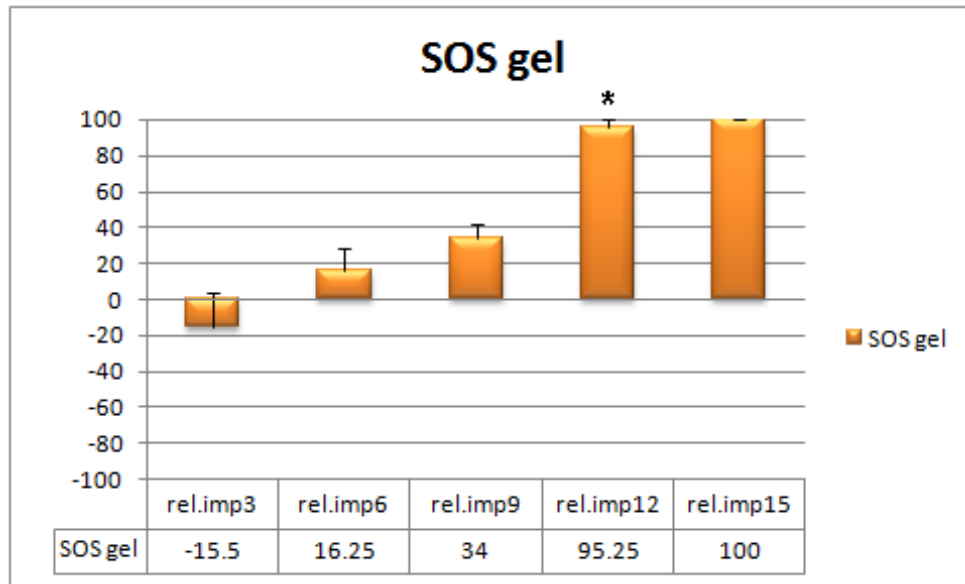


Figure 18. Comparison of relative improvement of SOS gel group. Star indicates significant difference between rel.imp of each day in comparison with three days earlier ($*P < 0.05$). The results are expressed as means $\pm$ SEM of two independent experiments.

The blank gel's graph shows healing improvement in all days, but statistically no significant alterations occurred (Figure 19).

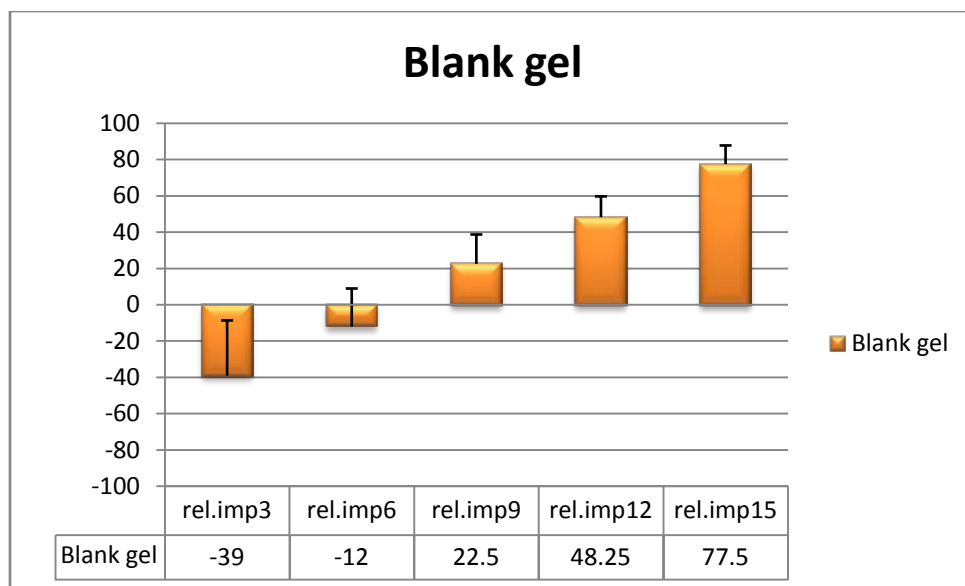


Figure 19. Comparison of relative improvement of blank gel group. The results are expressed as means $\pm$ SEM of two independent experiments.

The positive control group's graph reveals substantial decrease in wound area from day 3 to 15. Based on statistical analysis, day9 revealed significant difference in its relative improvement value in comparison to day 6 (Figure 20).

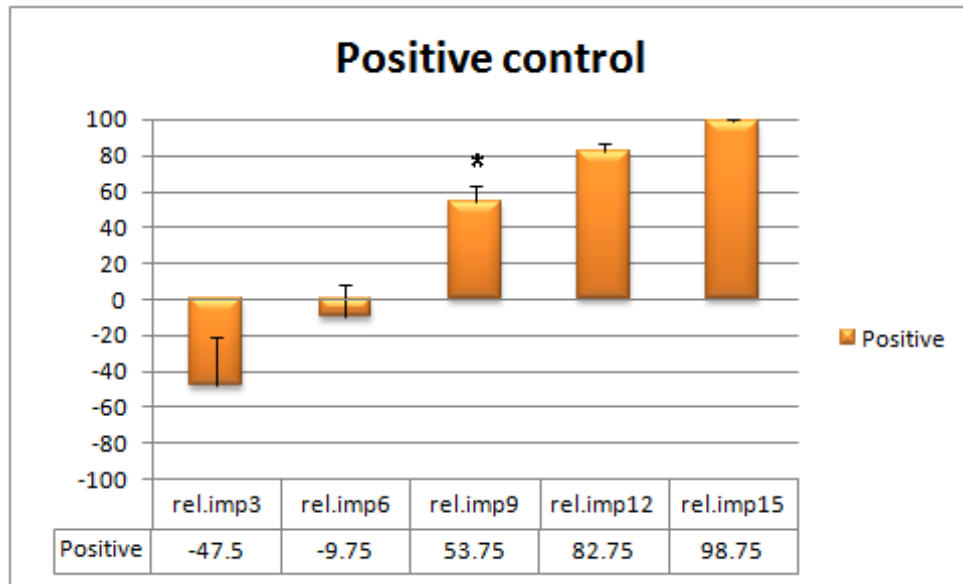


Figure 20. Comparison of relative improvement of positive control group. Star indicates significant difference between rel.imp of each day in comparison with three days earlier ($*P<0.05$). The results are expressed as means $\pm$ SEM of two independent experiments.

In negative control group, although there was a growth healing rate, no significant differences between days were observed (Figure 21).

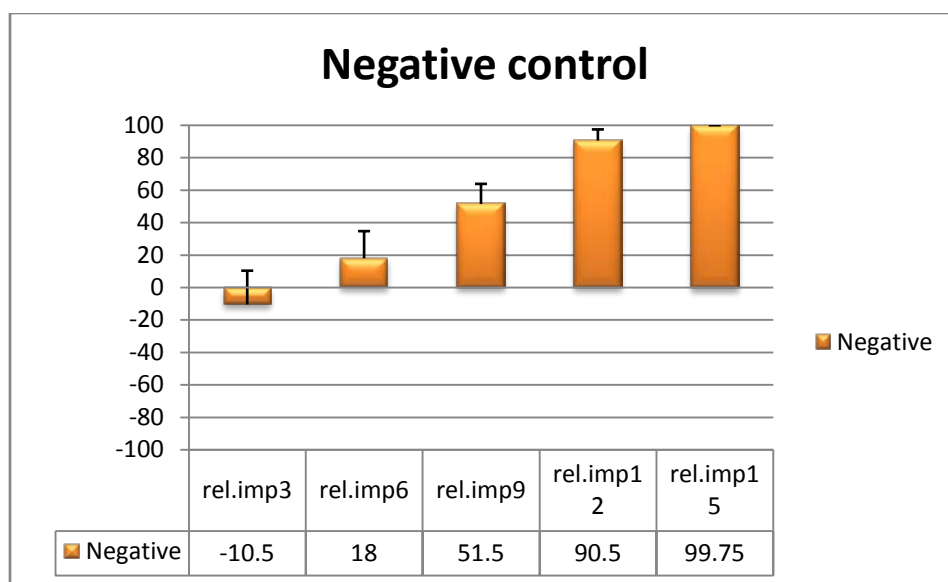
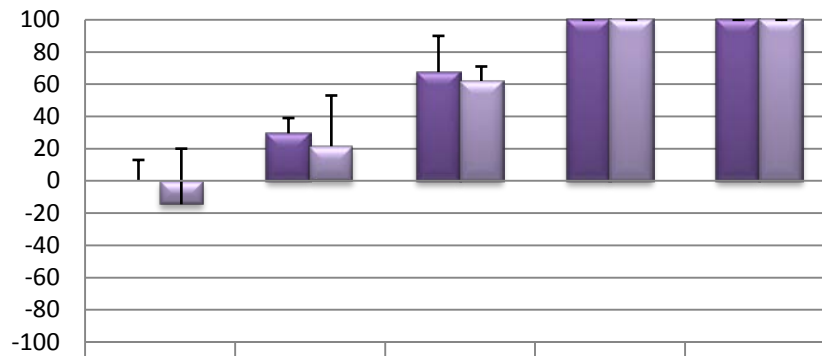


Figure 21. Comparison of relative improvement of negative control group. The results are expressed as means $\pm$ SEM of two independent experiments.

Based on the data obtained and summarized in Figures 17 to 21, relative improvement of SOS and positive control groups in day 9 and SOS gel group in day 12 were significantly different in comparison with corresponding value obtaining three days earlier. In contrast, in blank gel and negative control groups no significant change was observed.

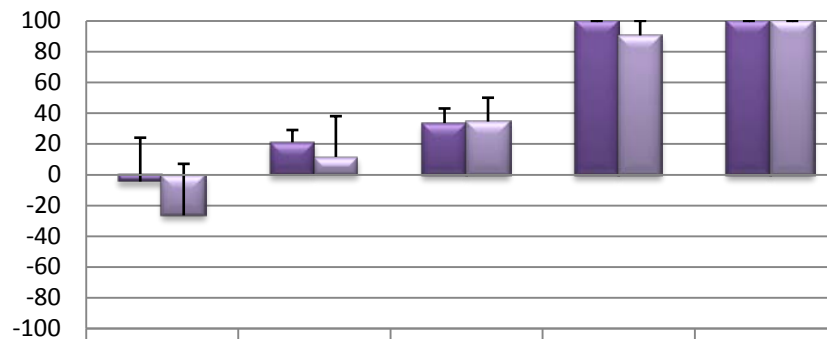
In order to investigate the effect of gender on wound healing process a comparison was done between male and female mice (two male and two female in each group). Statistical analysis was done to explore the effect of each group treatment on wound closure between male and female. The results are shown in Figure 22. In general, it seems that wound closure may occur faster in male than female, although there were not any significant differences between male and female in healing process in each group. Therefore additional experiments are needed to verify this hypothesis.

Comparing male & female in SOS group



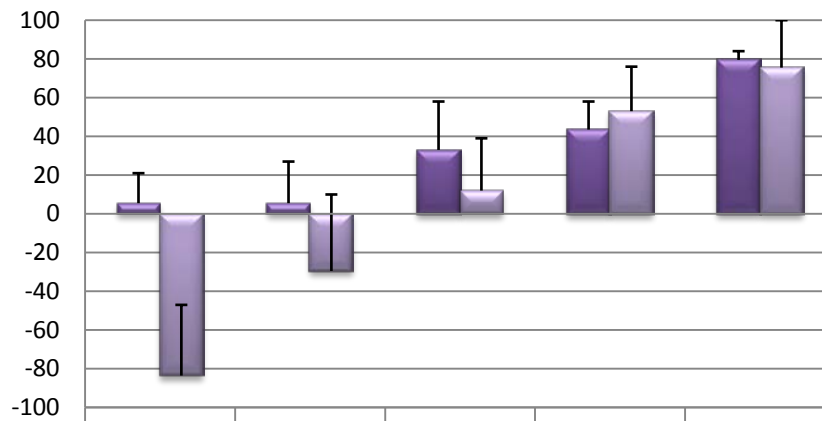
	rel.imp3	rel.imp6	rel.imp9	rel.imp12	rel.imp15
males	0	29.5	67.5	100	100
females	-14.5	21.5	62	100	100

Comparing male and female in SOS gel group



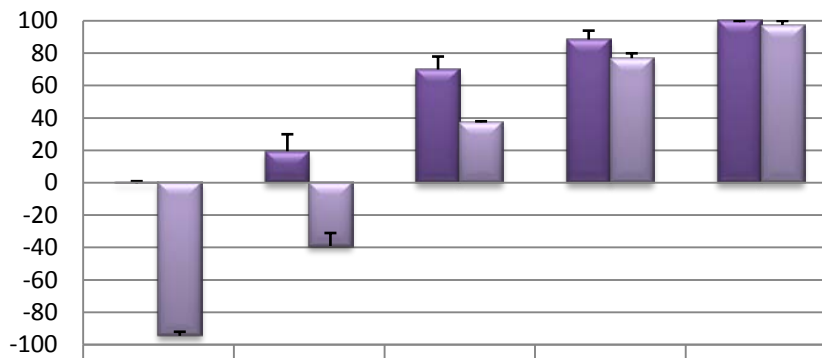
	rel.imp3	rel.imp6	rel.imp9	rel.imp12	rel.imp15
males	-4.5	21	33.5	100	100
females	-26.5	11.5	34.5	90.5	100

Comparing male and female in blank gel group



	rel.imp3	rel.imp6	rel.imp9	rel.imp12	rel.imp15
males	5.5	5.5	33	43.5	79.5
females	-83.5	-29.5	12	53	75.5

Comparing male and female in positive control group



	rel.imp3	rel.imp6	rel.imp9	rel.imp12	rel.imp15
males	-0.5	19.5	70	88.5	100
females	-94.5	-39	37.5	77	97.5

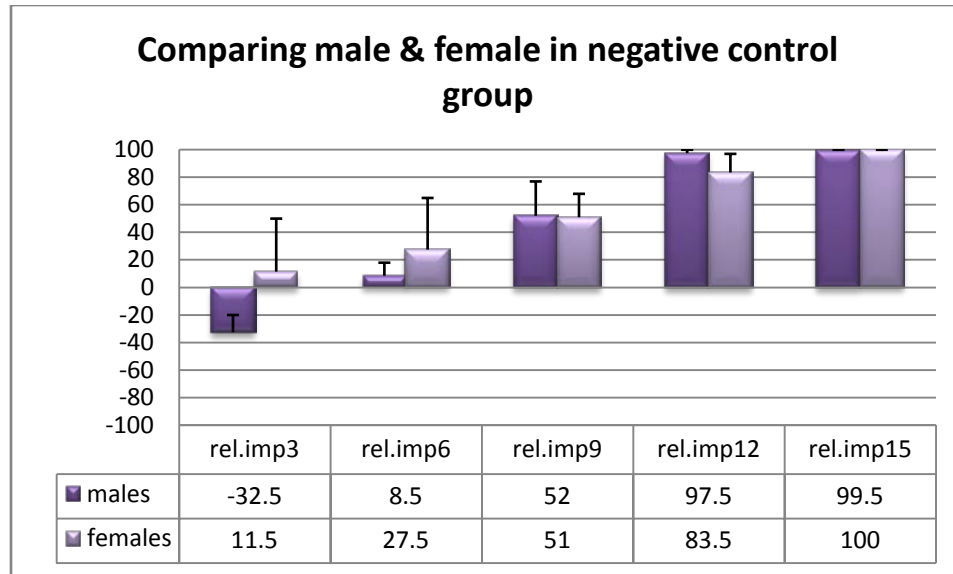


Figure 22. Comparison of wound closure between male and female in different groups. The results are expressed as means $\pm$ SEM of two independent experiments.

Below graph shows wound closure comparison between males and females irrespective of their groups (10 males and 10 females). Our statistical analysis shows a significant increase in relative improvement value at day 3(Rel.imp 3) for male mice regardless of their type of treatment. Totally, the data may reveal that the wound in males heal faster especially during the first three days following wound generation (Figure 23).

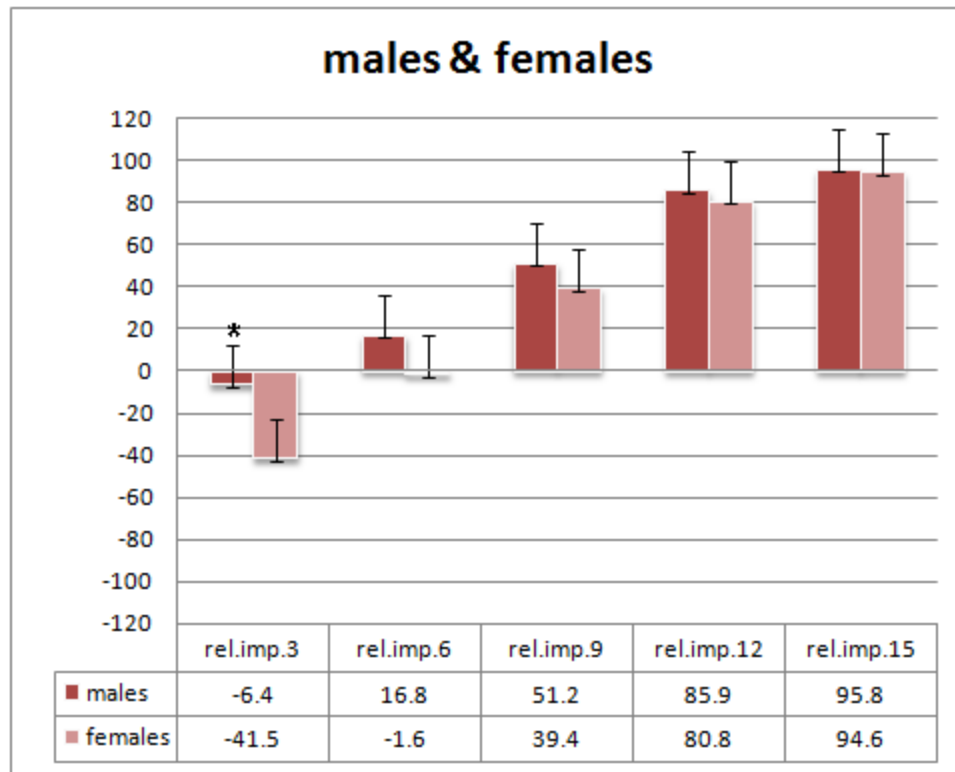


Figure 23. Wound closure comparison between males and females irrespective of their groups. Star indicates significant difference for rel.imp3 ($*P < 0.05$). The results are expressed as means $\pm$ SEM of two independent experiments.

Figure 24. Wound closure pictures. Pictures from left to right are from Day 0, Day 3, Day 6, Day 9, Day 12 and Day 15, respectively.

Negative control group/female



Negative control group/male



Positive control group/female



Positive control group/male



SOS group/female



SOS group/male



SOS gel group/female



SOS gel group/ male



Blank gel group/ female



Blank gel group/ male



Histological observation

In this section the results of microscopic analysis of tissue sections are described. Tissue sections are prepared on days 3, 6, 9, 12 and 15 of experiment. Each slide was checked to see whether it met the below criteria or not:

- . Presence of epithelium
- . Fibroblast formation
- . Collagen formation and maturity
- . Presence of angiogenesis
- . Formation of hair follicle and glands

Day 3

Negative control group

Observation of male and female of this group shows presence of fibroblast with average density (Figure 25). Both had intact scab.

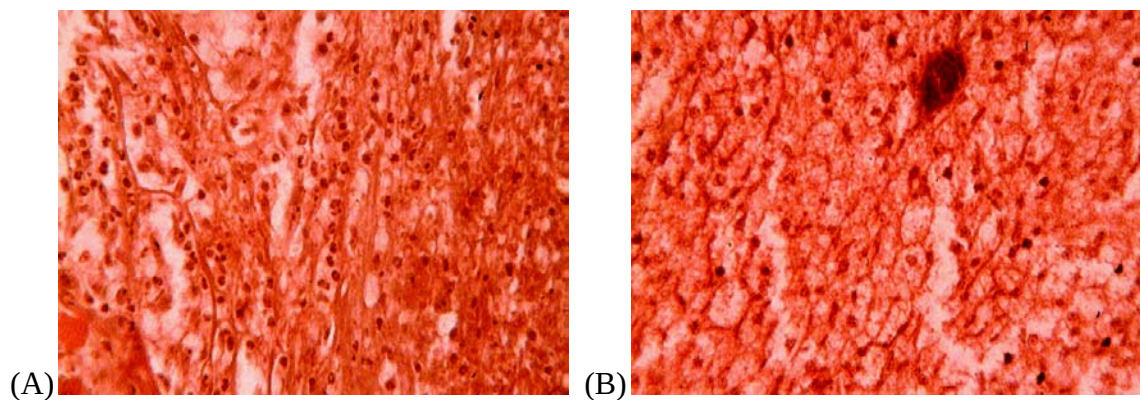


Figure 25. Microscopic view of 3-day-old wound tissue with no treatment. (A) Wound tissue of male. (B) Wound tissue of female.

SOS group

There was average density of fibroblast in both male and female, a few angiogenesis also was seen in female sample of first test (Figure 26). Scab was intact for male and female.

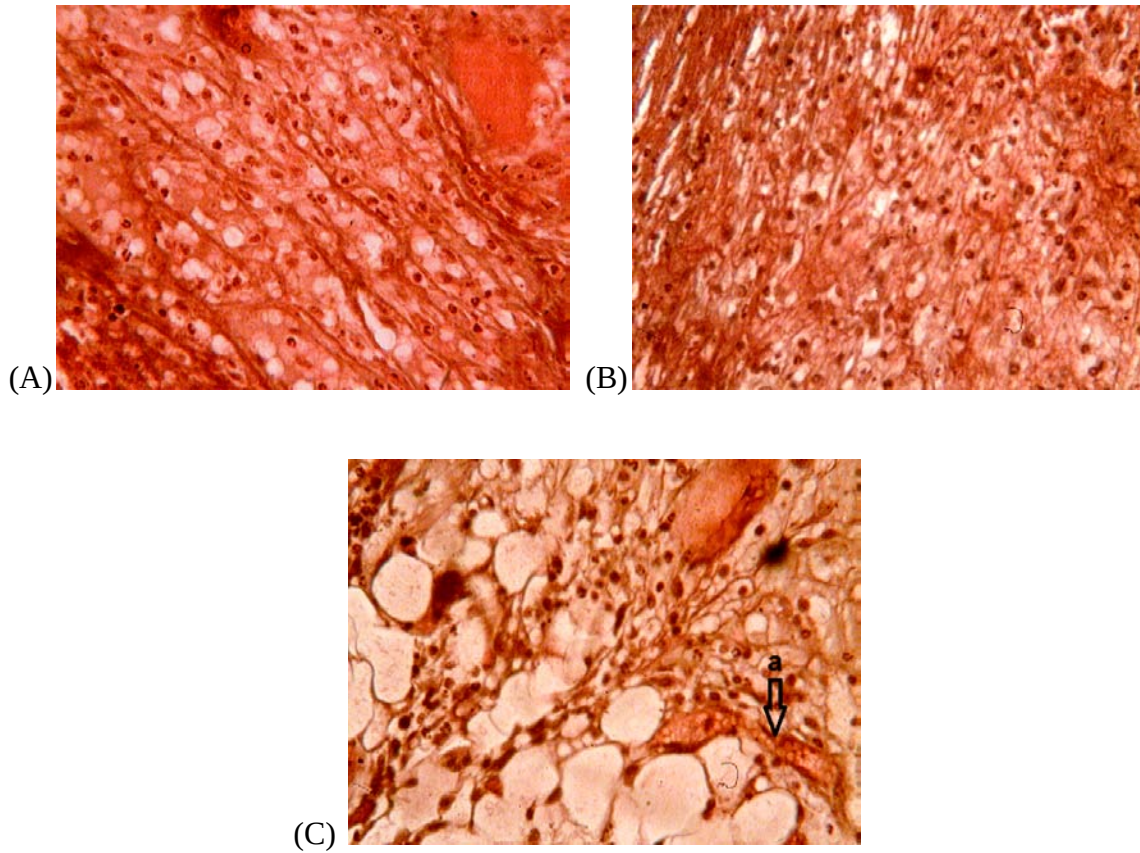


Figure 26. Microscopic view of 3-day-old wound tissue treated with SOS. (A) Male mouse dermal fibroblast. (B). Female mouse dermal fibroblast. (C) Microscopic view of 3-day-old female wound tissue treated with SOS in first test. (a) Angiogenesis.

SOS gel group

Histological sections of male and female both show medium number of fibroblasts and a few angiogenesis for male and female just in second test (Figure 27). Complete scab was also seen.

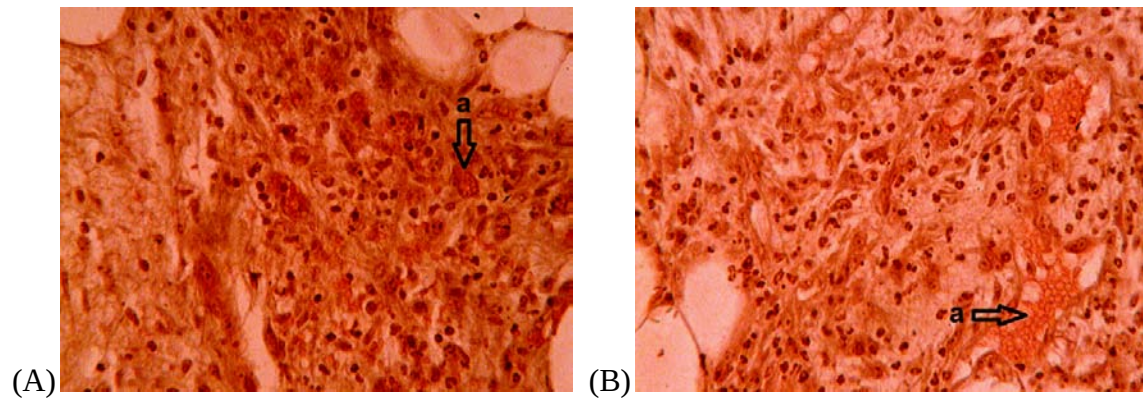
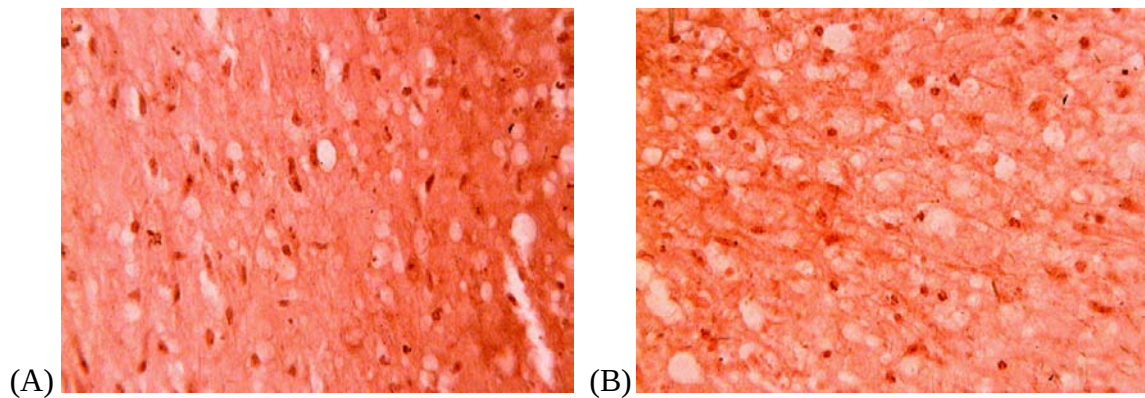


Figure 27. Microscopic view of 3-day-old wound tissue treated with SOS gel of test two. (A) Male mouse dermal fibroblast. (a) Angiogenesis (B). Female mouse dermal fibroblast. (a) Angiogenesis.

Blank gel group

It seems that the amount of fibroblast in this group was less than previous ones. Besides, they were more distributed. Both male and female also had intact scab. Just in second test, male animal showed angiogenesis (Figure 28).



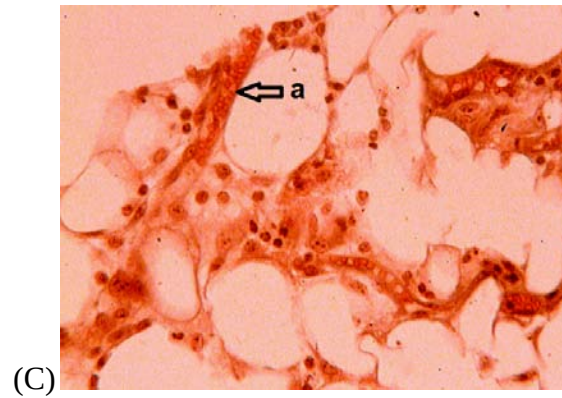
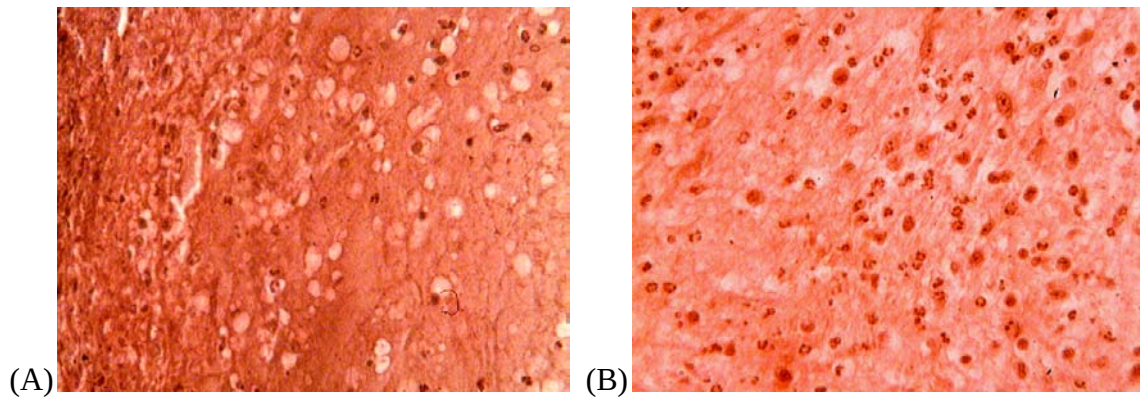


Figure 28. Microscopic view of 3-day-old wound tissue treated with blank gel. (A) Male mouse dermal fibroblast. (B). Female mouse dermal fibroblast. (C) Microscopic view of the section of 3-day-old male wound tissue treated with Blank gel. (a) Angiogenesis.

Positive control group

Tissue samples revealed distributed amount of fibroblast (Figure 29). In first test scab was seen in both male and female; however, in second test just females revealed some excretion on the surface of the wound.



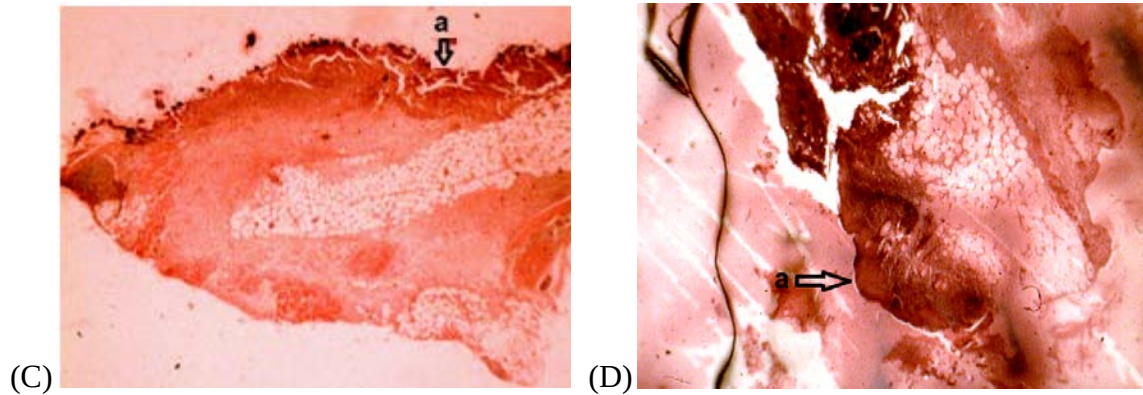


Figure 29. Microscopic view of 3-day-old wound tissue of positive control group. (A) Male mouse dermal fibroblast. (B). Female mouse dermal fibroblast. (C) Microscopic view of female tissue in second test. (a) Excretion on wound surface. (D) Microscopic view of female tissue in first test. (a) Intact scab on wound surface.

Day 6

Negative control group

In males and females the amount of fibroblasts increased in comparison with 3 day old wound tissue. In males of both tests and female of test one scarce and scattered amount of collagen was also seen which were pale gray and irregular (Figure 30). All had intact scab.

In test one both male and female's tissue revealed medium number of angiogenesis as well as immature epithelial cells that started proliferating to cover surface of the wound (Figure 31).

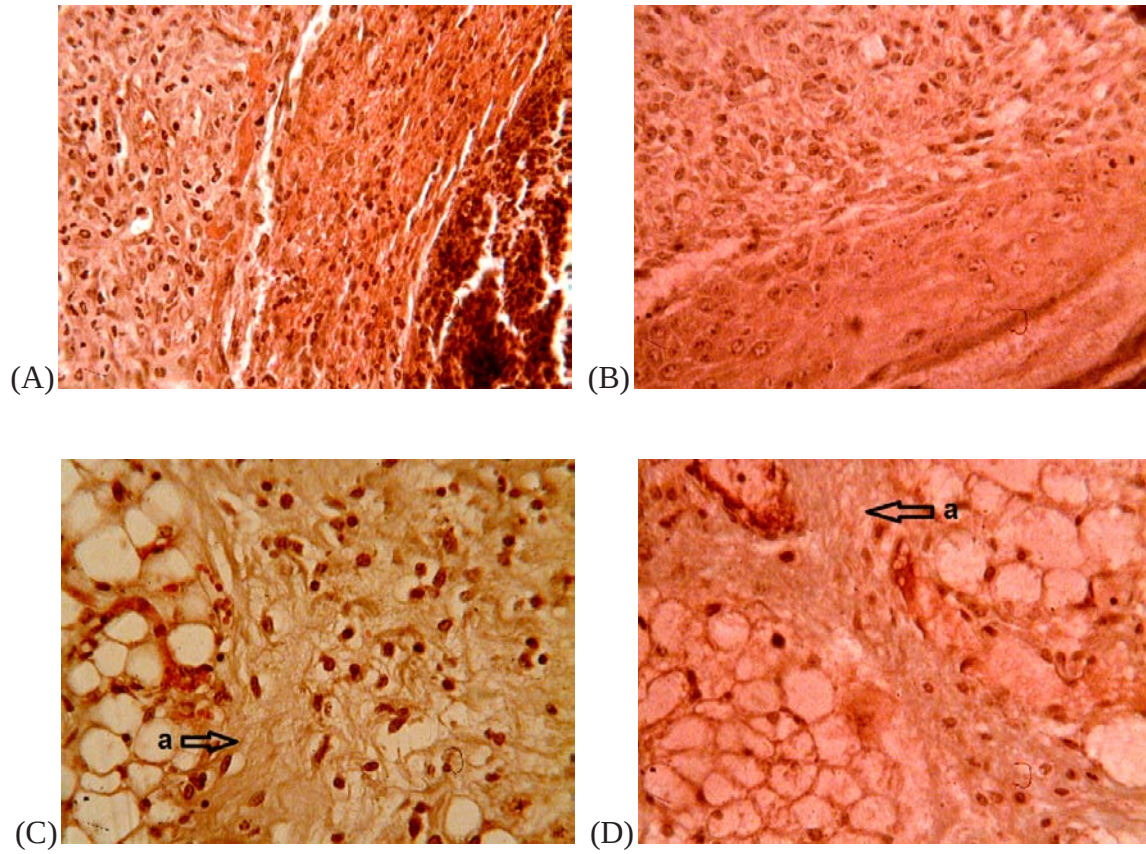
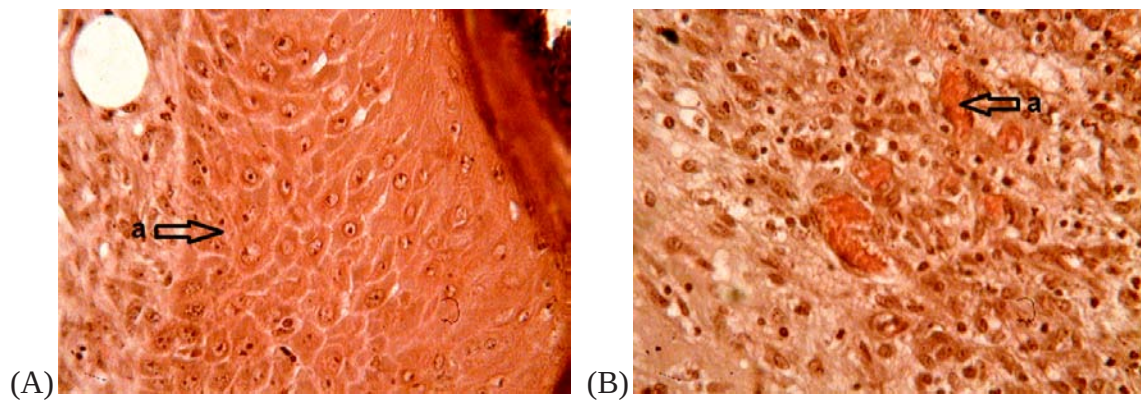


Figure 30. Microscopic view of 6-day-old wound tissue of negative control group. (A) Dispersion and amount of fibroblasts in male. (B) Dispersion and amount of fibroblasts in female. (C) Male section. (a) Collagens. (D) Female section of test one. (a) Collagens



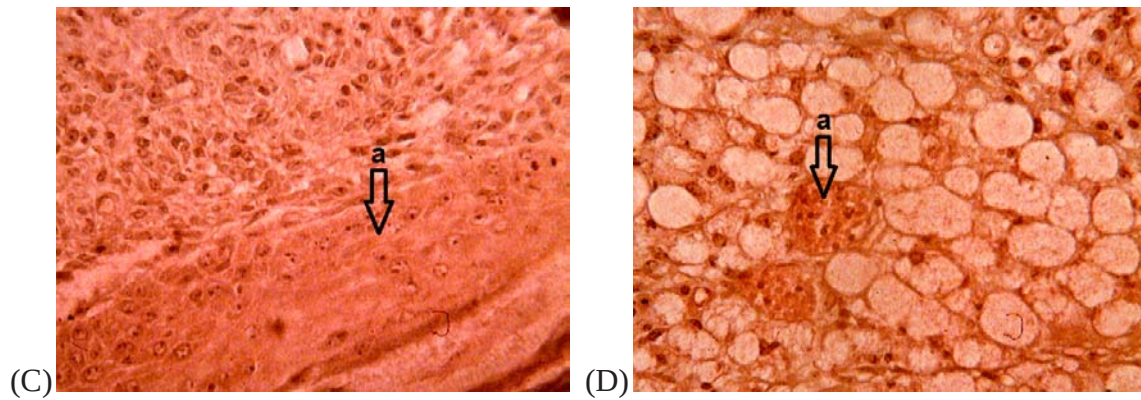


Figure 31. Microscopic view of 6-day-old wound tissue of negative control group in test one. (A) Male wound tissue. (a) Immature epithelial cells. (B) Dispersion of fibroblast in male. (a) Medium amount of angiogenesis. (C) Female wound tissue. (a) Epithelial cells. (D) Female wound tissue. (a) Angiogenesis.

SOS group

Fibroblasts were more in comparison with day 3 in both male and female. However, males demonstrated more number of fibroblasts than female. In both tests just males' tissue sections illustrated angiogenesis which were medium in test one and scarce in test two (Figure 32). All contained complete scab.

Tissue sections of male and female of second tests revealed thin epithelial layer drawing at one end of wound which seemed that have more mature cells in male than female. In first test irregular and disperse amount of collagen was seen in male tissue sections (Figure 33).

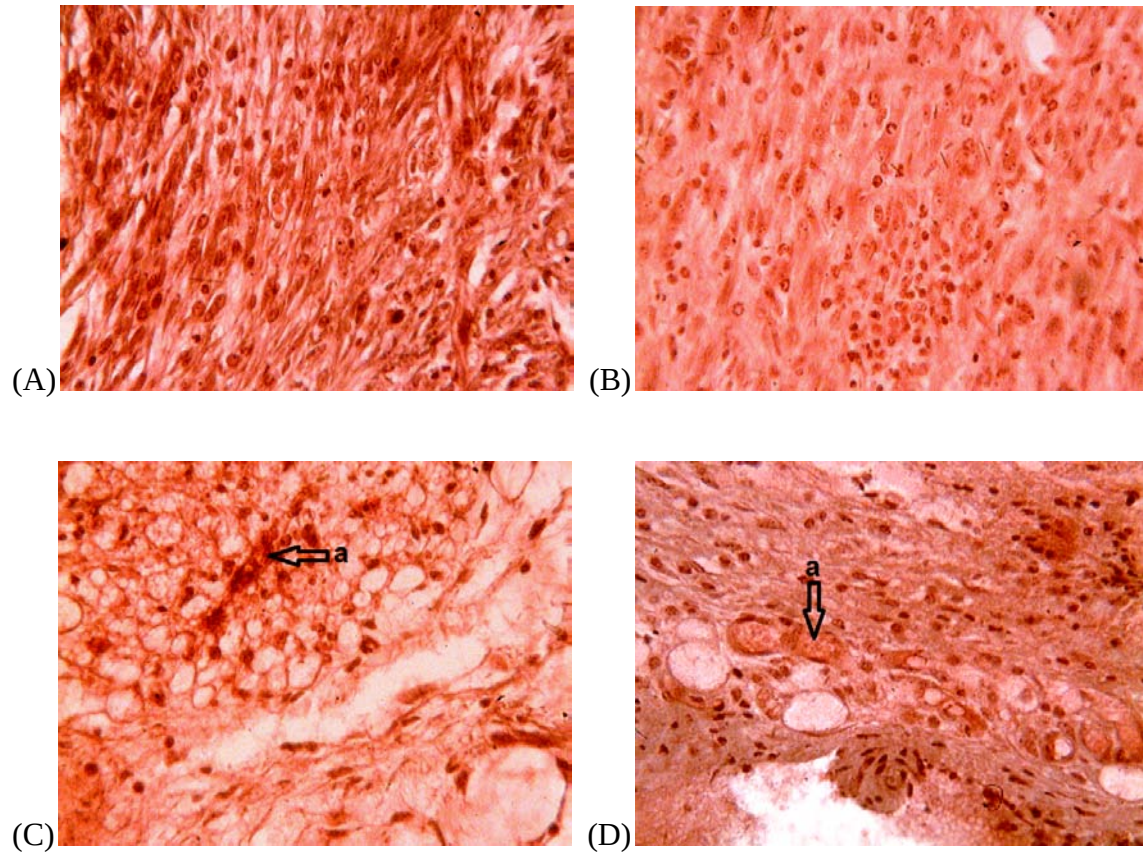
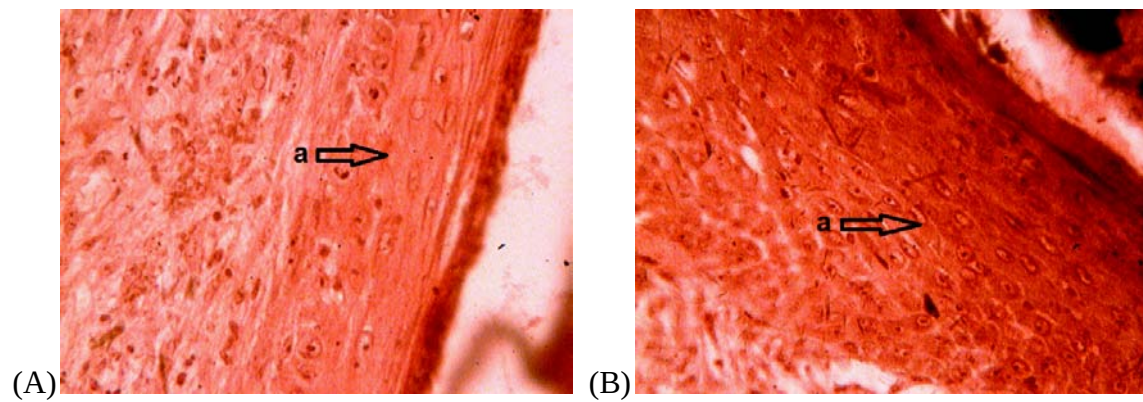


Figure 32. Microscopic view of 6-day-old wound tissue of SOS group.(A) Male's fibroblasts of wound. (B) Female's fibroblasts. (C) Scarce amount of angiogenesis in male in test two. (a) Angiogenesis. (D) Medium amount of angiogenesis in male of test one. (a) Angiogenesis.



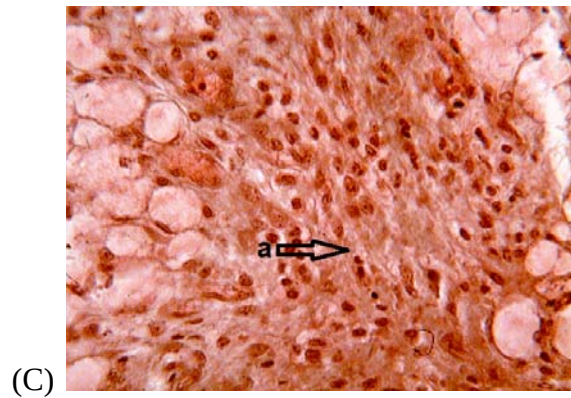
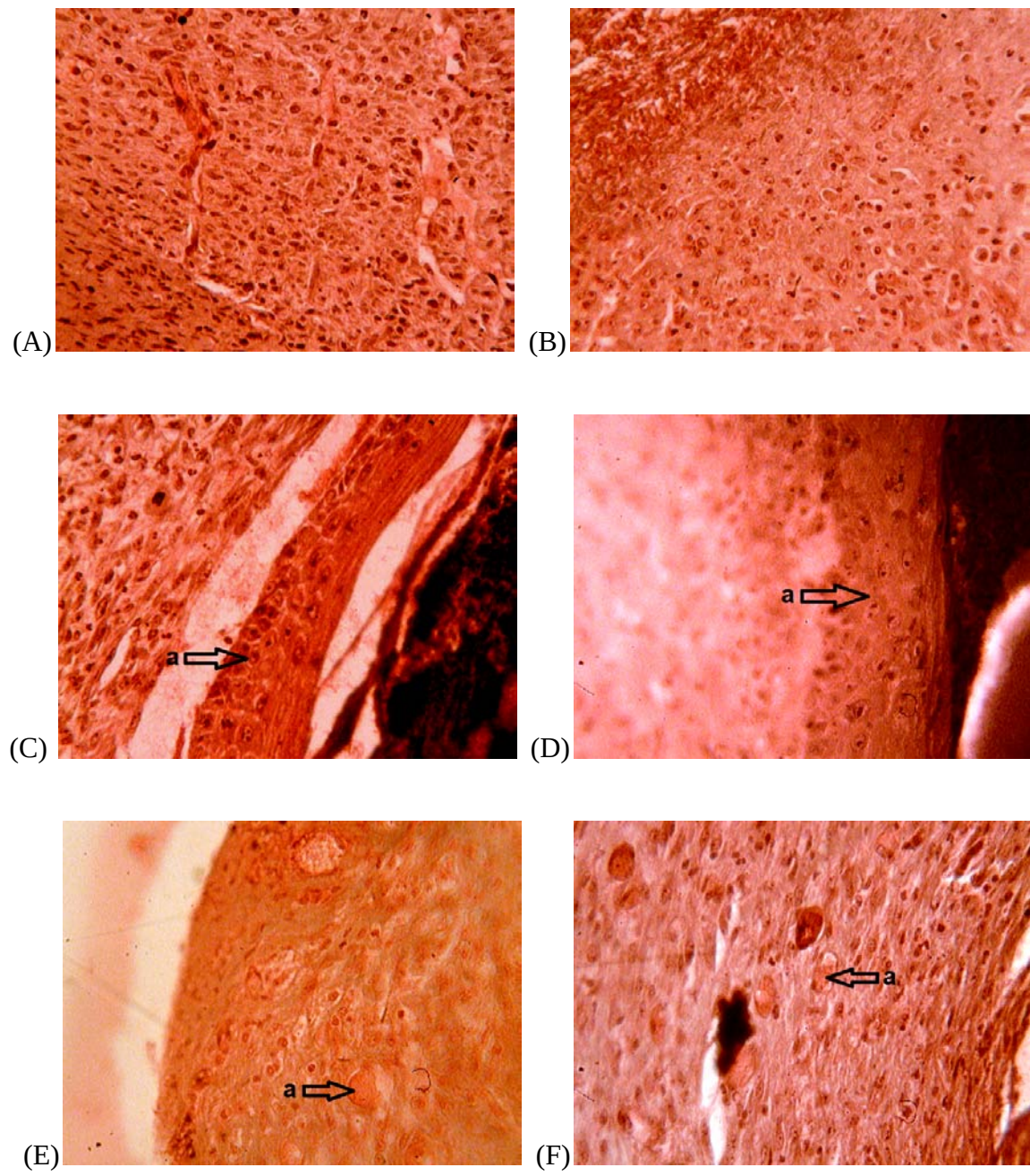


Figure 33. Microscopic view of 6-day-old wound tissue of SOS group. (A) Epithelial cells in female tissue section of test two. (a) Epithelial cells. (B) Epithelial cells in male tissue section of test two. (a) Epithelial cells. (C) Collagen in male tissue section of test one. (a) Collagen.

SOS gel group

Fibroblasts were more in comparison with day 3 in both male and female. However, it seemed that male's fibroblasts were more. In both tests males tissue sections demonstrated immature thin layer of epithelial cells which covered just one end of wound; in contrast, females of just test one had the same situation. Both male and female wound tissues had angiogenesis. In test one, number of angiogenesis in male and female were few and a lot respectively. In test two, male had medium number of angiogenesis and female had few numbers. Disperse and disorganized gray collagens were also seen in male and female of test two. However, male's collagens seemed to be more organized than female. In first test just male revealed collagens which were partially organized and oriented horizontally (Figure 34). Scabs were intact in all of them.



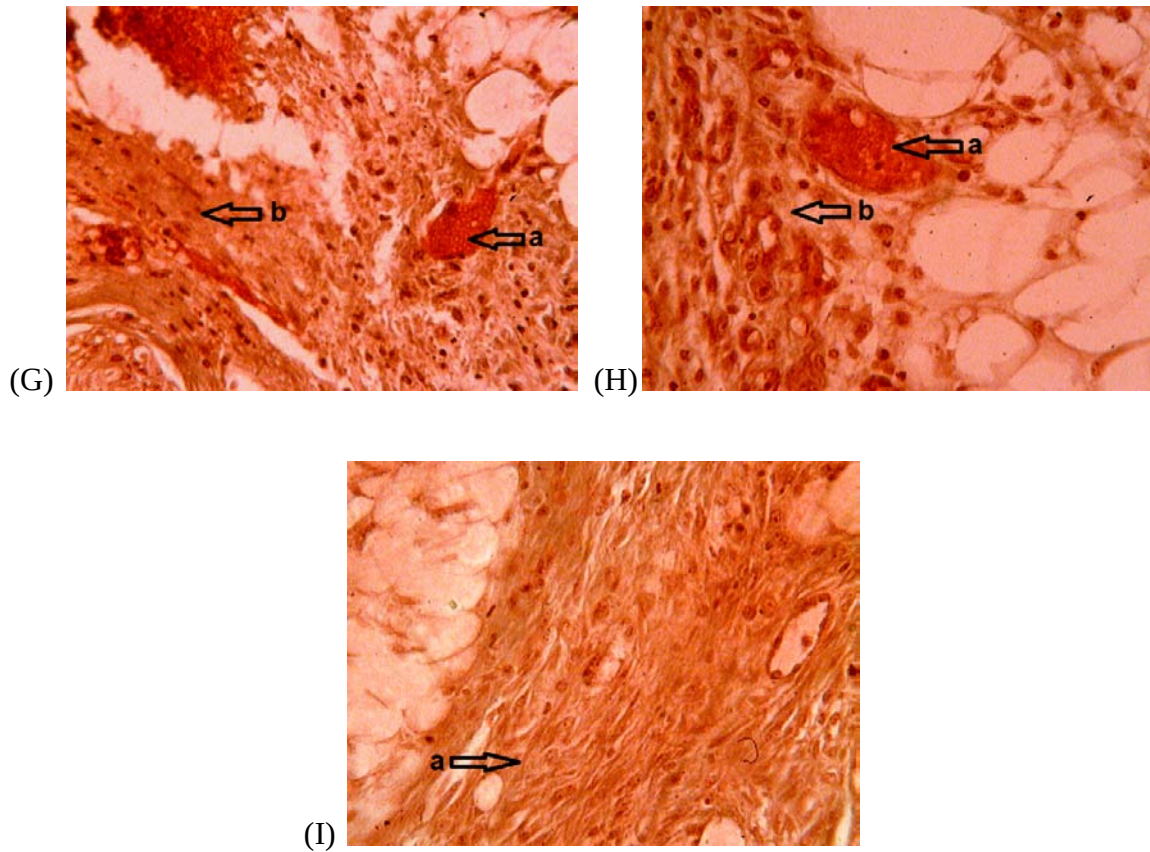


Figure 34. Microscopic view of 6-day-old wound tissue of SOS gel group. (A) Fibroblasts of male tissue section. (B) Fibroblasts of female tissue section. (C) Epithelial layer in male. (a) Epithelial cells. (D) Epithelial layer in female tissue section of first test. (a) Epithelial cells. (E) Number of angiogenesis in male of test one. (a) Angiogenesis. (F) Number of angiogenesis in female of test one. (a) Angiogenesis. (G) Amount and type of angiogenesis and collagen in male of test two. (a) Angiogenesis. (b). Collagen (H) Amount and type of angiogenesis and collagen in female of test two. (a) Angiogenesis. (b). Collagen. (I) Form of Collagen in male of test one. (a) Collagen.

Blank gel group

Tissue sections of all males and females illustrated fibroblasts more than day 3. Just male sections of test one had disperse disorganized collagens far from the cell layer of wound's surface. In addition, both tissue sections of male and female in test one and female in test two showed angiogenesis which were medium, large and scarce in number respectively (Figure 35). All had intact scab.

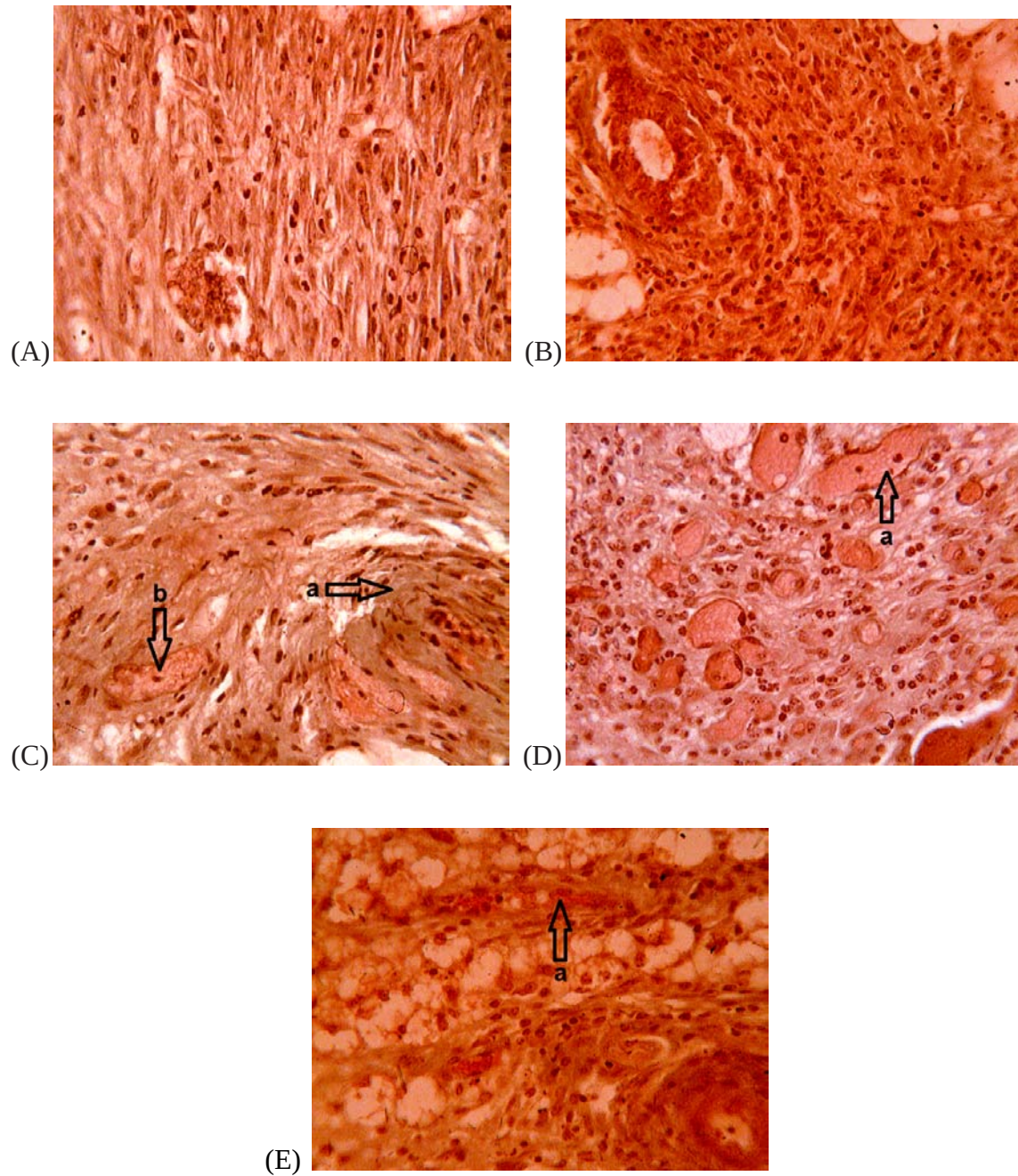
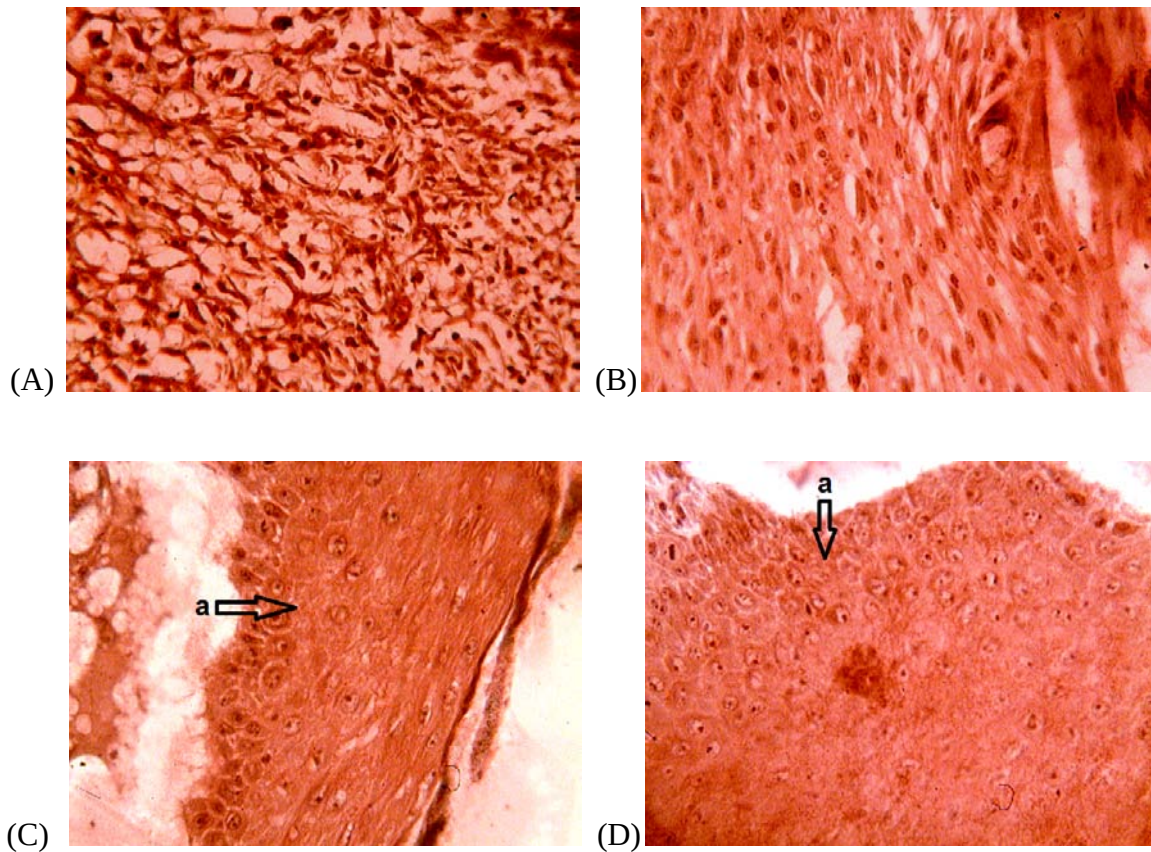


Figure 35. Microscopic view of 6-day-old wound tissue of Blank gel group.(A) Fibroblasts of male tissue section. (B) Fibroblasts of female tissue section. (C) Male tissue section of test one. (a) Collagen. (b). Angiogenesis. (D) Female tissue section of test one. (a) Angiogenesis. (E) Female tissue section of test two. (a) Angiogenesis.

Positive control group

In this group what was similar in first and second test was the presence of more amounts of fibroblasts in comparison with day 3; moreover, it seemed that male section had more fibroblasts than female. Other features were just seen in test one. Both tissue sections of male and female in test one demonstrated epithelial cell proliferation from one end of wound surface to the middle of it, epidermis diameter was thick and partially mature at one end; however, both features decreased gradually when it covered middle surface of the wound. Male tissue had scarce and totally disorganized collagen near the wound surface (Figure 36).



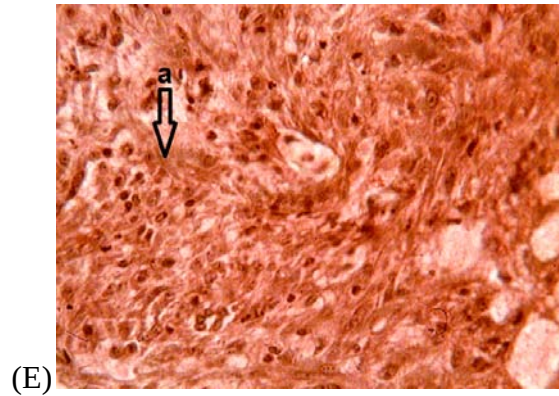
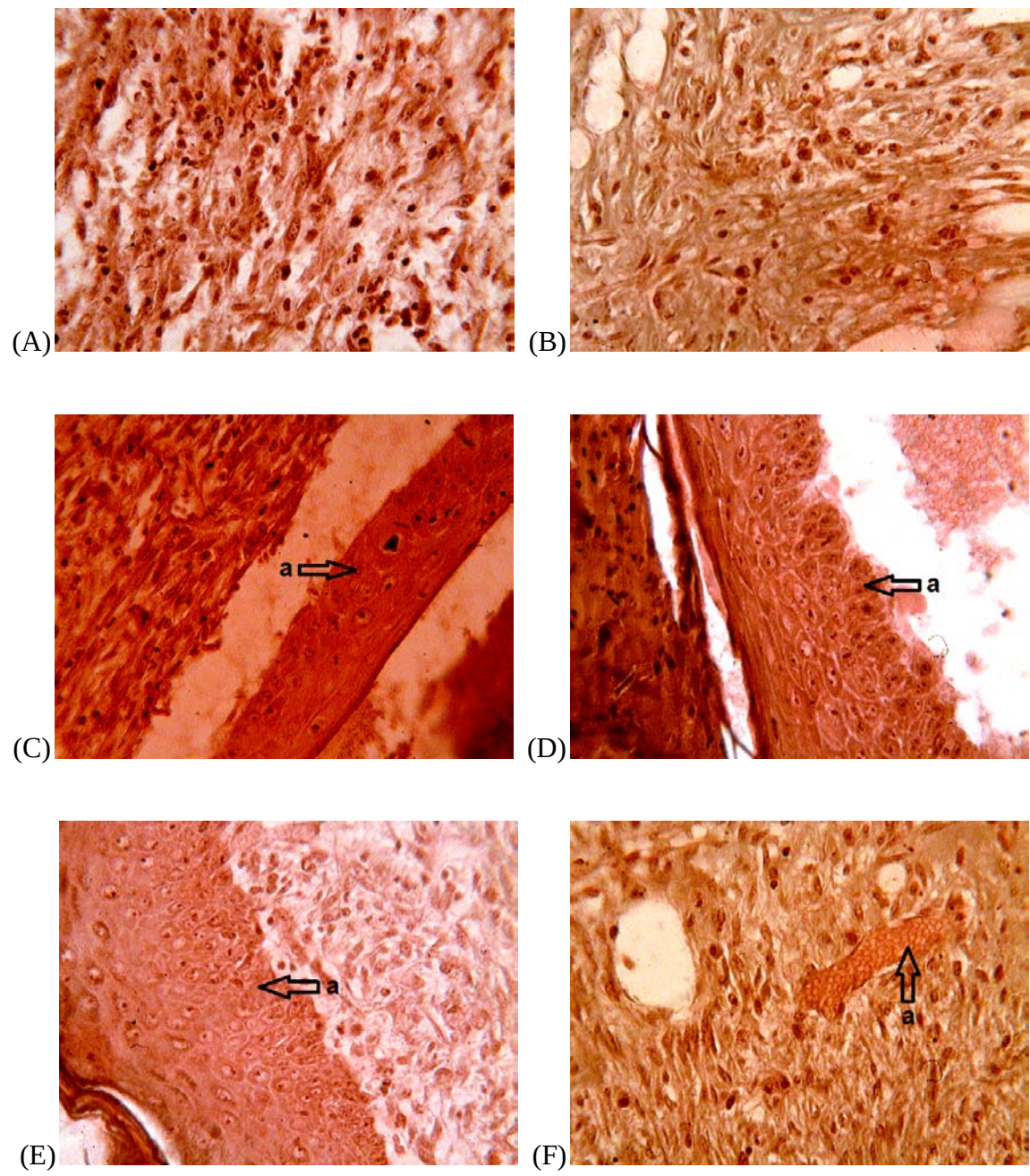


Figure 36. Microscopic view of 6-day-old wound tissue of positive control group. (A) Fibroblasts of male tissue section. (B) Fibroblasts of female tissue section. (C) Male tissue section of test one. (a) Epithelial cells. (D) Female tissue section of test one. (a) Epithelial cells. (E) Male tissue section of test one. (a) Collagens.

Day 9

Negative control group

Fibroblasts were seen in all tissue sections. Epithelium cells were also observed. In female of test two they covered slight part of one end of wound and they were completely immature, while epithelial cells of male and female wound tissue in test one covered half and the entire wound surface respectively. They were also partially mature. All sections revealed average number of angiogenesis; however, collagen synthesis was just seen in first test. The collagens concentration was far from the wound surface, they were partially organized especially in female (Figure 37). All female had intact scab, while male didn't.



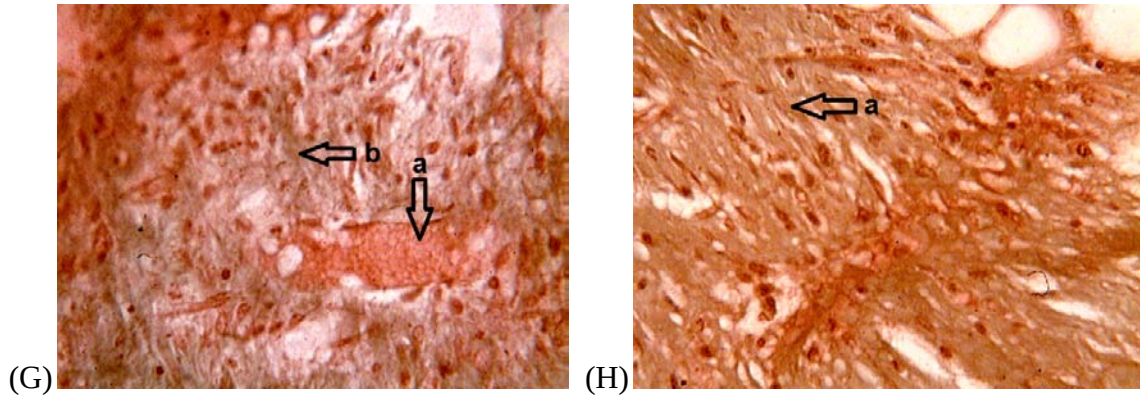
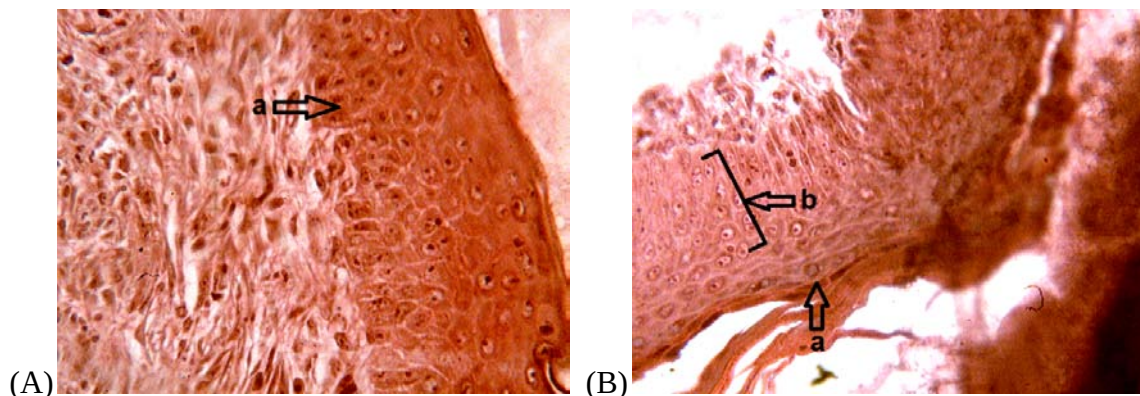


Figure 37. Microscopic view of 9-day-old wound tissue of negative control group. (A) Fibroblasts of male tissue section. (B) Fibroblasts of female tissue section. (C) Female tissue section of second test. (a) Epithelial cells. (D) Female tissue section of first test. (a) Epithelial cells. (E) Male tissue section of first test. (a) Epithelial cells. (F) Female wound tissue section. (a) Angiogenesis. (G) Male wound tissue section. (a) Angiogenesis. (b) Collagens. (H). Female tissue section of test one. (a) Collagens.

SOS group

All sections showed epithelial cells which covered whole surface of the wound, and their maturity was more in female than male. In addition, collagens disappear unequally all around the wound tissue section, and their concentration increase gradually from surface to the wound depth (in depth collagens were partially organized and gray in color). Average number of angiogenesis was also observed in all sections (Figure 38). All had intact scab above wound area.



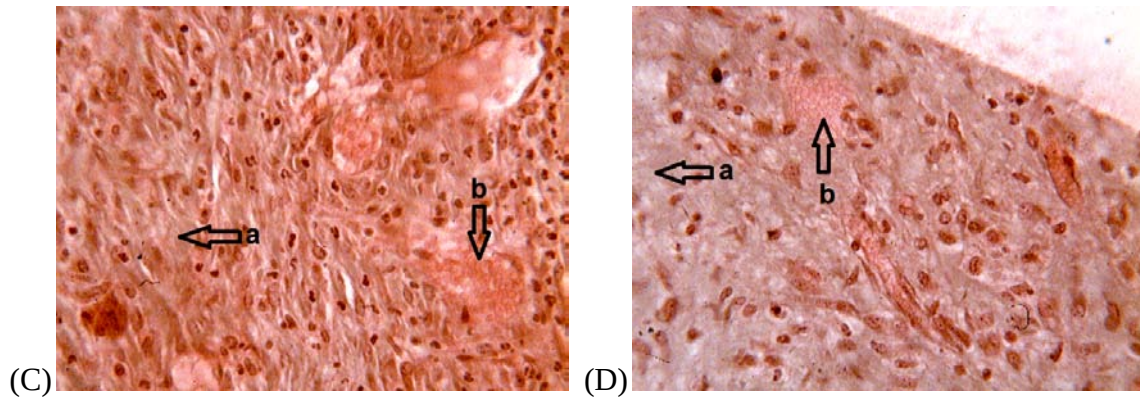


Figure 38. Microscopic view of 9-day-old wound tissue of SOS group. (A) Male tissue section. (a) Epithelial cells. (B) Female tissue section. (a) Stratum granulosum. (b) Stratum Malpighi. (C) Male tissue section. (a) Collagens. (b) Angiogenesis. (D) Female tissue section. (a) Collagens. (b). Angiogenesis.

SOS gel group

All samples revealed that epidermis layer covered whole surface of the wound, and the maturity of their cells was average. Diameter and maturity of epidermal layer of male animals were more at ends of wound than middle. However, epidermis of female tissue was approximately uniform with average maturity. Collagens were scattered throughout the dermis in males and the further we get from epidermis the more organized and dense collagens were observed, while collagens of female's sections were located just far from epidermis and they were less organized than males. Amount of angiogenesis was more than average in all samples (Figure 39). Female sections did not have any scab and they were just covered by thin layer of excretion, while males' sections had scab.

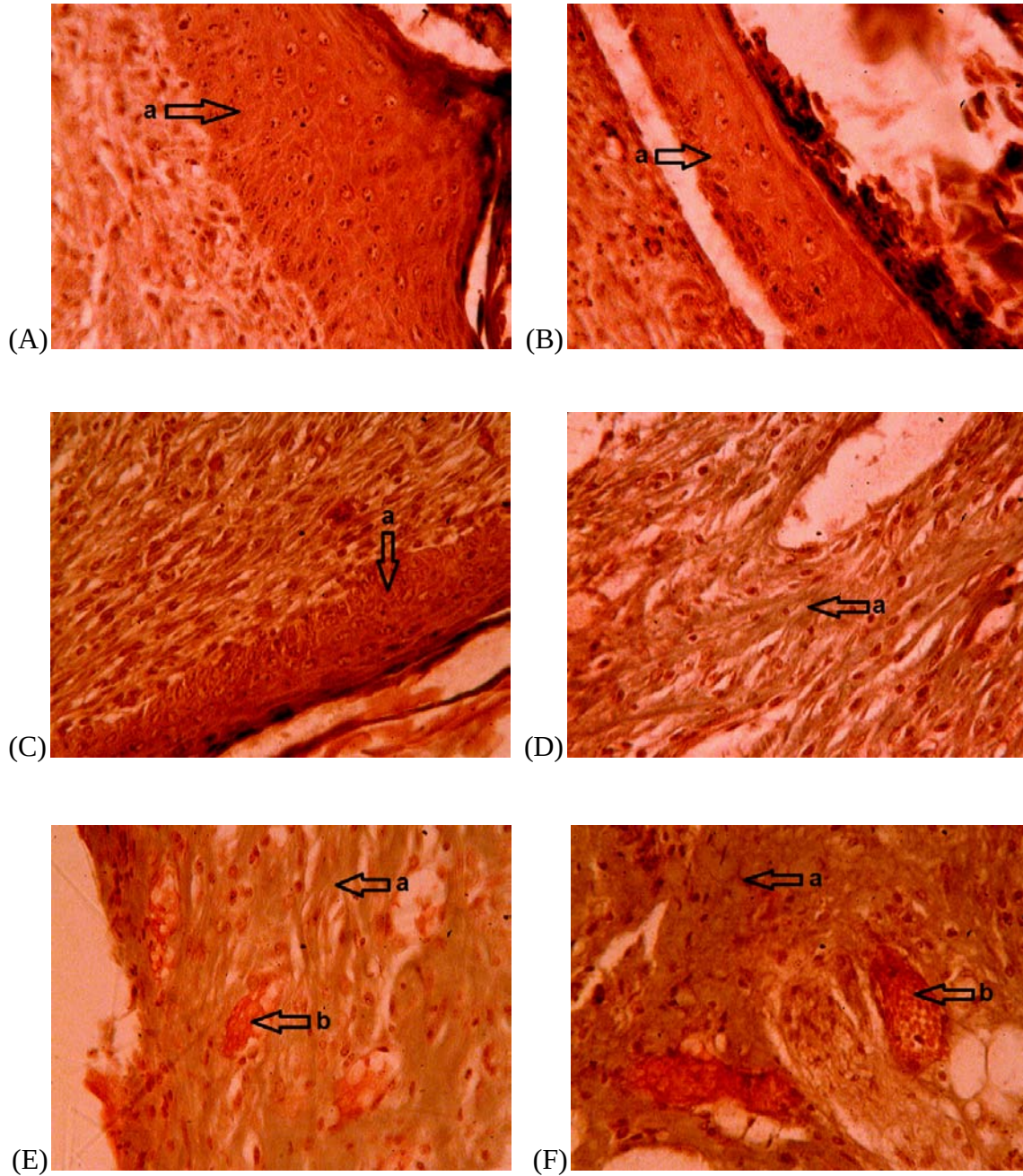
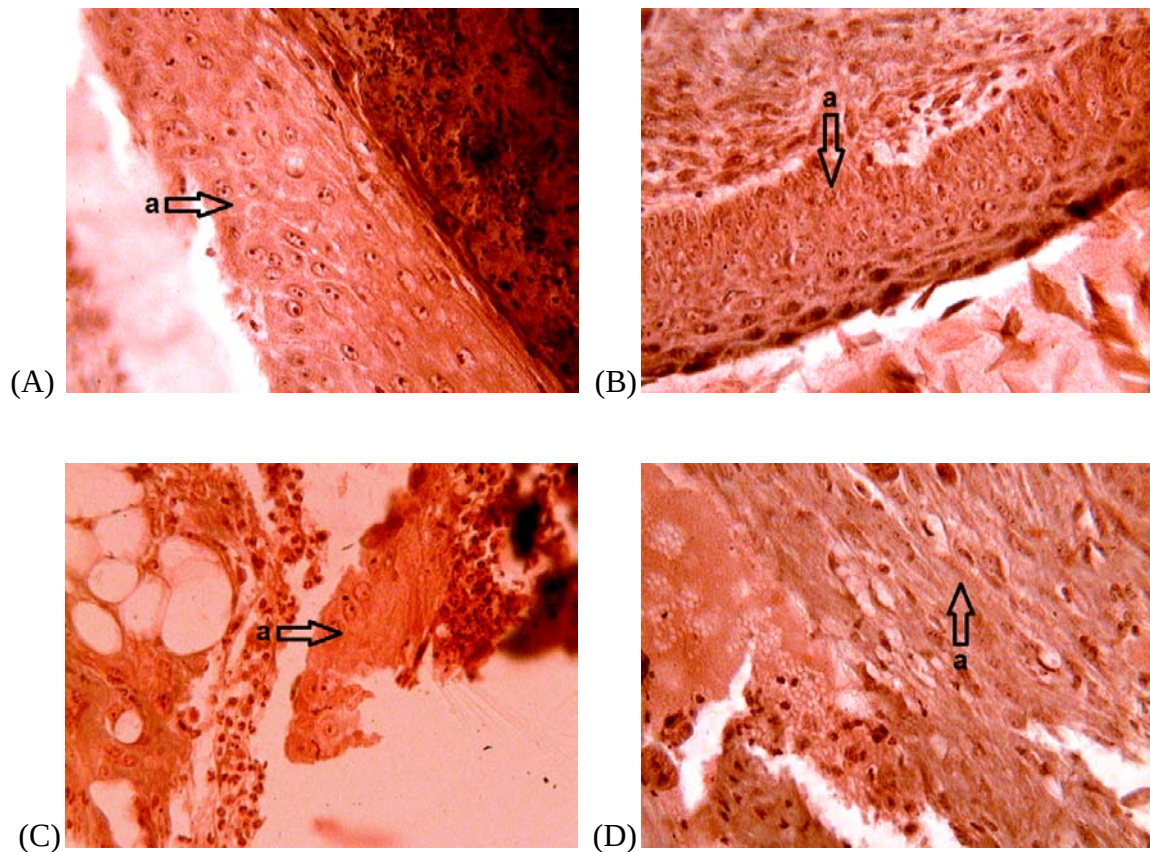


Figure 39. Microscopic view of 9-day-old wound tissue of SOS gel group. (A) Male tissue section. (a) Thick epithelial cell layers at both end of wound. (B) Male tissue section. (a) Thin epithelial cell layers at middle of wound. (C) Female tissue section. (a) Epithelial cells of epidermis. (D) Dermis of male section. (a) Partially organized collagens at the middle of tissue with average density. (E). Dermis of male section. (a) Dense collagens located far from the epidermal layer. (b) Angiogenesis. (F) Female dermal layer. (a) Collagens. (b) Angiogenesis.

Blank gel group

In test one male tissue section showed very thin immature layer of epidermis, while female of the same test revealed cells with average maturity. However, in test two a very small and thin immature epidermis could be observed at one end of tissue. Observation of smaples from test one male and female cleared that semi organized collagens were just concentrated in area far from epidermis. However in female sample of test two, collagens' concentration start from midle portion of tissue to its end, and the further we got from epidermis the more organized and dense collagens were observed. Angiogenesis was just observed in test one which was scarce for male and great in number for female (Figure 40).Test one male and test two both male and female had intact scab.



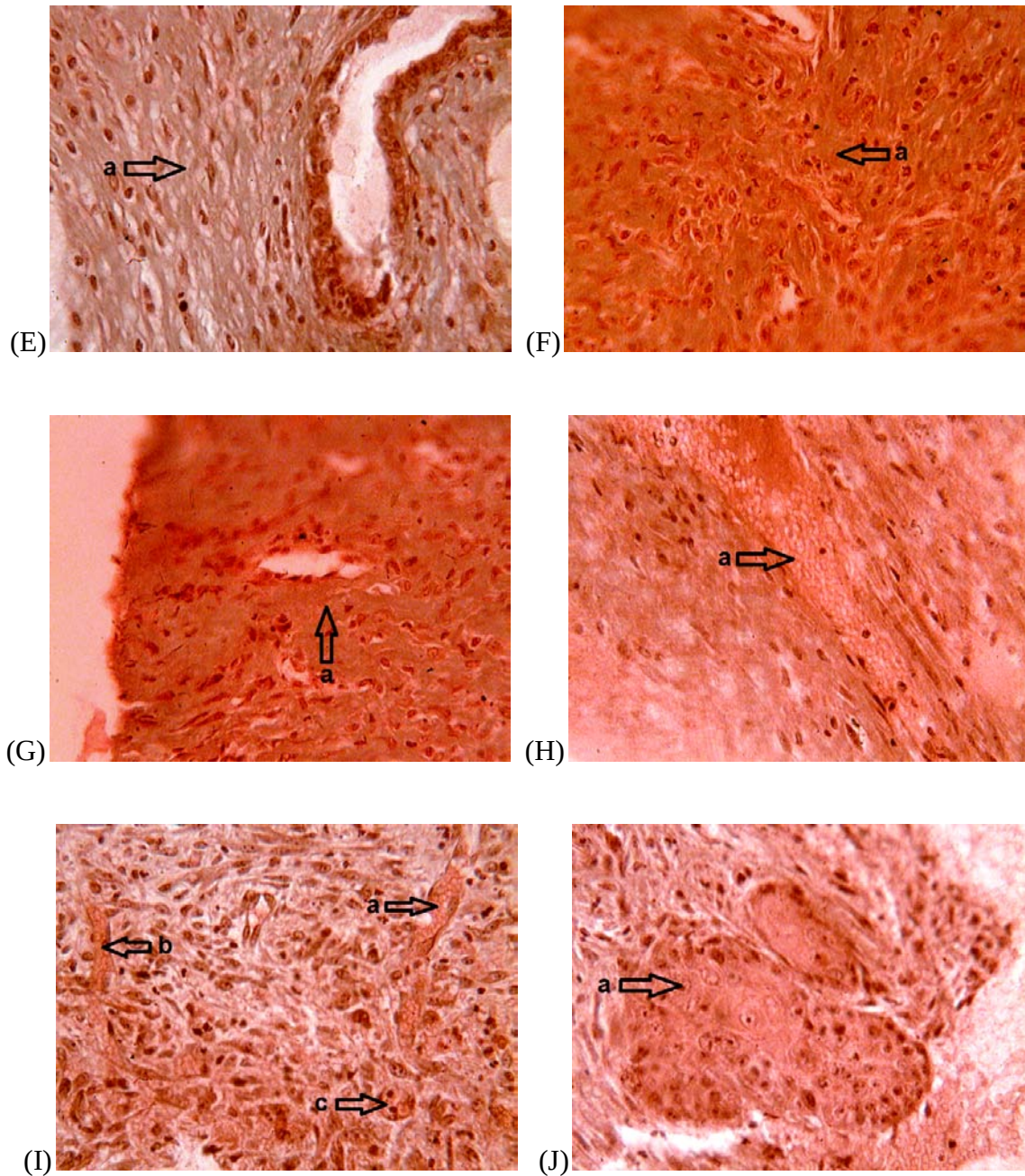
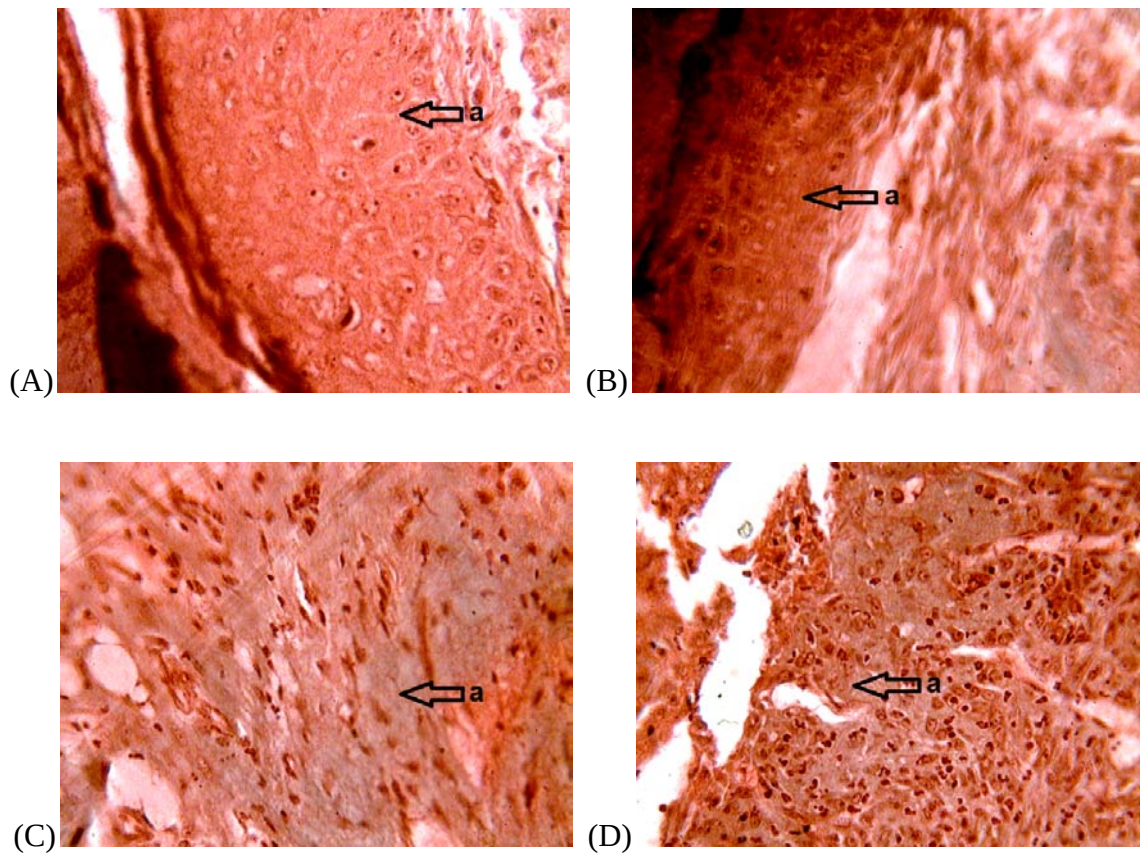


Figure 40. Microscopic view of 9-day-old wound tissue of Blank gel group. (A) Male tissue section of test one. (a) Epithelial cell of epidermis. (B) Female tissue section of test one. (a) Epithelial cells of epidermis. (C) Female tissue section of test two. (a) Epithelial cells of epidermis. (D) Dermal layer of male sample in test one. (a) Collagens. (E) Dermal layer of female sample in test one. (a) Collagens. (F) middle of dermis in female sample of test two. (a) Collagens. (G) End of dermis of female samples in test two. (a) Collagens. (H). Male's dermal layer of skin in test one. (a). angiogenesis. (I). Female's dermal layer in test one. (a)(b)(c) Angiogenesis. (J) male's skin dermal layer in test one. (a) Gland.

Positive control group

In test two no epidermis was constructed. However, one third of male tissue sample of first test was covered with thick immature epidermal layer and part of female tissue section showed medium size immature epidermal layer. Collagens covered all part of dermis in all samples. All males had average number of angiogenesis (Figure 41). However, just female belong to test one had scarce amount of angiogenesis. All had intact scab.



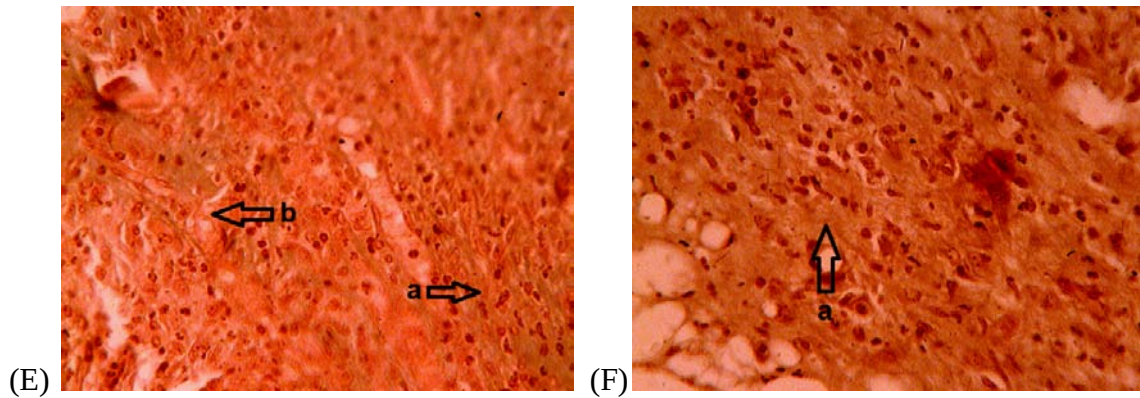


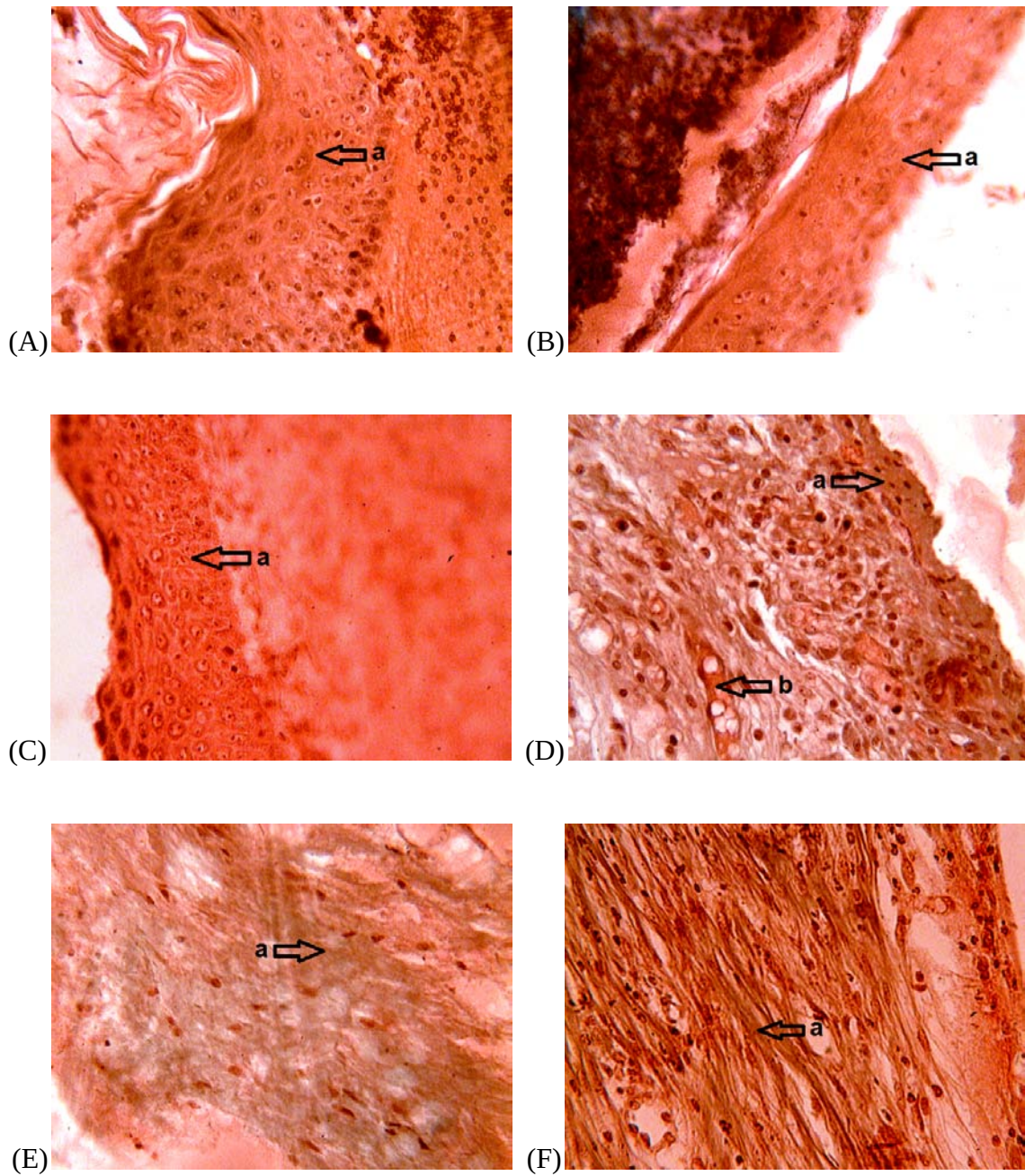
Figure 41. Microscopic view of 9-day-old wound tissue of positive control group. (A). Test one, male animal tissue's epidermal layer. (a) Epithelial cells. (B). Test one, female animal tissue's epidermal layer. (a) Epithelial cells. (C) Test one, male animal dermal layer. (a) Collagens. (D) Test one, female animal dermal layer. (a) Collagens. (E) Test two, Male animal dermal layer. (a) Collagens. (b) Angiogenesis. (F) Test two, female animal dermal layer. (a) Collagens.

Day 12

Negative control group

Tissue sections of male animal showed epidermal layer which covered whole surface of wound. Its thickness was more at both end of wound, while in middle of the wound its diameter became less. The thicker the epidermal layer was, the more mature cells it had. According to females, just the animal of test two had intact epidermis which was uniform with partially mature epithelial cells. Main density of collagens in male and female animal of test one were located in restricted area deeper and far from the epidermal layer, which were not organized. Collagens of test two tissue sections covered two thirds of dermis, and the farther from epidermis they were, the more density they had. They also were partially organized and oriented horizontally. Angiogenesis was observed scarcely in female animals tissue. Test two male animal did not have angiogenesis, while his counterpart in test one had a lot. Average number of hair follicles were

observed in female animal tissue section of test one (Figure 42). Intact scab was seen in female and male sections of first test, while in test two restricted part of wound was covered by scab and female sections were covered by excretion.



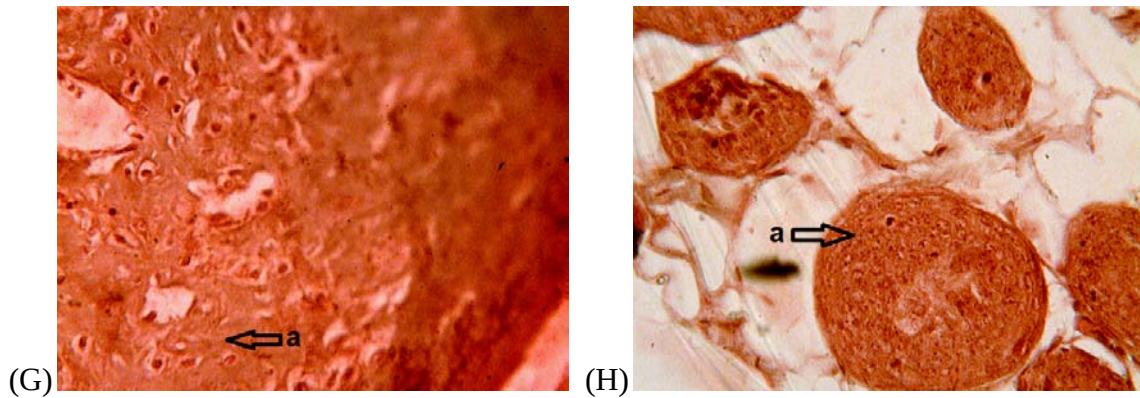
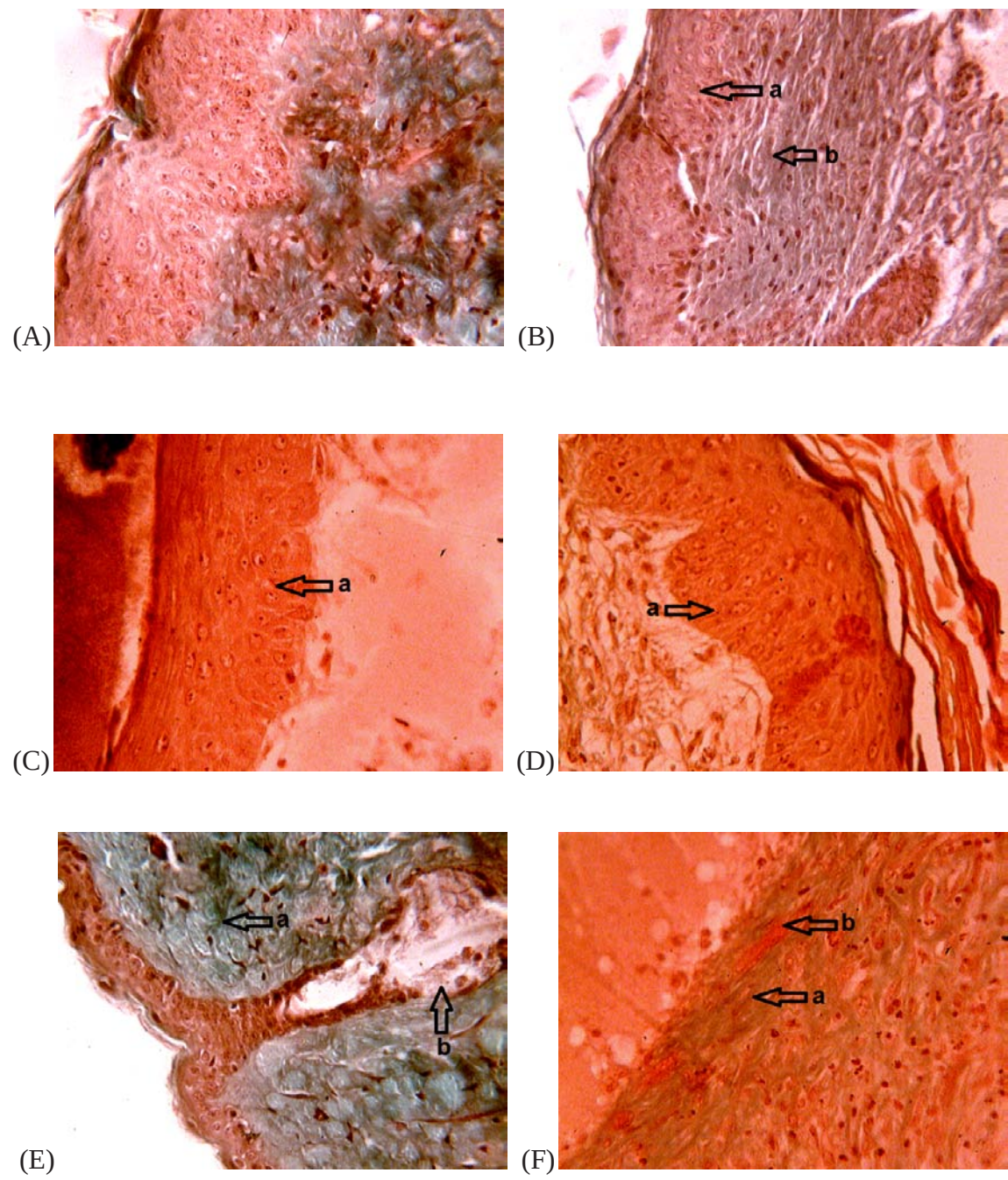


Figure 42. Microscopic view of 12-day-old wound tissue of negative control group. (A). Test one, male animal thick epidermal layer (a) Epithelial cells. (B) Test one, thin epidermal layer of male animal at the middle of wound. (a) Epithelial cells. (C). Test two, epidermal layer of female animal. (a) Epithelial cells. (D) Test one, dermis of male sample. (a) Collagens. (b) Angiogenesis. (E) Test one, dermis of female sample. (a) Collagens. (F) Test two, dermis of male sample. (a) Collagens. (G) Test two, dermis of female sample. (a) Collagens. (H) Test one, dermis of female sample. (a) Hair follicles.

SOS group

All samples had mature epidermis that covered whole surface of wound. Generally maturity of test one epidermis was more. Their dermis completely covered with collagens; still, their density and organization were more in test one than two. In test one, both male and female collagens were great in density and they were organized; however, in test two, collagens' concentration and organization were average. According to angiogenesis, just male animals contain angiogenesis far from epidermis, and average and scarce numbers of them were observed in test two and one respectively. Hair follicle and gland were just observed in test one, and they were scarce in male sample and great number in female ones (Figure 43). Scab just existed in test two male samples.



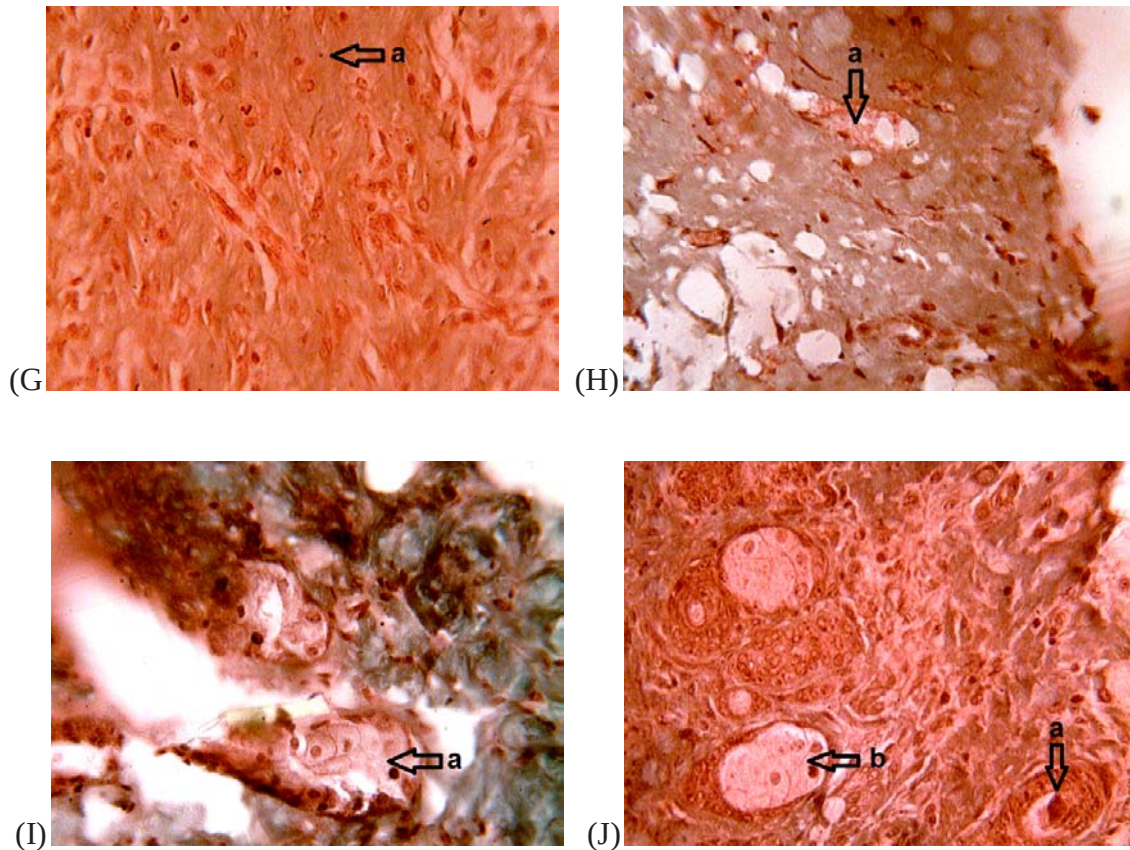
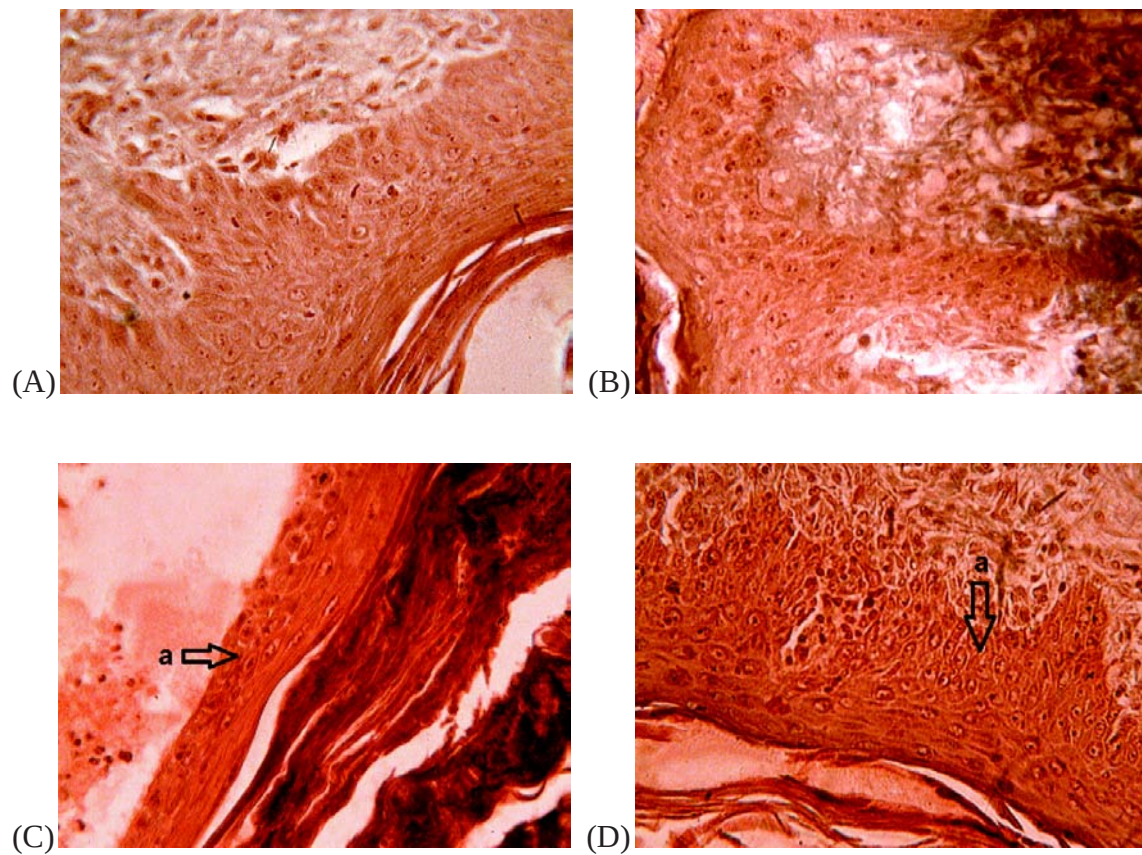


Figure 43. Microscopic view of 12-day-old wound tissue of SOS group. (A) Test one, male animal epidermal layer. (B) Test one, female animal epidermal and dermal layer. (a) Epithelial cells. (b) Collagens (C) Test two, male animal epidermal layer. (a) Epithelial cells. (D) Test two, female animal epidermal layer. (a) Epithelial cells. (E) Test one, male animal skin dermal layer. (a) Collagens. (b) Hair follicle. (F) Test two, male animal dermal layer. (a) Collagens. (b) Angiogenesis. (G) Test two, female animal dermal layer. (a) Collagens. (H) Test one, male animal skin dermal layer. (a) Angiogenesis. (I) Test one, male animal skin dermal layer. (a) Gland. (J) Test one, female animal skin dermal layer. (a) Hair follicle. (b) Gland.

SOS gel group

All tissue samples of wounds were covered by mature epidermal layers with projections, except male sample of test two which epithelial cells were not mature. Collagens presented from a little below the epidermis to the end of tissue, and their concentration gradually increased near the end

of dermal layer. Collagens were partially organized, except in female of test one sample whose dermis was covered completely by darker and more organized collagens. Male sample of test two and female sample of test one had average and scarce number of angiogenesis respectively. Hair follicles were observed in females in average number and just one was seen in male sample of test two. Glands were also observed in female sample of test one (Figure 44). Scab was just seen in female sample of test one and male sample of test two.



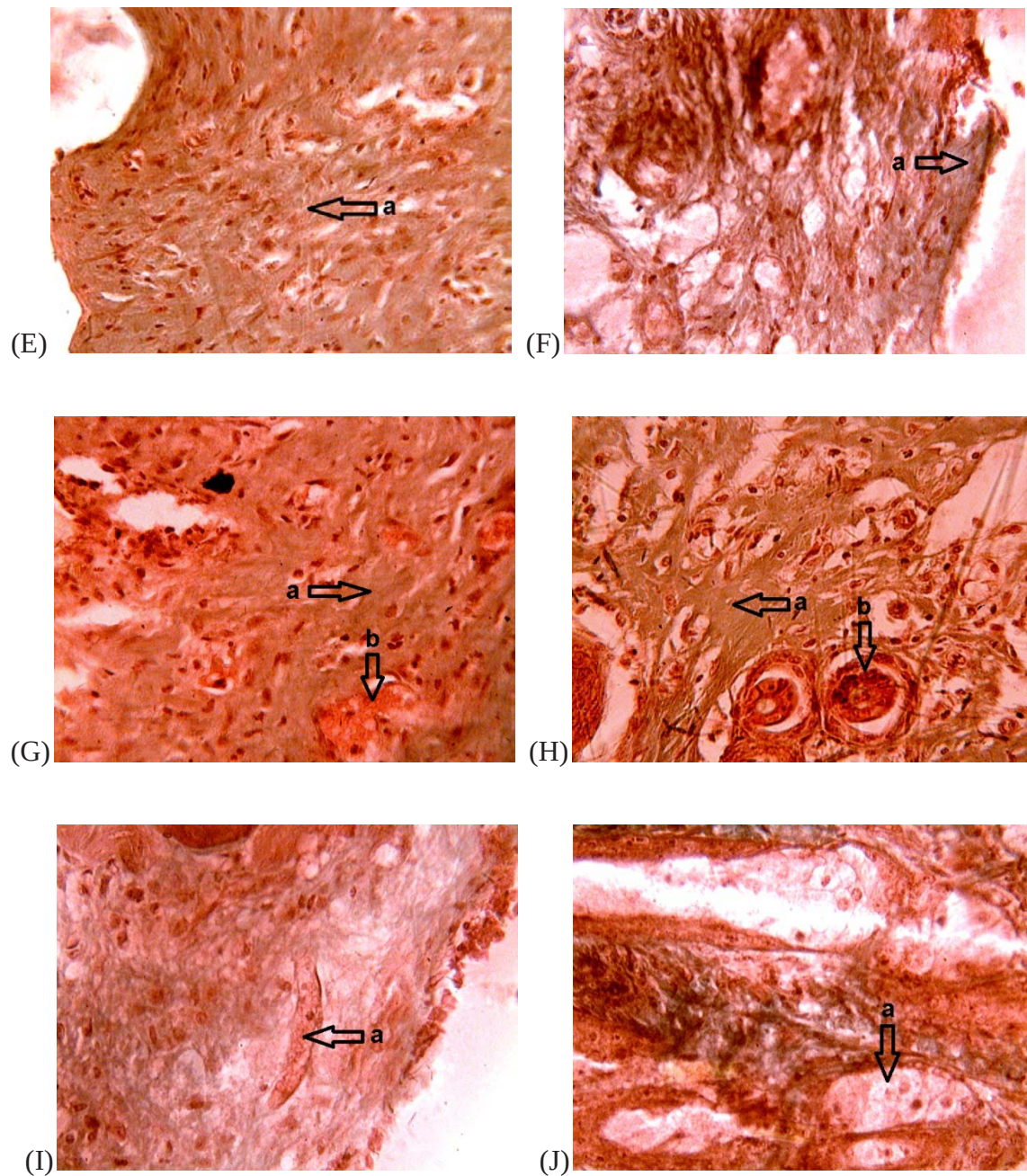
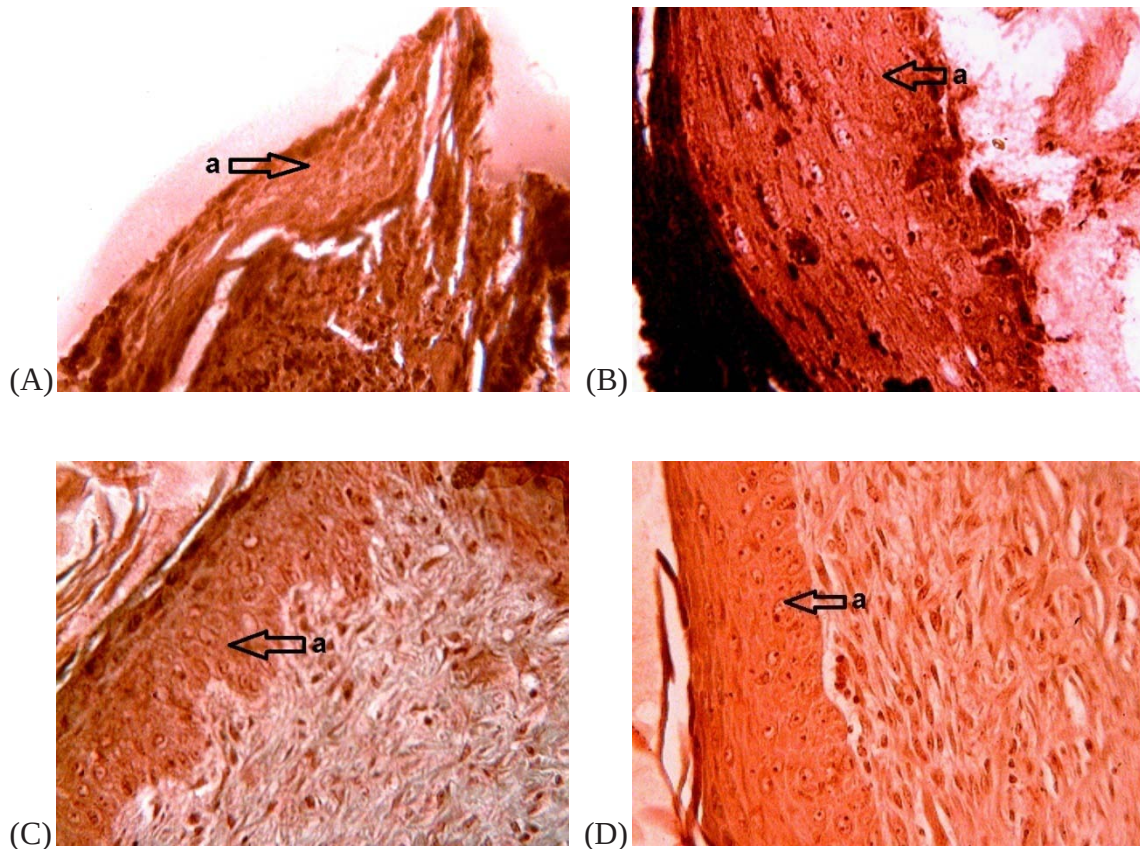
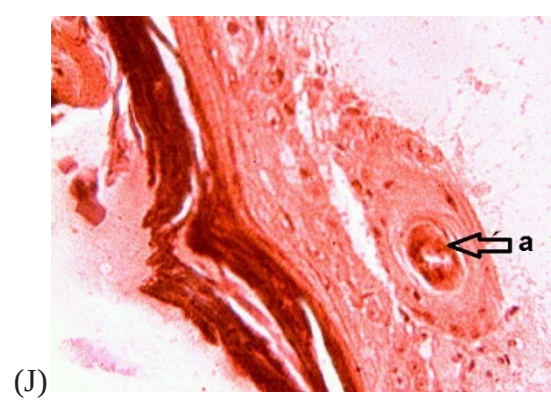
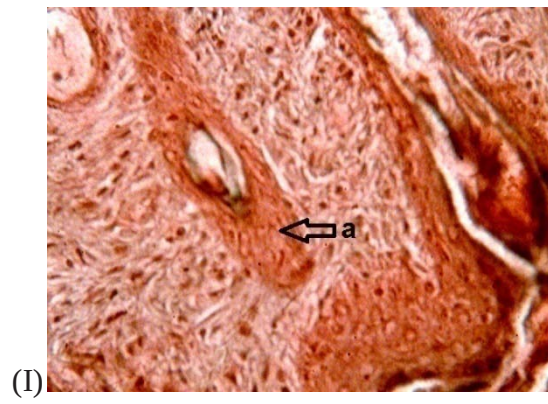
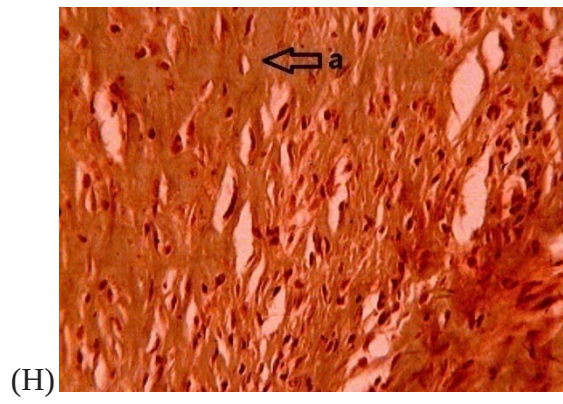
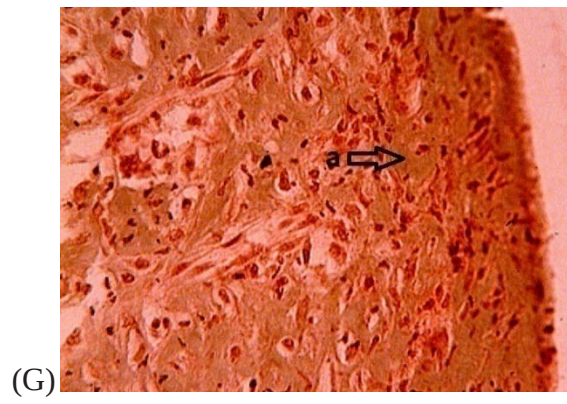
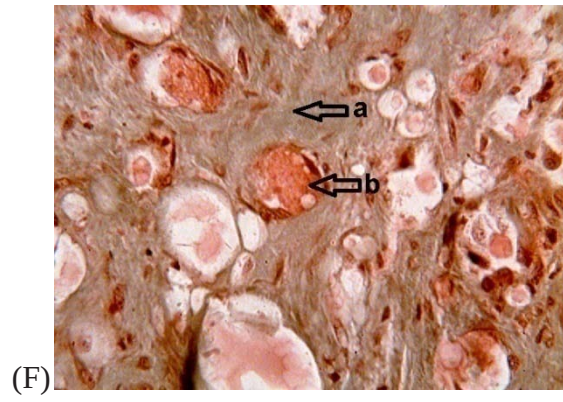
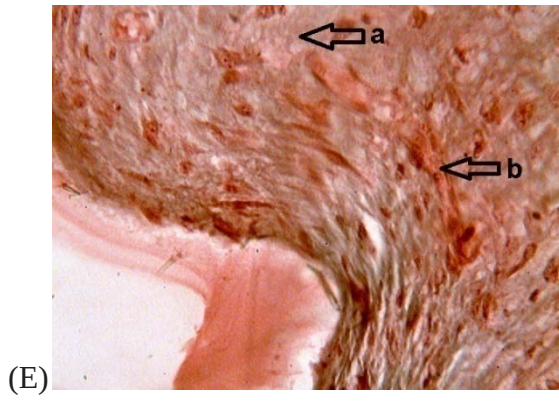


Figure 44. Microscopic view of 12-day-old wound tissue of SOS gel group.(A) Test one, male animal epidermal layer. (B) Test one, female animal epidermal and dermal layer. (C) Test two, male animal epidermal layer. (a) Epithelial cells. (D) Test two, female animal epidermal layer. (a) Epithelial cells. (E) Test one, male animal skin dermal layer. (a) Collagens (F) Test one, female animal skin dermal layer. (a) Collagens. (G) Test two, male animal skin dermal layer. (a) Collagens. (b) Angiogenesis. (H) Test two, female animal dermal layer. (a) Collagens. (b) Hair follicle. (I) Test two, male animal dermal layer. (a) Angiogenesis. (J) Test one, female animal dermal layer. (a) Gland.

Blank gel group

Epidermis in male sample of test one just cover small surface of wound. While other samples were completely covered by epidermis having immature cells in male samples and mature cells in female tissue samples. Collagens presented throughout the dermis, and their density and regularity were more in females. In addition, samples of test one showed more concentration of collagens in dermis than test two. Density of collagens grew gradually to the end of dermal layer in all samples. Few numbers of angiogenesis were observed in all samples. Few number of hair follicles and glands were also observed in male sample of test two and female sample of test one (Figure 45). Just males' tissue sample had scab.





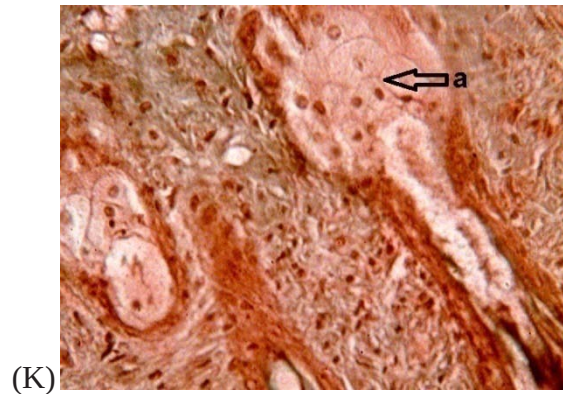


Figure 45. Microscopic view of 12-day-old wound tissue of Blank gel group. (A). Test one, male animal tissue's epidermal layer. (a) Epithelial cells. (B). Test two, male animal tissue's epidermal layer. (a) Epithelial cells. (C) Test one, female animal epidermal layer. (a) Epithelial cells. (D) Test two, female animal epidermal layer. (a) Epithelial cells. (E) Test one, Male animal dermal layer. (a) Collagens in farthest area from epidermis. (b) Angiogenesis. (F) Test one, female animal dermal layer. (a) Collagens in distant area from epidermis. (b) Angiogenesis. (G) Test two, male animal dermal layer. (a) Collagens in distant area from epidermis. (H) Test two, female animal dermal layer. (a) Collagens in distant area from epidermis. (I) Test one, dermis of female sample. (a) Hair follicle. (J). Test two, male sample. (a) Hair follicle. (K) Test one, dermis of female sample. (a) Gland.

Positive control group

All samples had complete epidermis with mature cells and small number. Their dermis had great amount of collagens which were dark gray and mostly organized and oriented horizontally. Small amount of Angiogenesis was just observed in male tissue sections of test one. Scarce amount of hair follicles were also observed in female of test one and male sample of test two (Figure 46). Test two samples all had scab, and test one just part of female tissue sections was covered with it.

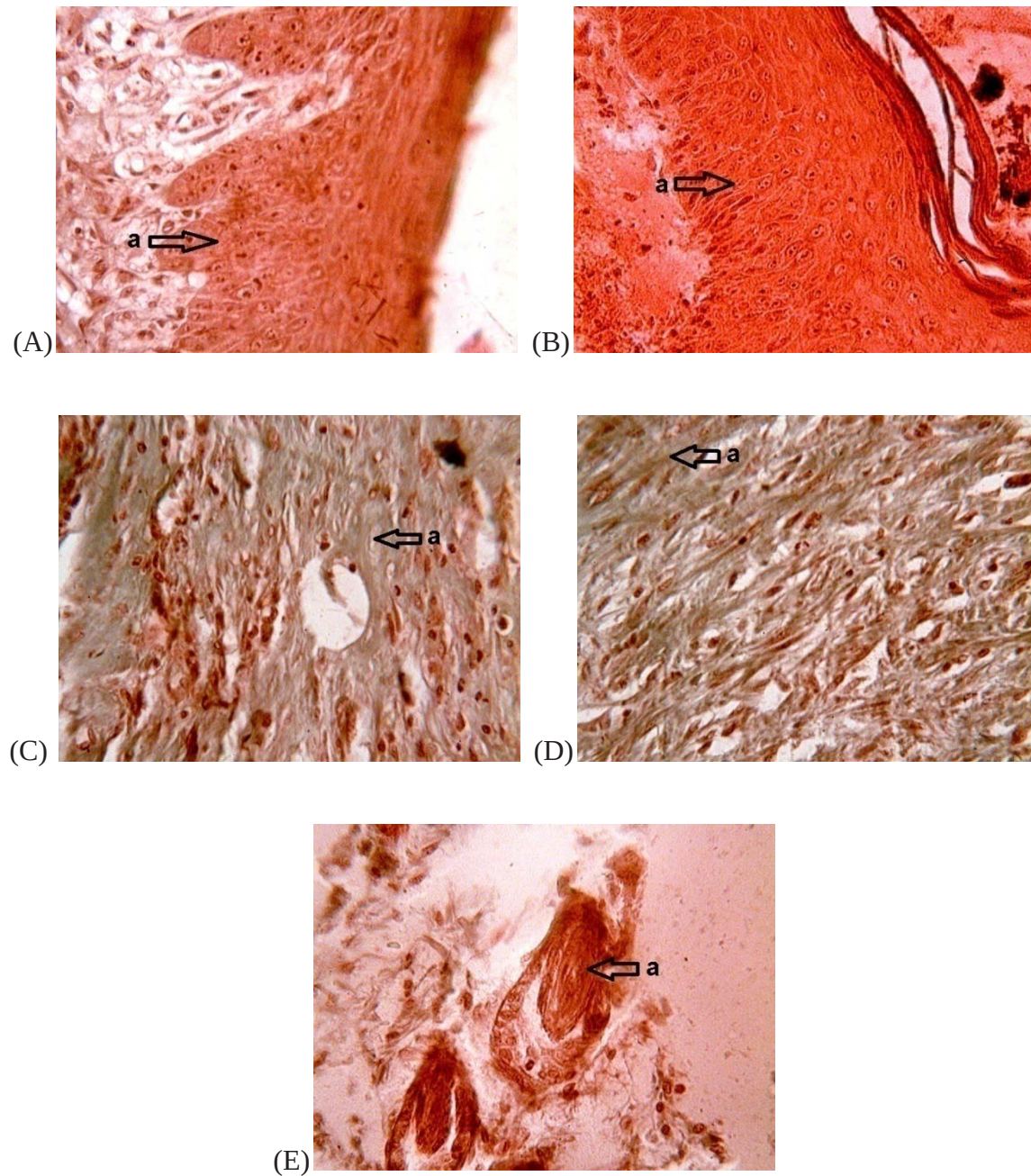


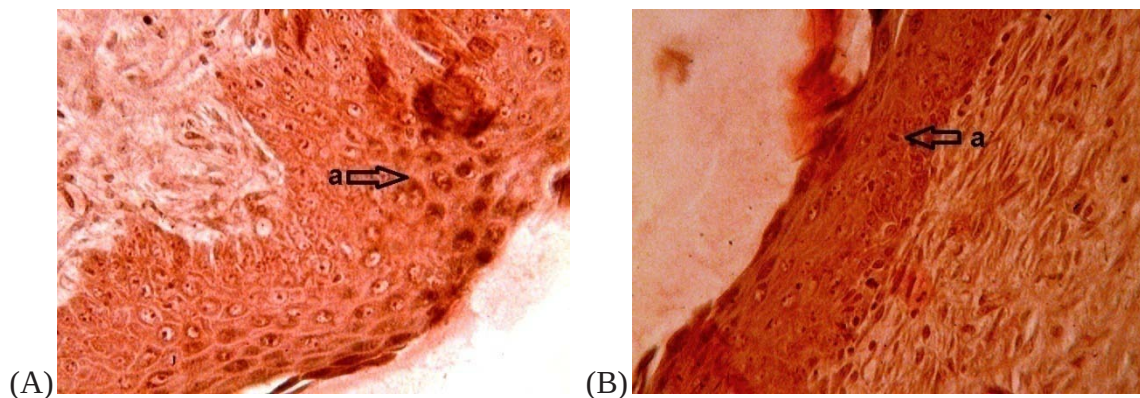
Figure 46. Microscopic view of the section of 12-day-old wound tissue of positive control group.

(A). Male animal tissue's epidermal layer. (a) Epithelial cells. (B). Female animal tissue's epidermal layer. (a) Epithelial cells. (C) Male animal dermal layer. (a) Collagens. (D) Female animal dermal layer. (a) Collagens. (E) Test one female sample. (a) Hair follicle.

Day 15

Negative control group

All tissue samples had complete epidermis containing mature epithelial cells except test two male samples whose epidermal cells were partially mature. Collagen presented throughout the dermis of all samples. The further we got from epidermis, the more concentration of collagens was observed. Overall, collagens of female samples were denser and their color was between dark gray and green. In addition, they were partially organized. Test one male sample showed dense collagen which were gray in color and partially organized, while its counterpart in test two have far less amount of collagens which was just clearly obvious in the distant part of dermis from wound surface and they were light gray and unorganized. All males and female of test one and male sample of test two showed average number of angiogenesis (Figure 47). Just female sample of first test had tiny scab covering small part of tissue.



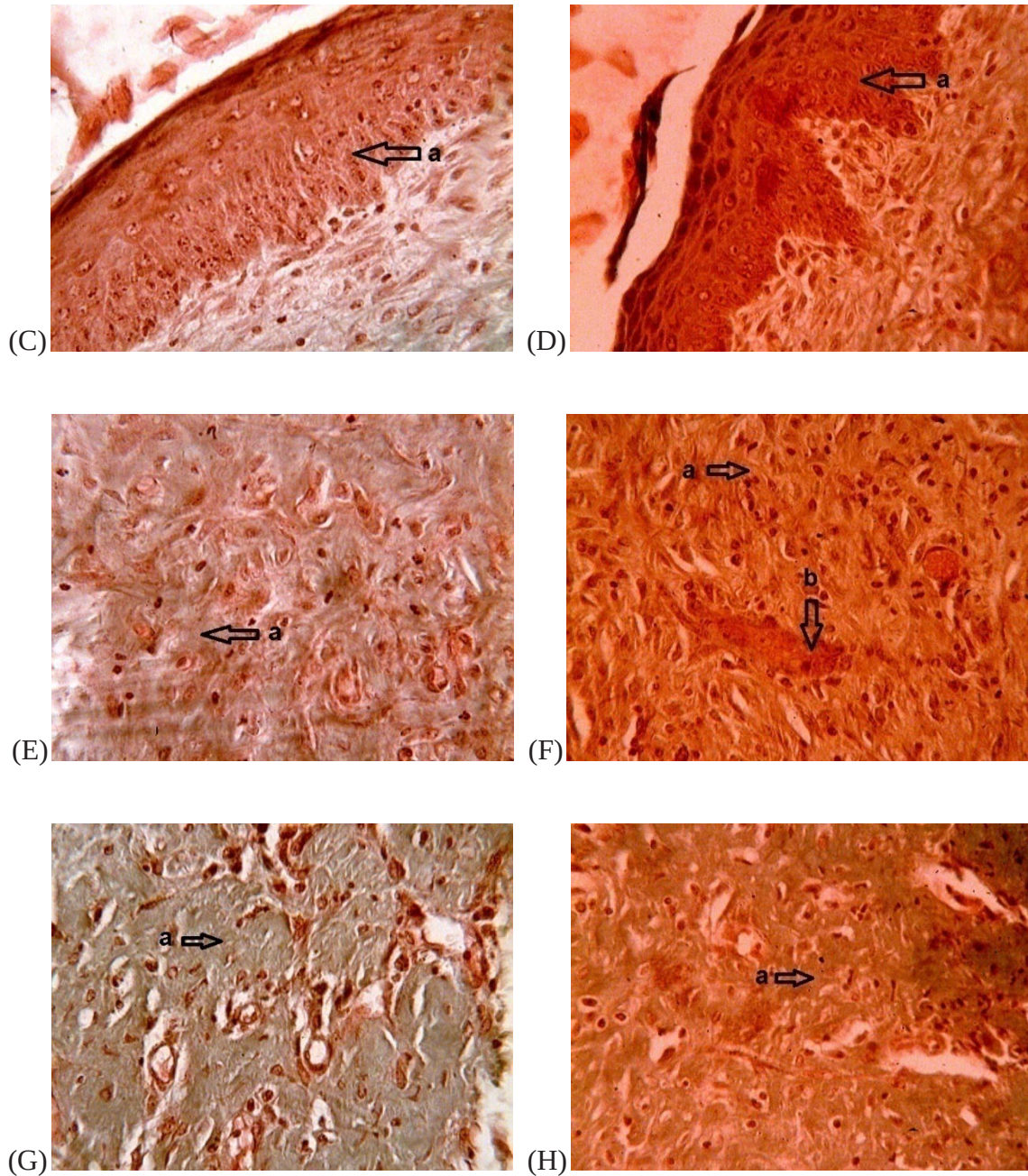
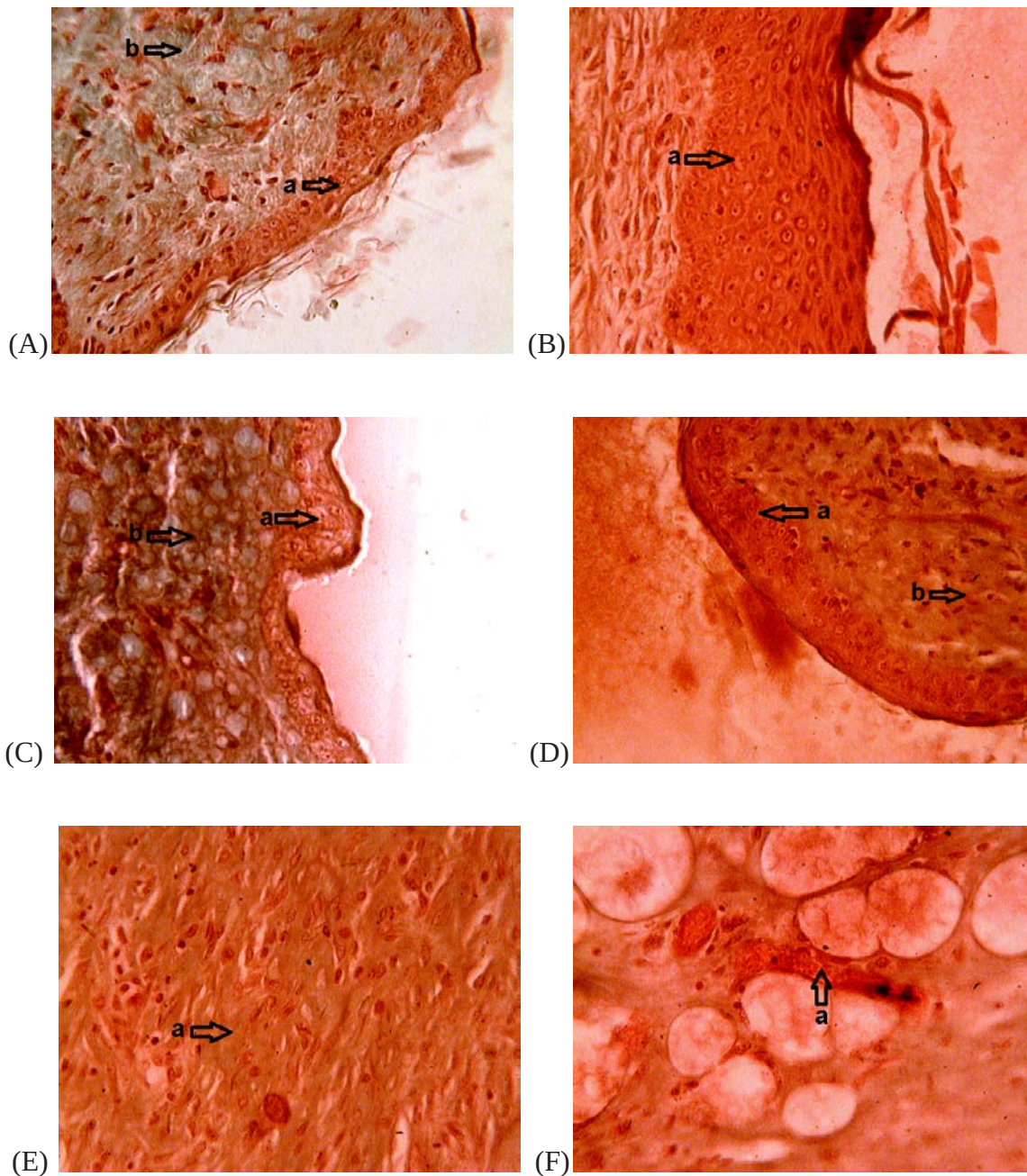


Figure 47. Microscopic view of 15-day-old wound tissue of negative control group.(A). Test one, epidermal layer of male. (a) Epithelial cells. (B) Test two, epidermal layer of female animal. (a) Epithelial cells.(C). Test two, epidermal layer of male. (a) Epithelial cells. (D). Test two, epidermal layer of female animal. (a) Epithelial cells. (E) Test one, Male animal dermal layer. (a) Collagens. (F) Test two, male animal dermal layer. (a) Collagens. (G) Test one, female animal dermal layer. (a) Collagens. (H) Test two, female animal dermal layer. (a) Collagens.

SOS group

All samples obtained complete epidermal layer. Collagen presented densely throughout the dermis of all samples which were gray- green in color and oriented horizontally. Few numbers of angiogenesis were just observed in female samples. Hair follicle and gland were also observed in all samples, while they are few in test two (Figure 48).



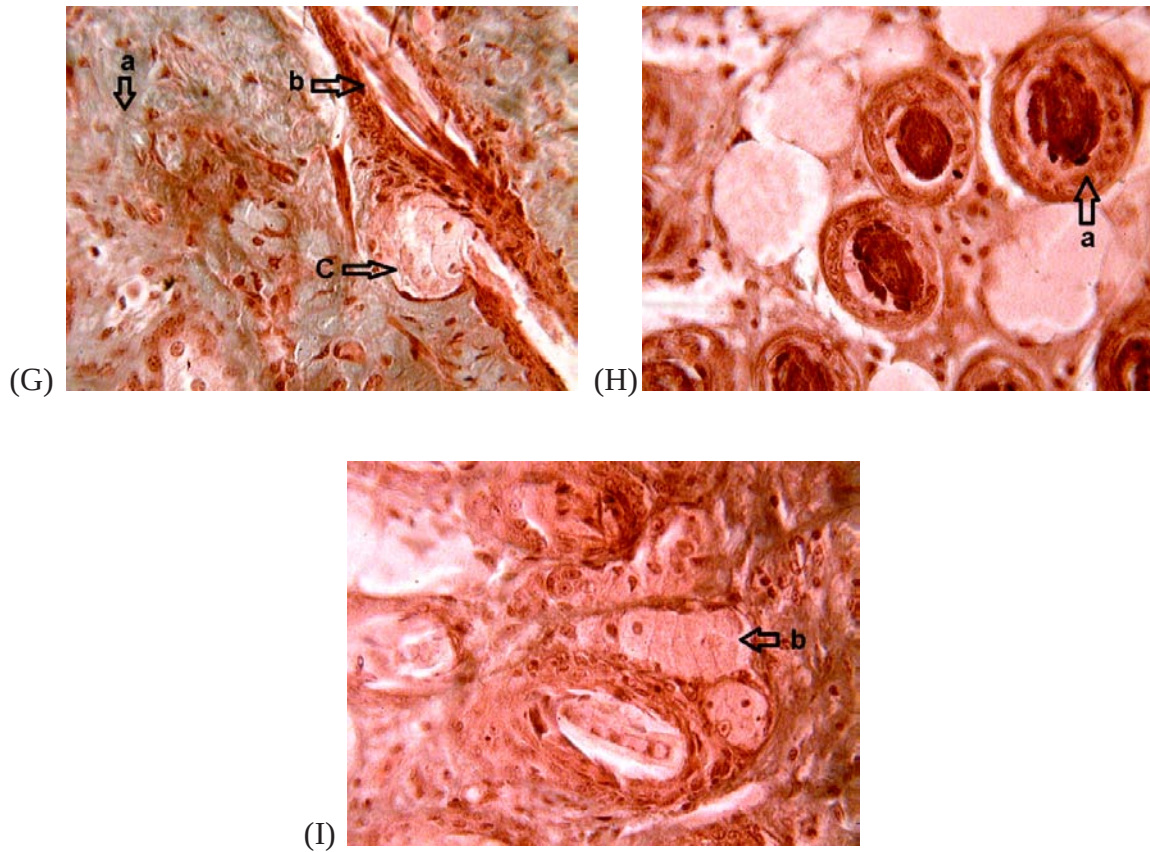
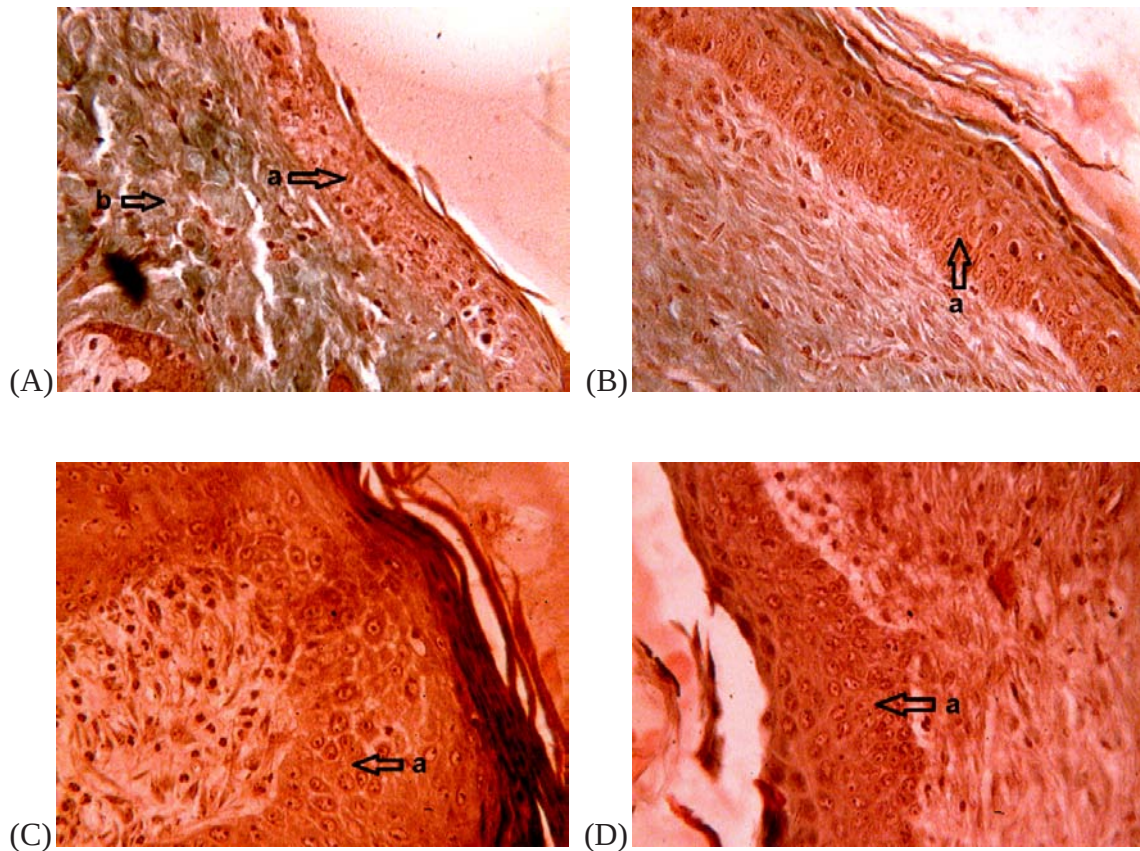


Figure 48. Microscopic view of 15-day-old wound tissue of SOS group. (A). Test one, epidermal and dermal layer of male sample. (a) Epithelial cells. (b) Collagens. (B). Test two, epidermal layer of male. (a) Epithelial cells. (C) Test one, epidermal and dermal layer of female tissue sample. (a) Epithelial cells. (b) Collagens. (D) Test two, epidermal and dermal layer of female tissue sample. (a) Epithelial cells. (b) Collagens. (E) Test two, dermal layer of male. (a) Collagens. (F) Test two, dermal layer of female. (a) Angiogenesis. (G) Test one, dermal layer of male. (a) Collagen. (b) Hair follicle. (c) Gland. (H) (I) Test one, dermal layer of female tissue sample. (a) Hair follicle. (b) Gland.

SOS gel group

Intact epidermal layer covered surface of all tissue sections. However, they were different in rate of maturity. In test one male had thin immature layer of epithelial cells while female had average size epidermis with mature cells. In test two, both male and female tissue sections had average

size epidermis which constructed by partially mature epithelial cells. Great amount of collagens covered whole area of dermis in test one which were gray-green and oriented horizontally. In test two although collagens covered total dermis area, their density and organization were less than test one. Male sample had denser collagens than female. All samples showed few numbers of angiogenesis except female sample of test one which had large number of it at middle of the tissue. Hair follicle was just observed in male sample, and glands were seen in all males and test one female (Figure 49). Just male sample in test two had small scab which covered half of it.



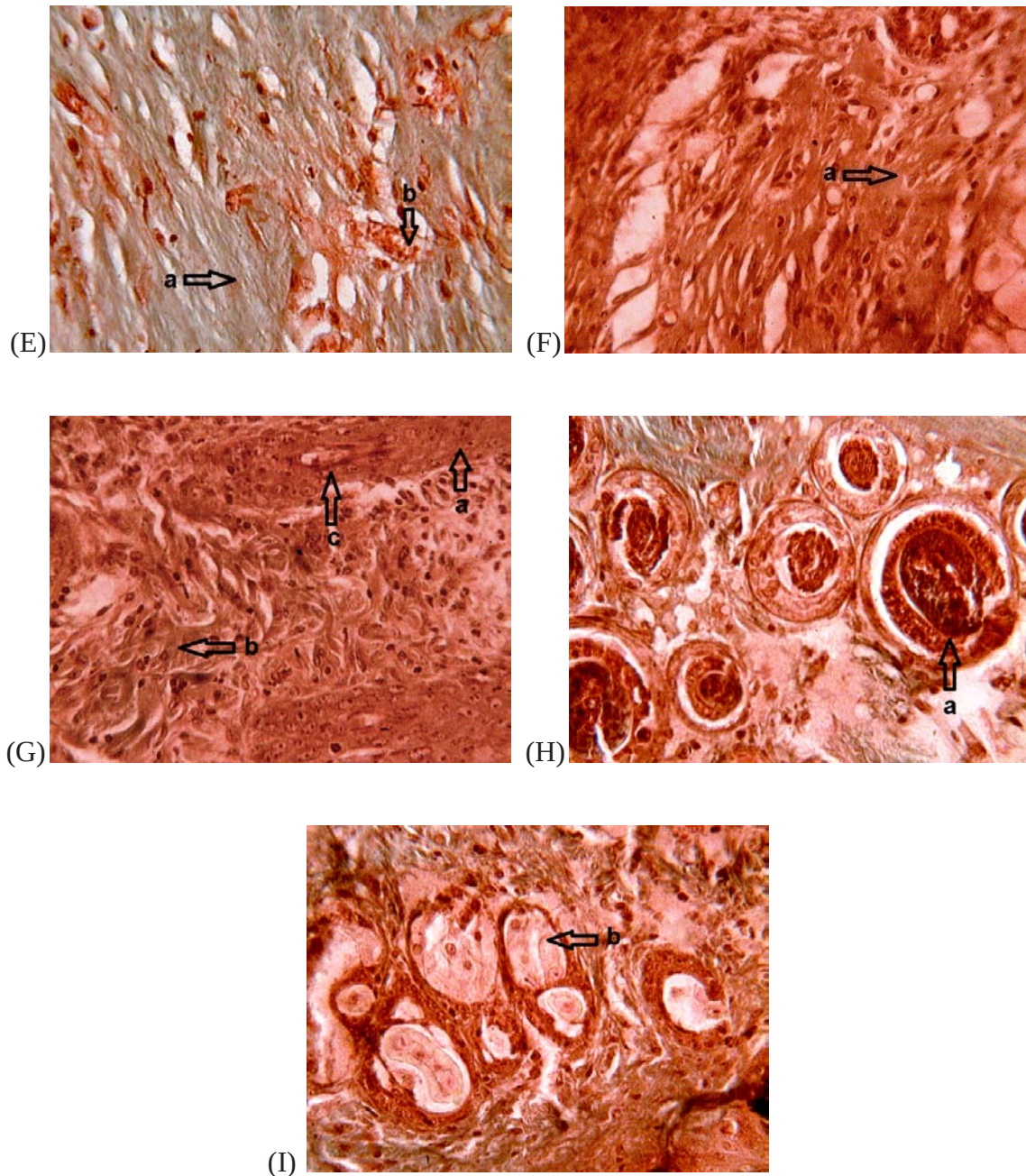
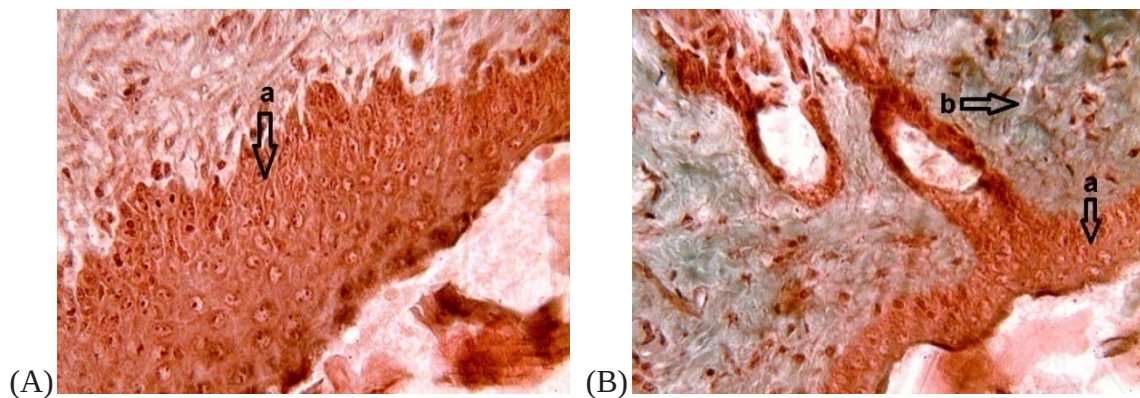
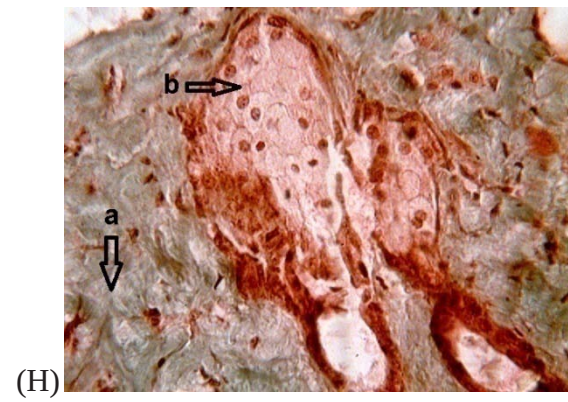
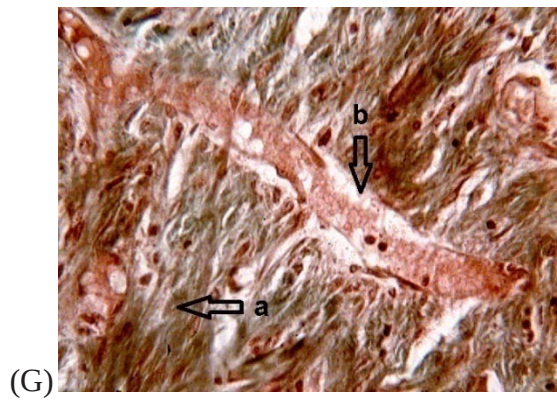
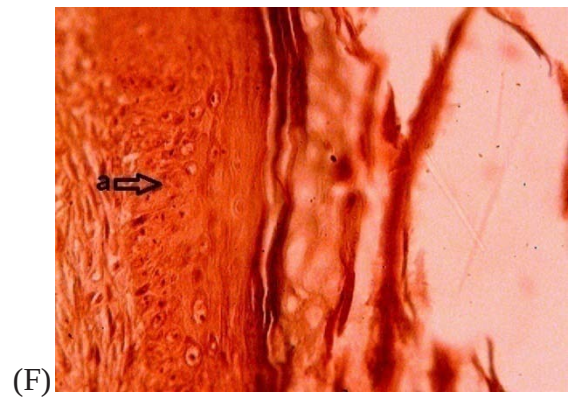
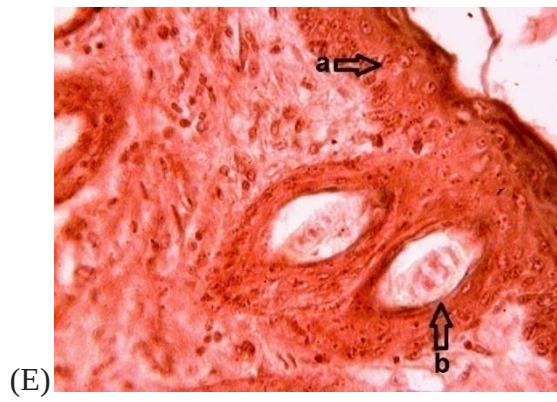
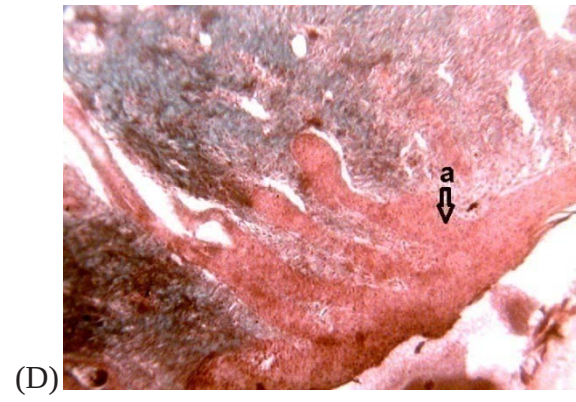
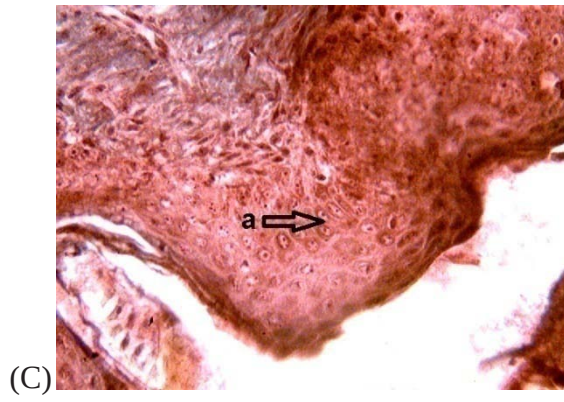


Figure 49. Microscopic view of 15-day-old wound tissue of SOS gel group. (A) Test one, epidermal and dermal layer of male (a) Epithelial cells. (b) Collagens (B) Test one, epidermal and dermal layer of female. (a) Epithelial cells. (C) Test two, epidermal layer of male. (a) Epithelial cells. (D) Test two, epidermal layer of female. (a) Epithelial cells. (E) Test one, female's dermal layer. (a) Collagens. (b) Angiogenesis. (F) Test two, female's dermal layer. (a) Collagens. (G) Test two, male's dermal layer. (a) Epithelial projection. (b) Collagens. (c) Emergence of hair follicle from epithelial cells. (H) (I) Test one, female's dermis. (a) Hair follicle. (b) Gland.

Blank gel group

Epidermis in all samples covered the total surface of wound except in male sample of test one, under the scab covering small part of middle of the tissue it did not exist. In test one in female sample half of the surface of the tissue was covered with thick epidermis with mature cells the other half had thin epidermis. Male sample epidermis was average in thickness and cells maturity with lots of projections. In test two female samples had thin layer of epidermis with average maturity with some folds, and in male samples, half of the tissue was covered with thin immature epidermis and the other half covered by average size epidermis with partially mature cells. Collagens, in test one, covered entire of dermis which were organized, dense and dark gray-green in color. In test two, dermal layer of male tissue sections had the same situation as test one, while, density and organization of collagens in female sample was less, and they did not covered entire area of dermis. They just presented at middle of the tissue to the end. Few numbers of angiogenesis were seen in test one, and average number in male of test two. Average number of hair follicles and glands were observed in test one samples and female of test two. All previous features were placed at the edges of wound near the intact skin where epithelialization started (Figure 50). Test two and small part of test one male samples had scab.





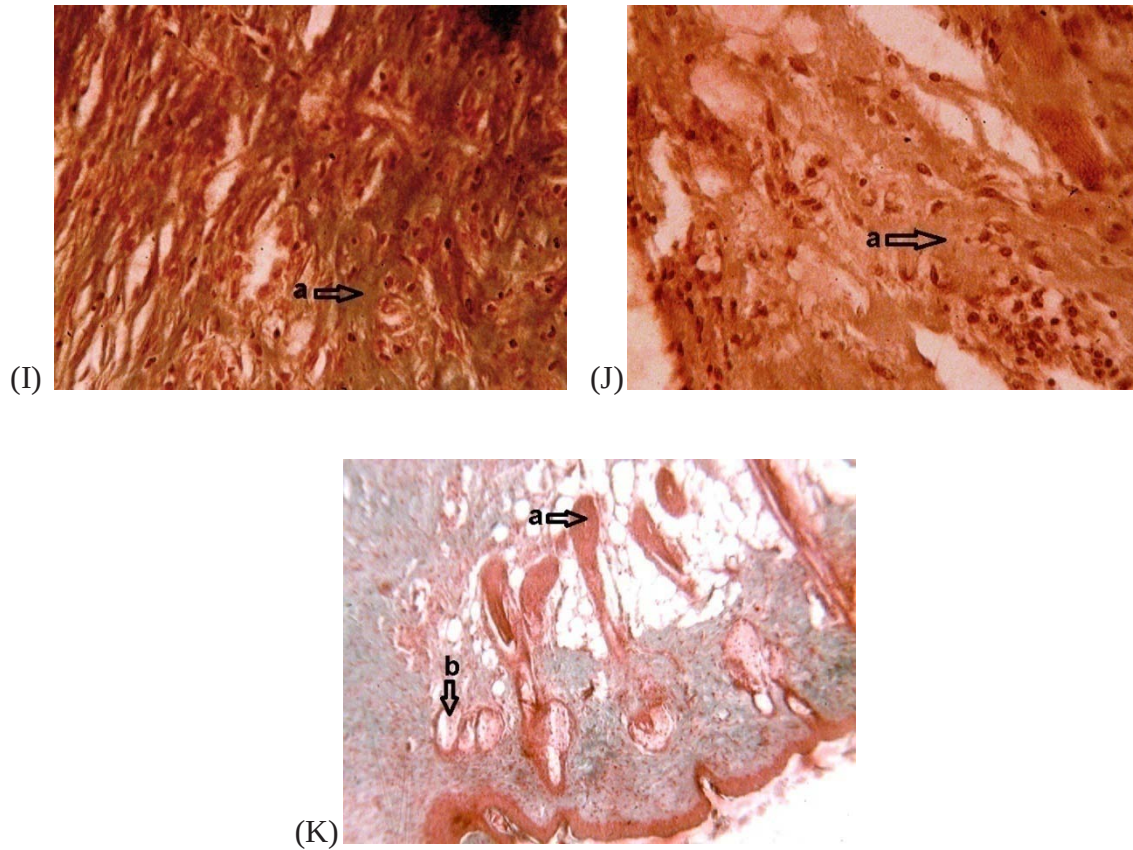
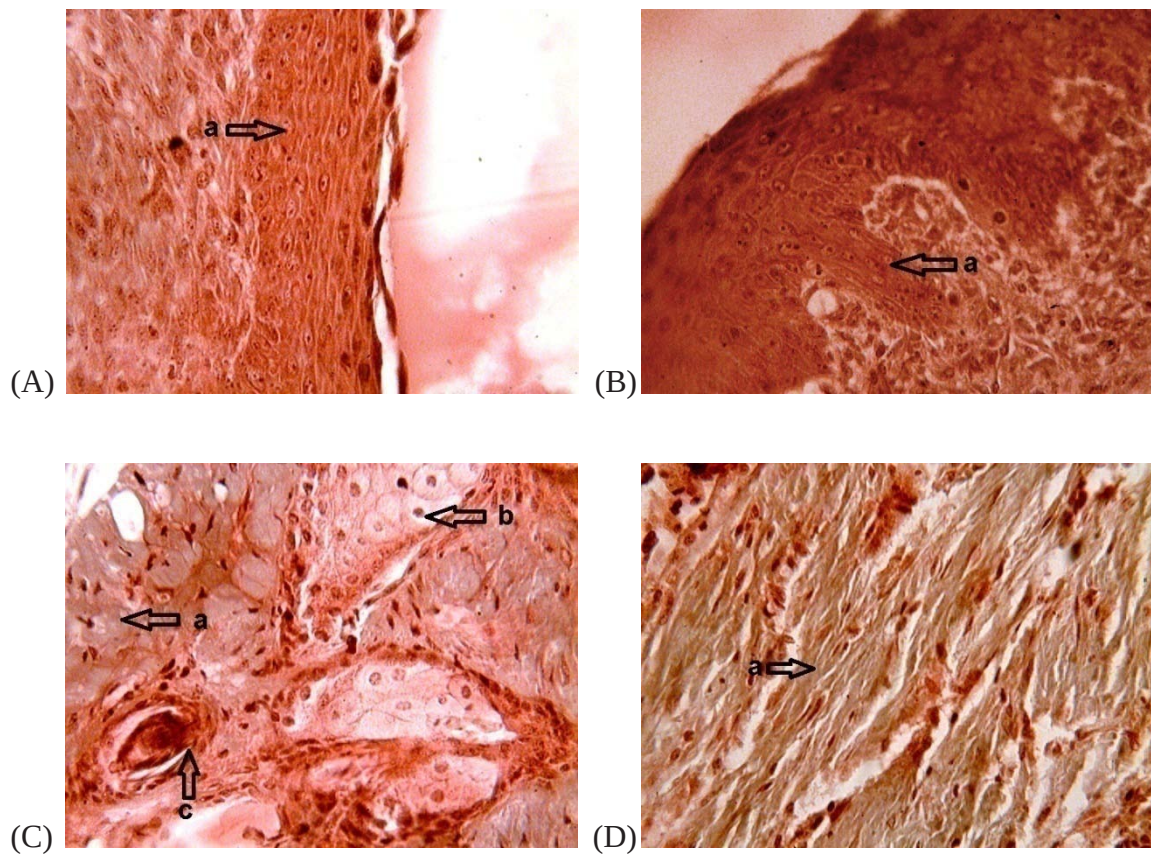


Figure 50. Microscopic view of 15-day-old wound tissue of blank gel group. (A) Test one, female animal thick epidermal layer. (a) Epithelial cells. (B) Test one, female animal thin epidermal layer. (a) Epithelial cells. (b) Collagens. (C) Test one, male animal epidermal layer. (a) Epithelial cells with emerging projections. (D) Test one, male animal epidermal and dermal layer. (a) Epithelial projections. (E) Test two, female animal thin epidermal layer. (a) Epithelial cells. (b) Hair follicle. (F) Test two, male animal epidermal layer. (a) Epithelial cells. (G) Test one male animal dermal layer. (a) Collagens. (b) Angiogenesis. (H) Test one female animal dermal layer. (a) Collagens. (b) Gland. (I) Test two, male animal's dermal layer. (a) Collagens. (J) Test two, female animal dermal layer. (a) Collagens. (K) Test one, female tissue section of wound margin. (a) Hair follicle. (b) Gland.

Positive control group

Epidermis covered entire area of wound in all samples, and it had average thickness with mature cells. In test one, whole part of dermis contained dense dark gray organized collagen, while in

test two scarce amounts of collagens were observed just beneath the epidermis, which increased gradually to the end of dermis. Overall, in test two male samples had collagens with average density and they were mostly organized; however, female sample had fewer amounts of collagens which were not organized and they were light gray. Male of test one and female of test two had few number of angiogenesis. Average number of glands and few number of hair follicle were just observed in male tissue sample of test one (Figure 51).



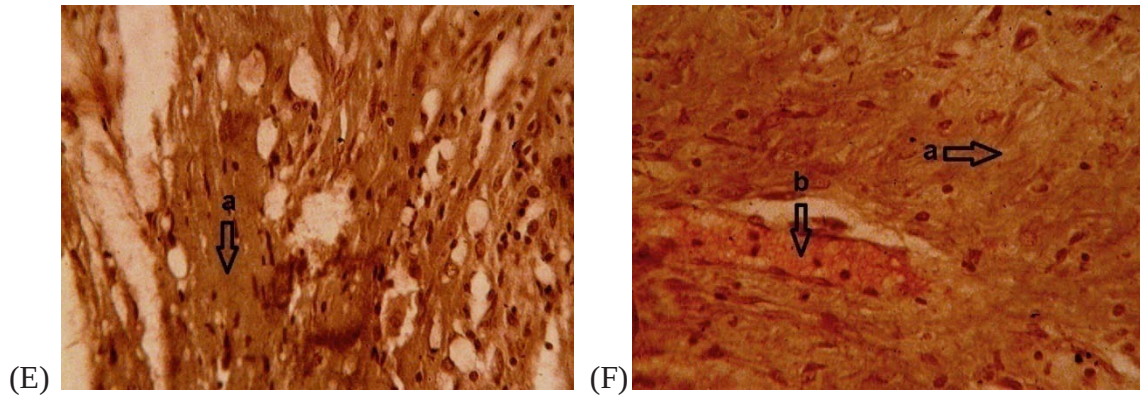


Figure 51. Microscopic view of 15-day-old wound tissue of positive control group. (A) Male sample epidermal layer. (a) Epithelial cells. (B) Female sample epidermal layer. (a) Epithelial projections. (C) Test one, dermis of male wound tissue. (a). Collagens. (b) Gland. (c) Hair follicle. (D) Test one, dermis of female wound tissue. (a) Collagens. (E) Test two, dermis of male wound tissue. (a) Collagens. (F) Test two, dermis of female wound tissue. (a) Collagens. (b) Angiogenesis.

Chapter 4:

Discussion and Conclusions

The ability of wound healing is a biologic necessity for all organisms. Scientists and researchers try to improve the ability to manage wounds and accelerate the process of healing. Superoxidized solution (SOS) has a long history in wound healing; still, most experiment was done to consider its impact on microorganism eradication and few of them cover its histological effect on wound tissue during the treatment. In addition, no scientific survey was done to examine the influence of SOS composition with a thickening agent. Therefore, in this study at first different experiment was done to find out a formulation containing the highest value of ORP and then its effects on animal models were observed during the experiment to see whether increasing durability and stability of SOS on wound via thickening agent has positive or negative effect and compare it with effect of SOS. Besides, histological repairing effect of SOS and SOS gel were compared with positive and negative control groups.

Morphological Evaluation

Results of wound area measurements did not indicate a healing potential for the test solution and gel. Statistically, area measurements showed that there was no significant difference between the different groups in comparison with negative control group from day 3 to 15 except for blank gel on day 12. However, the wound healing potential for SOS group was evident on day 12 (Figure 15), since complete epithelialization (rel.imp = 100) was just observed in this group and other groups did not show full healing at the same day. This potential was further confirmed in the histological evaluation (Figure 43). The second best healing was observed in SOS gel group on day 12, which relative improvement was just over 95%. Reduction in its effect in comparison with SOS group might be due to its formulation which will be discussed more in next

paragraphs. Therefore, it seems that SOS and SOS gel accelerate healing, while still further investigation is needed to obtain conclusive results.

During comparing rel.imp of all groups in each day, the statistical significant result emerged just from comparing blank gel with others on day 12 and 15. Relative improvement of blank gel (rel.imp12= 48.25) was about half of the negative control group (rel.imp12= 90.5) which led to significant difference in their wound area (Figure 15 and 16). Since, adverse impact of blank gel on wound might have been due to its ingredients, a brief review was done to see whether Carboxymethyl cellulose (CMC) or glycerin has negative effect on healing or not.

In a study, effect of six modified type of cellulose on the inflammatory response in macrophage-like THP-1 cells was assessed. The main focus of that research was the influence of those types of cellulose on inducing or inhibiting production of tumor necrosis factor(TNF)- α which is playing crucial role in acute and chronic wound inflammation by enhancing further inflammatory reactions. Thus, inhibition of the release of TNF- α can reduce the severity of inflammation. Transcription of TNF- α was measured in THP-1 which exposed to lipopolysaccharide (LPS) or LPS free (macrophage-like THP-1 cells exposed to LPS, which are similar to the classically activated macrophages (M1), expressing pro-inflammatory cytokines). The result illustrated that CMC does not induce TNF- α production in LPS free THP-1 cells, while it is not able to diminish the production of TNF- α in LPS induced cells. Thus, the survey revealed that CMC does not have pro and antiinflammatory effects based on its effect on TNF- α production (Fernandez *et al.*, 1996; Holtmann *et al.*, 2004; Gouwy *et al.*, 2005; Tesch, 2007). Another study evaluated the effect of the combination of purified carboxymethyl cellulose topical 2% gel on treating normal

ulcers and diabetic and burn ulcers in comparison with saline as control group. Results revealed that the wounds treated with CMC showed lower amount of hemorrhage and tissue necrosis in all days. In addition, the density of collagen fibers and epithelialization level was higher in day 12. Thus the length of healing was shorter and not only CMC had any adverse effect, but also it improved healing (Karami *et al.*, 2012). Another study examined ability of CMC used in artificial tear to interact with corneal-epithelial-cells (HCECs) and promoting epithelial wound healing. The potential of CMC was explored in both in vitro and in vivo (animal model) wound healing model. The result demonstrated that CMC induce in vitro closure of human cornea–limbal epithelial (HCLE) wound and re-epithelization of rabbit corneal epithelial scrape wound via stimulating epithelial cell migration through enhancing attachment of cells to matrix proteins (Garrett *et al.*, 2007). Other survey about impact of CMC on corneal epithelial wound on rabbits confirmed previous one since CMC group had significant reduction in wound area at 24 hr and 32 hr post-wounding and complete wound closure was observed by 48 hr in 100% of CMC-treated eyes compared to 45% of control group. Therefore, it suggested therapeutic effect of CMC on corneal epithelial defects (Garrett *et al.*, 2008). In Conclusion, due to the fact that not only CMC have adverse effect but also it assist wound repair, reduction in wound healing in blank gel group might be because of another factor.

Since other ingredient of blank gel was glycerin, a brief review was done to see whether previous studies found any negative effect of it on wound or not. Glycerol or glycerin is a humectant that has been used in dermatological preparations for many years. Glycerin attracts holds and binds water to itself or into the product that it is used in like hydrogel dressing. The concentration of glycerin in a product has relation with its stability on the wound because of the moist environment that it creates; products with higher concentration of glycerin have longer duration

on the wound (Fluhr *et al.*, 2008). Glycerin has many effects on the skin. It prevents water loss and improves skin barrier properties by preventing the phase transition of the stratum corneum lipids from liquid to solid crystalline structure (Froebe, 1990). It plasticizes stratum corneum to maintain its flexibility and avoid damage due to mechanical stress (Batt *et al.*, 1986). In addition, it has keratolytic effect, softening and shedding of the horny outer layer of the skin, by enhancement of desmosomal digestion (Rawlings *et al.*, 1995), as well as antiirritant (AI) properties (Andersen *et al.*, 2006b; Andersen *et al.*, 2006a), antimicrobial effect (Saegeman *et al.*, 2007) and accelerating wound healing process (Hara *et al.*, 2002; Hara-Chikuma *et al.*, 2008a). Glycerol significantly increase stratum corneum hydration in a long-term usage of glycerin-based cream compared with placebo group (Gloor *et al.*, 2001). Improvement of skin hydration by glycerin in comparison with placebo was also observed in a study on eczematous skin (Breternitz *et al.*, 2008). It was shown in another survey that patients, who were treated with glycerin cream, felt significantly less skin reactions like smarting than patients treated with saline cream (Loden *et al.*, 2002). As previously mentioned, glycerin accelerates wound healing. In a study on aquaporin3 (AQP3) knockout keratinocyte cultures showed reduction in epidermal cell proliferation and migration, use of glycerin corrected this deficiency of cell proliferation and migration (Hara-Chikuma *et al.*, 2008b). Antimicrobial effects of glycerin have been shown by incubating different strains of bacteria with glycerin at different concentrations and temperatures. Comparing the rate of viable bacterial cell reduction between glycerin treated with untreated cultures demonstrated its antimicrobial effect (Saegeman *et al.*, 2007). Virucidal effect of glycerin was also shown on different pathogenic agents (van Baare *et al.*, 1994). As long as, glycerin has positive effect on wound repair similar to CMC, it seems that we cannot relate significant healing difference between blank gel and other groups to ingredient of blank gel.

Thus, it might be due to the specific reaction of Swiss Webster breed to the blank gel or other reasons which needs more investigation.

Data analysis of Figures 17, 18, 19, 20 and 21, which compare healing rate of each animal in proportion to its three days earlier, revealed that repair of SOS and positive control group were accelerated significantly in day 9 in comparison with day 6. While it happened three days later for SOS gel group in day 12. Other groups did not exhibit any significant betterment. In general, our data may demonstrate that the wound exposed to SOS may heal faster especially between day 6 and 9 similar to positive control group, which, as previously mentioned, is due to its different species of ions, including chlorine, ozone, and active oxygen. The active oxygen species in SOS function as bio-signals and activate the enzymes and growth factors for promoting the healing mechanism (Alimi, 2005). Therefore, by using SOS more amount of active oxygen is supplied to the wound site and then greater amount of bio-signals are secreted which result in further promotion of growth of cells and rapid tissue reconstruction (Yahagi *et al.*, 2002). Oxygen species also have an important role in collagen synthesize and promoting the tissue growth. These ions, specially chlorine species, also function as sterilizer for the wound sites (Najafi *et al.*, 2002). Furthermore, high ORP of SOS solution also causes microorganism destruction by unbalancing the osmotic pressure between the cell and hypotonic SOS, which alters integrity of cell membrane (Landa-Solis *et al.*, 2005; Martí'nez *et al.*, 2005). Besides, chlorine and oxygen species of SOS cause imbalanced osmolarity which lead to damage to the integrity of the cell membrane and cause microorganism to denature (Fuqua *et al.*, 1994; Kapur *et al.*, 2011). In terms of SOS gel, inasmuch as prime effects of SOS are according to its high ORP level, the reason why SOS gel did not reveal the same level of recovery as SOS and it accelerated healing three days later (in day 12) might be due to the fact that it had substantial

decrease in its ORP because of incorporation of SOS to other ingredients for preparation of proper formulation (Table 23). Therefore, even though the most suitable thickening agent was used among others in our study, more investigations are needed to find more proper formulation which could maintain higher level of ORP.

The next results obtained from investigation of impact of gender on wound healing and also effect of each group treatment on each gender. Since comparison between male and female irrespective of their group revealed that males' healing was significantly faster on day 3 (Figure 23), it is suggested that generally males' wounds heal faster than females in this study. However, the type of treatment can affect the wound healing activity for each gender (Figure 22). Although comparison of repairing between male and female in each group for different type of treatment demonstrated no significant changes, their differences were obvious especially on days 3 and 6 which are also confirmed by histological evaluation (Figure 22). Male treatment in SOS and positive control group was faster in all days, while it was more striking on days 3 and 6. Furthermore, from day 3 onwards amount of difference between male and female healing declined and on day 15 they had almost same amount of healing (Figure 22). SOS gel and blank gel approximately followed the same pattern as previous groups. However, females had less improvement on day 9 for SOS gel and day 12 for blank gel. In contrast, negative control group revealed opposite trend, female had faster healing on days 3 and 6, and the amount of their differences declined until the last day when they were the same (Figure 22). Since none of the group-based results were significant, the survey could just suggest that there might be interaction between gender and SOS, SOS gel, blank gel and positive control group which accelerate healing in male animals in first 6 days. Moreover, as negative control group received no treatment and female in this group had faster healing it could be concluded that in normal situation (no healing

material) females heal faster than males (Figure 22). The idea that healing in female, without any treatment, is faster than males is also demonstrated by gender-related wound healing studies, which promoted the suggestion that circulating sex hormones are key modulators of the response to cutaneous injury (Gilliver *et al.*, 2006). It was revealed that although estrogens have a positive effect on wound healing by reducing inflammation and accelerating wound closure, testosterone appears to have an adverse effect on it (Gilliver *et al.*, 2003). It was demonstrated that elderly men have been observed to heal wounds more slowly than women through reduced matrix deposition and an enhanced inflammatory response (Ashcroft *et al.*, 1999a; Ashcroft *et al.*, 2002; Gilliver *et al.*, 2006), which could be due to the fact that local levels of estrogen are altered in the elderly due to a combination of decreased circulating gonadal estrogen and highly reduced levels of the adrenal sex steroid precursor dehydroepiandrosterone (DHEA) (Perry, 1999; van den Beld *et al.*, 2000). In addition, the reason why elderly men's wound repair more slowly than elderly women even after similar estrogen treatment is owing to the fact that level of testosterone is generally maintained in aging men with gradual reduction with increasing age (Ashcroft *et al.*, 1999a). Furthermore, declining amount of estrogen in both elder men and women result in loss of protection existing in youth (Labrie *et al.*, 2001; Gilliver *et al.*, 2007).

In contrast to our results, in another study which investigated the effect of wound healing potential of 10% hydrogel of *Lavandula angustifolia* ethanolic extract on excision wounds in BALB/c mice suggest that generally females are healed faster than males but the type of treatment can affect the wound healing activity for each gender (Hemmati *et al.*, 2011)

Estrogens are steroidal hormones that are mainly produced by ovaries and they are responsible for secondary sexual characteristics. Various studies have been done about in vivo and in vitro

effects of estrogen on wound healing in animal or human. Estrogen affects cutaneous wound repairing by adjusting the inflammatory response, cytokine expression, accelerating reepithelialization, increasing matrix deposition, stimulating angiogenesis and wound contraction and modulating proteolysis (Ashcroft *et al.*, 1997; Ashcroft *et al.*, 1999a). A study revealed that ovariectomized (Ovx) rat exhibit a marked reduction in healing in comparison with intact animals, their collagen deposition was less than control group and there was a delay in reepithalization, with an increase in wound size (Ashcroft *et al.*, 1997). Although the exact effect of estrogen on different healing phases are not yet understood, another study showed that estrogen has an antiinflammatory property by its suppressive effect on chemotaxis of polymorphonuclear leukocytes (PMN) via a receptor-mediated mechanism (Josefsson *et al.*, 1992; Margolis *et al.*, 2002). Inhibition of PMN chemotaxis caused early reduction of neutrophils and neutrophil elastase activity, a protease degrading structural and functional protein deposited in wounds such as proteoglycan, collagen and mainly fibronectin. Therefore, downregulation of elastase activity by estrogen decreases fibronectin degradation and influences proliferative phase (McDonald *et al.*, 1980; Miyagi *et al.*, 1992; Ito *et al.*, 1995; Kafienah *et al.*, 1998; Ashcroft *et al.*, 1999a). Another study about antiinflammatory impact of estrogen also suggested that Macrophage migration inhibitory factor (MIF) which is a proinflammatory cytokine produced by skin macrophages, monocytes, neutrophils, keratinocytes and endothelial cells is being downregulated by estrogen to reduce inflammation and induce matrix deposition (Nishihira *et al.*, 1998; Ashcroft *et al.*, 2003b; Baumann *et al.*, 2003; Hardman *et al.*, 2005). Moreover, mitogenic effect of estrogen on keratinocytes causes reepithalization induction; furthermore, it inhibits oxidative stress-induced apoptosis of human keratinocytes (Ashcroft *et al.*, 1997; Ashcroft *et al.*, 1999b; Kanda *et al.*, 2003; Kanda *et al.*, 2004). Effect of

estrogen on angiogenesis might be through direct influence on endothelial cells. Besides, it stimulates the expression of platelet-derived growth factor (PDGF) by monocytes and macrophages having chemotactic and mitogenic impact on dermal fibroblast and induces fibroblast driven wound contraction. Estrogen also stimulates secretion of TGF- β 1 by dermal fibroblast which respectively induce and inhibit matrix deposition and degradation to promote granulation tissue formation and collagen deposition (Rappolee *et al.*, 1988; Katz *et al.*, 1991; Shanker *et al.*, 1995; Ashcroft *et al.*, 1997). Another indication of estrogen positive effect on wound healing is estrogen reduction effect on postmenopausal female. Its decline protease activity, and reduced matrix deposition. (Ashcroft *et al.*, 1997; Son *et al.*, 2005). Since depletion of both estrogen and progesterone occur in postmenopausal phase, wound healing effect seen after Ovx could not only be due to the deprivation of estrogen, but also progesterone. A murine model of incision wound also revealed beneficial effects of estrogen, and to a lesser extent progesterone in wound repairing (Routley *et al.*, 2009).

In contrast to the beneficial effects of estrogen, most recent studies suggested that androgen has an adverse impact on wound repair. Androgens are steroids cholesterol synthesized mainly in the male testes and small amount of them are also produced in ovary and adrenal glands in female (Gilliver *et al.*, 2003). A study investigating wound healing in animals indicated that wound healing was significantly accelerated in the castrated mice compared with the intact animals (Ashcroft *et al.*, 2002). Androgens have proinflammatory effect contributing to delayed wound repair by increasing proteolytic destruction of fibronectin and collagen. One effect of androgens is through Smad3 and intracellular mediator of TGF- β inhibiting wound healing by stimulating inflammatory response (Williams *et al.*, 1996; Ashcroft *et al.*, 2003a). In addition,

androgen was found to induce inflammatory phase via TNF- α whose downstream effect is upregulation of proinflammatory cytokines; thus, upregulation of TNF- α expression by testosterone leads to inflammatory induction (Ashcroft *et al.*, 2002). It was also shown that androgens reduce proteolytic activity of matrix metalloproteinases by negatively regulating its expression, which leads to an increase in collagen deposition in remodeling phase which may cause keloid formation and hypertrophic scarring (Schneikert *et al.*, 1996). Although androgens have adverse impact on healing via their proinflammatory function, they are reported to stimulate keratinocyte proliferation by enhancing expression of keratinocyte growth factors; as a result, reduction in androgen level would delay epithelization. Besides, androgen induces dermal epithelial proliferation. Therefore, its adverse impact on wound repairing is due to its effects on inflammatory cells and matrix deposition (Planz *et al.*, 1998; Perry, 1999; Ashcroft *et al.*, 2002). In addition, a research on castrated adult rats, suggested that testosterone stimulates angiogenesis and vascular regrowth by inducing the synthesis of an angiogenic factor, or by decreasing the synthesis of an angiogenesis inhibitor, or through up-regulating the expression of endothelial receptors of angiogenic factors (Franck-Lissbrant *et al.*, 1998), and it was illustrated that testosterone levels modulate human mucosal healing rates by impacting on the proliferative phase of healing which involves immune processes like angiogenesis and reepithelialization (Engeland *et al.*, 2009).

Histological Evaluation

Observation of second test's tissue sections highlighted the point that complete epithelialization (epidermis cover whole surface of the wound tissue) in SOS and SOS gel group occurred three days earlier than other groups at day 9 (Figure 38 and Figure 39). This finding is supported by other studies. In a study involving 200 patients suffering from different wounds, 100 patients

were treated with SOS and 100 patients with povidone iodine (betadine). The results revealed that the average length of hospital stays in SOS treated patients were less than betadine group. The granulation tissue formation and epithelialization occurred earlier in the SOS group compared to betadine group (Kapur *et al.*, 2011). In another study the effectiveness of electrolyzed strong acid aqueous solution with high ORP and high concentrations of chloride and oxygen was examined on the three-degree burn rats which received physiological saline, electrolyzed solution and no treatment. As a result, epithelialization occurred significantly earlier in group of electrolyzed aqueous solution (Nakae *et al.*, 2000b). The study, which was conducted by Yahagi (2002), correlated the promoting growth of tissue cells at wound sites to the effect of active oxygen. Active oxygen species function as bio-signals for the growth of the cells; hence, by using SOS solution, greater amount of active oxygen is supplied to the wound site. Thus, greater amount of bio-signals are secreted and it resulted in cell growth promotion which leads to earlier reconstruction of tissue. The active oxygen concentration should be between 1ppm to 500ppm, while below 1 ppm, it does not function as bio-signal, and above 500ppm or more, it produces undesired effects on the cells because of its toxicity (Yahagi *et al.*, 2002).

Based on ours and previous mentioned studies, it could be suggested that SOS stimulates epithelialization in wound healing process which is also preserved in the form of gel. Hence, it seems that using CMC as a thickening agent to produce SOS gel does not have a negative impact on its epithelialization promoting effect.

Considering histological changes at day 6, it illustrated that SOS, SOS gel and the positive control group had more fibroblast proliferation in male animals than female (Figure 32, 34 and 36). Inasmuch as statistical study showed that SOS, SOS gel and positive control group

accelerate healing in male animals in first 6 days (Figure 22), it seems that interaction between gender and three mentioned group might enhance fibroblast proliferation to facilitate repairing. However, more studies are needed to confirm these results.

Technical Issues

There were some technical issues during the experiments which may have influenced our results. One of these problems was the population studied. If the number of mice in each group was higher, more accurate and significant statistical results would have been obtained.

Inasmuch as wound should be created on a certain area of mouse body to avoid scratching, they were created on interscapular region. Observations done during experiments showed that size and shape of the wound varied by neck position during the first night when the wound is still fresh. Thus, it would be better to locate the wound where there is less flexibility.

Another problem owed to the use of an inhalant anesthetic drug, ethyl ether, due to the lack of facility. Since anesthetic effect of ethyl ether was too short, about 30 to 60 seconds, animals should be exposed to ethyl ether twice or three times for wound creation and size measurement. Besides, it is reported that sequential anesthesia has adverse effect on animal such as loss of appetite, concentration disorders, exhaustion, sleepiness, excitability, psychic disorders and changes in the blood counts (Arvidson, 1992). In addition, although skin would not be damaged through brief contact with ethyl ether, as ethyl ether is a good solvent for fats, repeated contact could make the skin dry and cracked (Kirwin *et al.*, 1981). Therefore, it could be better to use parenteral anesthetics or other inhalation anesthetic method which keeps animal in anesthesia for a longer period.

Conclusion

Based on our results, it may be concluded that epithelialization is accelerated by both SOS and SOS gel. In addition, SOS gel formulation used in this study has neither adverse nor positive effect on wound healing in comparison with SOS. Besides, as SOS and positive control group accelerate healing in three days earlier in comparison with SOS gel, it could be suggested that increasing stability of SOS on wound through making CMC-based formulation does not accelerate rate of healing, which could be according to the fact that ORP level was significantly decreased during gel production. Therefore, more research is required to find a new formulation which could retain higher level of ORP.

Although our results may suggest that male mice may heal faster than female animals, previous work in our department obtained an opposite finding (Hemmati *et al.*, 2011).

Overall, these opposite findings may suggest there is an interaction between gender and wound healing process. Further research is required to come to conclusive results.

Future Work

Some suggestions could be made for future related works. In order to obtain more accurate and significant statistical results further population of laboratory animals are needed. In addition, inasmuch as SOS has disinfectant effect, it might be more proper to use infected wound model, which might clarify further healing differences between SOS and SOS gel groups in comparison to other groups.

Since CMC-based formulation of SOS gel did not have high value of ORP, further investigations are required to find appropriate formulation.

As the results obtained from this study revealed faster healing in male than female which is opposite to another study done in the same department on different outbred mice (Hemmati *et al.*, 2011), it is suggested to design an experiment which focuses on effect of gender on rate of healing.

Chapter5:

References

Abhyankar SV, Venkatesh V, Karnad S, Kulkarni KP, Juneja M, Nanda BS, *et al.* (2009). Efficacy and safety of oxum in treatment of chronic wounds. *Journal of the Indian Medical Association* **107**(12): 904-906.

Al-Haq M, Seo Y, Oshita S, Kawagoe Y (2002). Disinfection effects of electrolyzed oxidizing water on suppressing fruit rot of pear caused by *Botryosphaeria berengeriana*. *Food Res Int* **35**(7): 657-664.

Alimi H (2005). Oxidative reductive potential water solution and methods of using the same, A01N 25/00 edn. US: Oculus Inoovative Sciences, Inc.

Alimi H (2006). Method of treating second and third degree burns using oxidative reductive potential water solution, A61N 1/30 edn. US: Oculus Innovative Sciences, Inc., Petaluma, CA.

Alimi H (2011). Oxidative reductive potential water solution, processes for producing same and methods of using the same, C02F 1/461 edn. US: Oculus Innovative Sciences, Inc.

Alimi H, Gutierrez A (2006). Method of treating skin ulcer using oxidative reductive potential water solution US: Oculus Innovative Sciences, Inc.

Alimi H, Gutierrez A (2007). Methods of treating or preventing inflammation and hyper sensitivity with oxidative reductive water potential solution, A61K 33/14 edn. US: Oculus Innovative sciences, Inc.

Alonso JE, Lee J, Burgess AR, Browner BD (1996). The management of complex orthopedic injuries. *The Surgical clinics of North America* **76**(4): 879-903.

Andersen F, Hedegaard K, Petersen TK, Bindslev-Jensen C, Fullerton A, Andersen KE (2006a). Anti-irritants I: Dose-response in acute irritation. *Contact dermatitis* **55**(3): 148-154.

Andersen F, Hedegaard K, Petersen TK, Bindslev-Jensen C, Fullerton A, Andersen KE (2006b). Anti-irritants II: Efficacy against cumulative irritation. *Contact dermatitis* **55**(3): 155-159.

Arnold F, West DC (1991). Angiogenesis in wound healing. *Pharmacology & therapeutics* **52**(3): 407-422.

Arrigo AP, Firdaus WJ, Mellier G, Moulin M, Paul C, Diaz-latoud C, *et al.* (2005). Cytotoxic effects induced by oxidative stress in cultured mammalian cells and protection provided by Hsp27 expression. *Methods* **35**(2): 126-138.

Artuc M, Hermes B, Steckelings UM, Grutzkau A, Henz BM (1999). Mast cells and their mediators in cutaneous wound healing--active participants or innocent bystanders? *Experimental dermatology* **8**(1): 1-16.

Arvidson B (1992). Ethyl ether. NEG and NIOSH basis for an occupational health standard. *Arbete Och Hälsa* **30**.

Ashcroft GS, Dodsworth J, van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, *et al.* (1997). Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. *Nature medicine* **3**(11): 1209-1215.

Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM, Ferguson MW (1999a). Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. *The American journal of pathology* **155**(4): 1137-1146.

Ashcroft GS, Mills SJ (2002). Androgen receptor-mediated inhibition of cutaneous wound healing. *The Journal of clinical investigation* **110**(5): 615-624.

Ashcroft GS, Mills SJ, Flanders KC, Lyakh LA, Anzano MA, Gilliver SC, *et al.* (2003a). Role of Smad3 in the hormonal modulation of in vivo wound healing responses. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **11**(6): 468-473.

Ashcroft GS, Mills SJ, Lei K, Gibbons L, Jeong MJ, Taniguchi M, *et al.* (2003b). Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. *The Journal of clinical investigation* **111**(9): 1309-1318.

Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, *et al.* (1999b). Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nature cell biology* **1**(5): 260-266.

Assoian RK, Sporn MB (1986). Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *The Journal of cell biology* **102**(4): 1217-1223.

Attinger CE, Bulan EJ (2001). Debridement. The key initial first step in wound healing. *Foot and ankle clinics* **6**(4): 627-660.

Attinger CE, Janis JE, Steinberg J, Schwartz J, Al-Attar A, Couch K (2006). Clinical approach to wounds: debridement and wound bed preparation including the use of dressings and wound-healing adjuvants. *Plastic and reconstructive surgery* **117**(7 Suppl): 72S-109S.

Ayello EA, Thomas DR, Litchford MA (1999). Nutritional aspects of wound healing. *Home healthcare nurse* **17**(11): 719-729; quiz 730.

Bahadori M (1991). *Histopathological Techniques*. 2nd edn. Tehran University Publication: Tehran.

Bancroft JD, Gamble M (2002). *Theory and Practice of Histological Techniques*. edn. Churchill and Livingstone: New York.

Barker TH (2011). The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. *Biomaterials* **32**(18): 4211-4214.

Barlett RH (1981). Skin substitutes. *J Trauma* **21**.

Barratt L, Lloyd B, Graham J (1990). Comparative evaluation of two novel disinfection methods for small-community water treatment in developing countries. *Aqua* **39**(6): 396-404.

Barrette WC, Jr., Hannum DM, Wheeler WD, Hurst JK (1989). General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. *Biochemistry* **28**(23): 9172-9178.

Batt MD, Fairhurst E (1986). Hydration of the stratum corneum. *International journal of cosmetic science* **8**(6): 253-264.

Battegay EJ (1995). Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. *Journal of molecular medicine* **73**(7): 333-346.

Bauer SM, Bauer RJ, Velazquez OC (2005). Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. *Vascular and endovascular surgery* **39**(4): 293-306.

Baum CL, Arpey CJ (2005). Normal cutaneous wound healing: clinical correlation with cellular and molecular events. *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al.]* **31**(6): 674-686; discussion 686.

Baumann R, Casaulta C, Simon D, Conus S, Yousefi S, Simon HU (2003). Macrophage migration inhibitory factor delays apoptosis in neutrophils by inhibiting the mitochondria-dependent death pathway. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**(15): 2221-2230.

Boateng JS, Matthews KH, Stevens HN, Eccleston GM (2008). Wound healing dressings and drug delivery systems: a review. *Journal of pharmaceutical sciences* **97**(8): 2892-2923.

Bolton L, van Rijswijk L (1991). Wound dressings: meeting clinical and biological needs. *Dermatology nursing / Dermatology Nurses' Association* **3**(3): 146-161.

Bongiovanni CM (2006). Superoxidized Water Improves Wound Care Outcomes in Diabetic Patients. *Diabetic Microvascular Complications Today*: 11-14.

Bowler PG (2003). The 10(5) bacterial growth guideline: reassessing its clinical relevance in wound healing. *Ostomy/wound management* **49**(1): 44-53.

Breternitz M, Kowatzki D, Langenauer M, Elsner P, Fluhr JW (2008). Placebo-controlled, double-blind, randomized, prospective study of a glycerol-based emollient on eczematous skin in atopic dermatitis: biophysical and clinical evaluation. *Skin pharmacology and physiology* **21**(1): 39-45.

Broughton G, 2nd, Janis JE, Attinger CE (2006a). The basic science of wound healing. *Plastic and reconstructive surgery* **117**(7 Suppl): 12S-34S.

Broughton G, 2nd, Janis JE, Attinger CE (2006b). Wound healing: an overview. *Plastic and reconstructive surgery* **117**(7 Suppl): 1e-S-32e-S.

Burns T, Breathnach S, Griffiths C, Cox N (eds) (2010). *Rook's Textbook of Dermatology*. Wiley-Blackwell: UK.

C P, J WH, S S, al e (2001). bacterial wall products induce down regulation of vascular endothelial growth factor receptors on endothelial cells via a CD 14 dependent mechanism: implications for surgical wound healing. *Sur Res* **101**: 138-145.

Cai Z Characterisation of electrochemically activated solutions for use in environmental remediation Ph.D. thesis, University of the West of England, Bristol, 2005.

Ceri H, Olson ME, Buret A (1996). The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. **37**: 1771-1776.

Chittoria RK, Yootla M, Sampatrao LM, Raman SV (2007). The role of super oxidized solution in the management of diabetic foot ulcer: our experience. *Nepal Medical College journal : NMCJ* **9**(2): 125-128.

Chu HQ, Xiong H, Zhou XQ, Han F, Wu ZG, Zhang P, *et al.* (2006). Aminoglycoside ototoxicity in three murine strains and effects on NKCC1 of stria vascularis. *Chinese medical journal* **119**(12): 980-985.

CKJ AP (2000). *Text book of microbiology*. 6th edn. Orient Longman Publishers: Hyderabad.

Clark RA (1993). Regulation of fibroplasia in cutaneous wound repair. *The American journal of the medical sciences* **306**(1): 42-48.

Cloete TE, Thantsha MS, Maluleke MR, Kirkpatrick R (2009). The antimicrobial mechanism of electrochemically activated water against *Pseudomonas aeruginosa* and *Escherichia coli* as determined by SDS-PAGE analysis. *Journal of applied microbiology* **107**(2): 379-384.

Cohen IK, Diegelman RF, Dome RY, etal (1999). Wound care and wound healing. In: Schwartz SI, Shires GT, Spencer FC, etal (eds). *Principles of surgery*, Seventh edn. New York: McGraw-Hill. p^pp 263-295.

Cohen IK, Diegelman RF, Dorne RY, etal (1993). Wound healing. In: Greenfield LJ, Mulholland MW, Oldham KT, etal (eds). *Surgery: scientific principles and practice*, Third edn. Philadelphia: Lippincott-Raven. p^pp 86.

Cooper ML, Laxer JA, Hansbrough JF (1991). The cytotoxic effects of commonly used topical antimicrobial agents on human fibroblasts and keratinocytes. *J Trauma* **31**(6): 775-782; discussion 782-774.

Cort WM (1974). Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid and their mode of action. *Journal of the American Oil Chemists' Society* **51**(7): 321-325.

Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G (2003). The application of biofilm science to the study and control of chronic bacterial infections. *The Journal of clinical investigation* **112**(10): 1466-1477.

Cutting KF (2003). Wound Healing, Bacteria and Topical Therapies. *EWMA journal* **3**: 17-19.

Cutting KF, White RJ (2005). Criteria for identifying wound infection--revisited. *Ostomy/wound management* **51**(1): 28-34.

de Beer D, Stoodley P, Roe F, Lewandowski Z (1994). Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnology and bioengineering* **43**(11): 1131-1138.

Degreef HJ (1998). How to heal a wound fast. *Dermatologic clinics* **16**(2): 365-375.

Delafuente JC, Prendergast JM, Modigh A (1986). Immunologic modulation by vitamin C in the elderly. *International journal of immunopharmacology* **8**(2): 205-211.

Denyer S (1995). Mechanisms of action of antibacterial biocides. *Int Biodeterior Biodegrad* **36**(3-4): 227-245.

Diegelmann RF, Cohen IK, Kaplan AM (1981). The role of macrophages in wound repair: a review. *Plastic and reconstructive surgery* **68**(1): 107-113.

Diegelmann RF, Evans MC (2004). Wound healing: an overview of acute, fibrotic and delayed healing. *Frontiers in bioscience : a journal and virtual library* **9**: 283-289.

DiPietro LA, Polverini PJ (1993). Role of the macrophage in the positive and negative regulation of wound neovascularization. *Behring Institute Mitteilungen* (92): 238-247.

Dissemond J, Goos M (2004). [Options for debridement in the therapy of chronic wounds]. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* **2**(9): 743-751.

Doughty D (1994). A rational approach to the use of topical antiseptics. *Journal of wound, ostomy, and continence nursing : official publication of The Wound, Ostomy and Continence Nurses Society / WOCN* **21**(6): 224-231.

Dow G, Browne A, Sibbald RG (1999). Infection in chronic wounds: controversies in diagnosis and treatment. *Ostomy/wound management* **45**(8): 23-27, 29-40; quiz 41-22.

Drury RAB, Wallington EA (1967). *CARLETON'S HISTOLOGICAL TECHNIQUE*. 4th edn. Oxford University Press: Great Britain.

E D, S M, U T, J. W (2007). Chemical and Physical Methods for Characterisation of Biofilms. *Microchimica Acta* **158**(1-2): 1-27.

E HK, E DC, G NR, al e (2003). A prospective characterisation of microbiology of chronic venous leg ulcers: the clinical predictive value of tissue biopsies and swabs re-evaluated [abstract]. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **11**.

Eaglstain WH, Davis SC, Mehle AL, Mertz PM (1988). Optimal use of an occlusive dressing to enhance healing. Effect of delayed application and early removal on wound healing. *Archives of dermatology* **124**(3): 392-395.

Eccleston GM (2007). *Pharmaceutics: The science of dosage form design. 3rd edition*. 3rd edn. Churchill Livingstone: UK.

Eckes B, Nischt R, Krieg T (2010). Cell-matrix interactions in dermal repair and scarring. *Fibrogenesis & tissue repair* **3**: 4.

Edwards R, Harding KG (2004). Bacteria and wound healing. *Current opinion in infectious diseases* **17**(2): 91-96.

Endrich B, Menger MD (2000). [Regeneration of the microcirculation during wound healing?]. *Der Unfallchirurg* **103**(11): 1006-1008.

Engeland CG, Sabzehei B, Marucha PT (2009). Sex hormones and mucosal wound healing. *Brain, behavior, and immunity* **23**(5): 629-635.

Enoch S, Grey J, Harding K (2006). Non-surgical drug treatments. *British medical journal* **332**: 900-903.

Enoch S, Leaper DJ (2007). Basic science of wound healing. *Surgery* **26**(2): 31-37.

F CK, G HK (1994). Criteria for identifying wound infection. *Wound Care* **3**: 198-201.

Falabella AF (2006). Debridement and wound bed preparation. *Dermatologic therapy* **19**(6): 317-325.

Falanga V (2005). Wound healing and its impairment in the diabetic foot. *Lancet* **366**(9498): 1736-1743.

Fauci AS (1996). Resistance to HIV-1 infection: its in the genes. *Nature medicine* **2**: 996-997.

Fernandez MC, Walters J, Marucha P (1996). Transcriptional and post-transcriptional regulation of GM-CSF-induced IL-1 beta gene expression in PMN. *Journal of leukocyte biology* **59**(4): 598-603.

Flanagan M (2000). The physiology of wound healing. *Journal of wound care* **9**(6): 299-300.

Fluhr JW, Darlenski R, Surber C (2008). Glycerol and the skin: holistic approach to its origin and functions. *The British journal of dermatology* **159**(1): 23-34.

Folkman J, Klagsbrun M (1987). Angiogenic factors. *Science* **235**(4787): 442-447.

Fowler E, Vesely N, Pelfrey M, Jordan S, Amberry T (1999). Wound care for persons with diabetes. *Home Health Nurse* **17**: 437-444.

Franck-Lissbrant I, Haggstrom S, Damber JE, Bergh A (1998). Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. *Endocrinology* **139**(2): 451-456.

Froebe CL (1990). Prevention of stratum corneum lipid phase transition in vitro by glycerol – an alternative mechanism for skin moisturization. *Soc Cosmet Chem* **41**: 51-65.

Fuqua WC, Winans SC, Greenberg EP (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of bacteriology* **176**(2): 269-275.

G K, J K (1996). *Nutrition almanac*. edn. McGraw Hill.

Gabbiani G, Ryan GB, Majne G (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* **27**(5): 549-550.

Gardner SE, Frantz RA, Doebbeling BN (2001). The validity of the clinical signs and symptoms used to identify localized chronic wound infection. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **9**(3): 178-186.

Garrett Q, Simmons PA, Xu S, Vehige J, Zhao Z, Ehrmann K, *et al.* (2007). Carboxymethylcellulose binds to human corneal epithelial cells and is a modulator of corneal epithelial wound healing. *Investigative ophthalmology & visual science* **48**(4): 1559-1567.

Garrett Q, Xu S, Simmons PA, Vehige J, Xie RZ, Kumar A, *et al.* (2008). Carboxymethyl cellulose stimulates rabbit corneal epithelial wound healing. *Current eye research* **33**(7): 567-573.

Ge L, Zhang X, Cao C, Gu Z, Liu Z, Liu L, *et al.* (2012). Feasibility study of the sterilization of pigskin used as wound dressings by neutral electrolyzed water. *The journal of trauma and acute care surgery* **72**(6): 1584-1587.

Gilliver SC, Ashworth JJ, Ashcroft GS (2007). The hormonal regulation of cutaneous wound healing. *Clinics in dermatology* **25**(1): 56-62.

Gilliver SC, Ashworth JJ, Mills SJ, Hardman MJ, Ashcroft GS (2006). Androgens modulate the inflammatory response during acute wound healing. *Journal of cell science* **119**(Pt 4): 722-732.

Gilliver SC, Wu F, Ashcroft GS (2003). Regulatory roles of androgens in cutaneous wound healing. *Thrombosis and haemostasis* **90**(6): 978-985.

Glat PM, Longaker MT (1997). Wound healing. In: Aston SJ, Besley RW, Thorn CH (eds). *Grabb and Smith's plastic surgery*, Fifth edn. Philadelphia: Lippincott-Raven. p[^]pp 3.

Gloor M, Gehring W (2001). Increase in hydration and protective function of horny layer by glycerol and a W/O emulsion: are these effects maintained during long-term use? *Contact dermatitis* **44**(2): 123-125.

Goldman R (2004). Growth factors and chronic wound healing: past, present, and future. *Advances in skin & wound care* **17**(1): 24-35.

Gonzalez-Espinosa D, Perez-Romano L, Guzman-Soriano B, Arias E, Bongiovanni CM, Gutierrez AA (2007). Effects of pH-neutral, super-oxidised solution on human dermal fibroblasts in vitro. *International wound journal* **4**(3): 241-250.

Goodman N, Scales JT, Towers AG (1956). Development and evaluation of a porous surgical dressing. *British medical journal* **2**(4999): 962-968.

Gouwy M, Struyf S, Proost P, Van Damme J (2005). Synergy in cytokine and chemokine networks amplifies the inflammatory response. *Cytokine & growth factor reviews* **16**(6): 561-580.

Greenhalgh DG (1998). The role of apoptosis in wound healing. *The international journal of biochemistry & cell biology* **30**(9): 1019-1030.

Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, *et al.* (2009). Topographical and temporal diversity of the human skin microbiome. *Science* **324**(5931): 1190-1192.

Grice EA, Segre JA (2011). The skin microbiome. *Nature reviews. Microbiology* **9**(4): 244-253.

Grinnell F (1984). Fibronectin and wound healing. *Journal of cellular biochemistry* **26**(2): 107-116.

Gurtner GC, Evans GR (2000). Advances in head and neck reconstruction. *Plastic and reconstructive surgery* **106**(3): 672-682; quiz 683.

Gutierrez AA (2006). The Science Behind Stable, Super-Oxidized Water. Exploring the various applications of super-oxidized solutions. *WOUNDS* **18**: 7-10.

Hara-Chikuma M, Verkman AS (2008a). Aquaporin-3 facilitates epidermal cell migration and proliferation during wound healing. *Journal of molecular medicine* **86**(2): 221-231.

Hara-Chikuma M, Verkman AS (2008b). Prevention of skin tumorigenesis and impairment of epidermal cell proliferation by targeted aquaporin-3 gene disruption. *Molecular and cellular biology* **28**(1): 326-332.

Hara M, Ma T, Verkman AS (2002). Selectively reduced glycerol in skin of aquaporin-3-deficient mice may account for impaired skin hydration, elasticity, and barrier recovery. *The Journal of biological chemistry* **277**(48): 46616-46621.

Hardman MJ, Waite A, Zeef L, Burow M, Nakayama T, Ashcroft GS (2005). Macrophage migration inhibitory factor: a central regulator of wound healing. *The American journal of pathology* **167**(6): 1561-1574.

Hart J (2002). Inflammation. 1: Its role in the healing of acute wounds. *Journal of wound care* **11**(6): 205-209.

Hemila H, Douglas RM (1999). Vitamin C and acute respiratory infections. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* **3**(9): 756-761.

Hemmati H, Sedaghat K (2011). In vivo evaluation of the wound healing properties of ethanolic extract from *Lavandula angustifolia*

BIHE.

Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Mille SI (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**: 1171-1175.

Holtmann MH, Neurath MF (2004). Differential TNF-signaling in chronic inflammatory disorders. *Current molecular medicine* **4**(4): 439-444.

Holton J, Shetty N (1997). In-use stability of Nu-Cidex. *J Hosp Infect* **35**(3): 245-248.

Howell-Jones RS, Wilson MJ, Hill KE, Howard AJ, Price PE, Thomas DW (2005). A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. *The Journal of antimicrobial chemotherapy* **55**(2): 143-149.

Huang YR, CHung Y, Hsu SY, Huang YW, Hwang DF (2008). Application of electrolyzed water in the food industry. *Food Control* **19**(4): 329-345.

Hunt TK (1988). The physiology of wound healing. *Annals of emergency medicine* **17**(12): 1265-1273.

Hunt TK, Hopf H, Hussain Z (2000). Physiology of wound healing. *Advances in skin & wound care* **13**(2 Suppl): 6-11.

Hynes RO (1986). Fibronectins. *Scientific American* **254**(6): 42-51.

Hynes RO (1987). Integrins: a family of cell surface receptors. *Cell* **48**(4): 549-554.

Ito I, Hayashi T, Yamada K, Kuzuya M, Naito M, Iguchi A (1995). Physiological concentration of estradiol inhibits polymorphonuclear leukocyte chemotaxis via a receptor mediated system. *Life sciences* **56**(25): 2247-2253.

JAM R, JA W, V G (1995). Collagen based biomaterials. *Biotechnol Rev* **13**: 336-382.

James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, *et al.* (2008). Biofilms in chronic wounds. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **16**(1): 37-44.

James WD, Berger TG, Elston. DM (2011). *Andrews' Diseases of the skin : clinical dermatology*. 11 edn. Elsevier: US.

Jespersen J (1988). Pathophysiology and clinical aspects of fibrinolysis and inhibition of coagulation. Experimental and clinical studies with special reference to women on oral contraceptives and selected groups of thrombosis prone patients. *Danish medical bulletin* **35**(1): 1-33.

Jnanadev KR, Satish Babu CL, Shilpa Shetty S, Surendra Kumar GP, Sheetal HS (2011). Disinfecting the acrylic resin plate using electrolyzed acid water and 2% glutaraldehyde: a comparative microbiological study. *Journal of Indian Prosthodontic Society* **11**(1): 36-44.

Josefsson E, Tarkowski A, Carlsten H (1992). Anti-inflammatory properties of estrogen. I. In vivo suppression of leukocyte production in bone marrow and redistribution of peripheral blood neutrophils. *Cellular immunology* **142**(1): 67-78.

Joseph-Silverstein J, Rifkin DB (1987). Endothelial cell growth factors and the vessel wall. *Seminars in thrombosis and hemostasis* **13**(4): 504-513.

Kafienah W, Buttle DJ, Burnett D, Hollander AP (1998). Cleavage of native type 1 collagen by human neutrophil elastase. *The Biochemical journal* **330**: 897-902.

Kanda N, Watanabe S (2003). 17beta-estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bcl-2 expression. *The Journal of investigative dermatology* **121**(6): 1500-1509.

Kanda N, Watanabe S (2004). 17beta-estradiol stimulates the growth of human keratinocytes by inducing cyclin D2 expression. *The Journal of investigative dermatology* **123**(2): 319-328.

Kapur V, Marwaha AK (2011). Evaluation of effect and comparison of superoxidised solution (oxum) v/s povidone iodine (betadine). *The Indian journal of surgery* **73**(1): 48-53.

Karami MY, Zekavat OR, Amanat A (2012). Excisional wound healing activity of Carboxymethyle cellulose in diabetic rat. *Journal of Jahrom University of Medical Sciences* **9**: 48-57.

Katz MH, Alvarez AF, Kirsner RS, Eaglstein WH, Falanga V (1991). Human wound fluid from acute wounds stimulates fibroblast and endothelial cell growth. *Journal of the American Academy of Dermatology* **25**(6 Pt 1): 1054-1058.

Khalil EA, Afifi FU, Al-Hussaini M (2007). Evaluation of the wound healing effect of some Jordanian traditional medicinal plants formulated in Pluronic F127 using mice (*Mus musculus*). *Journal of ethnopharmacology* **109**(1): 104-112.

Kim C, Hung YC, Brackett RE (2000). Roles of oxidation-reduction potential in electrolyzed oxidizing and chemically modified water for the inactivation of food-related pathogens. *Journal of food protection* **63**(1): 19-24.

Kim WJ, Gittes GK, Longaker MT (1998). Signal transduction in wound pharmacology. *Archives of pharmacal research* **21**(5): 487-495.

Kingsley A (2001). A proactive approach to wound infection. *Nursing standard* **15**(30): 50-54, 56, 58.

Kirwin JCJ, Sandmeyer EE (1981). Ether. In. *Patty's Industrial Hygiene and Toxicology*, edn, Vol. 2A. New York: John Wiley & Sons. p[^]pp 2491–2511.

Knighton DR, Hunt TK, Scheuenstuhl H, Halliday BJ, Werb Z, Banda MJ (1983). Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science* **221**(4617): 1283-1285.

Komarcevic A (2000). [The modern approach to wound treatment]. *Medicinski pregled* **53**(7-8): 363-368.

Konturek PC, Brzozowski T, Konturek SJ, Kwiecien S, Dembinski A, Hahn EG (2001). Influence of bacterial lipopolysaccharide on healing of chronic experimental ulcer in rat. *Scandinavian journal of gastroenterology* **36**(12): 1239-1247.

Koseki S, Yoshida K, Isobe S, Itoh K (2001). Decontamination of lettuce using acidic electrolyzed water. *Journal of food protection* **64**(5): 652-658.

Kraft A (2008). Electrochemical water disinfection: a short review. *Platinum Metals Rev* **52**(3): 177-185.

Krasner D, Kennedy KL, Rolstad BS, Roma AW (1993). The ABCs of wound care dressings. *Ostomy/wound management* **39**(8): 66, 68-69, 72 passim.

Krasner D, Rodeheaver GT, Sibbald RG (2001). *Chronic Wound Care: A Clinical Source Book for Healthcare Professionals* edn. HMP Communications.

Kujath P, Michelsen A (2008). Wounds - from physiology to wound dressing. *Deutsches Arzteblatt international* **105**(13): 239-248.

Kumar MS, Kirubanandan S, Sripriya R, Sehgal PK (2008). Triphala promotes healing of infected full-thickness dermal wound. *The Journal of surgical research* **144**(1): 94-101.

Kurkinen M, Vaheri A, Roberts PJ, Stenman S (1980). Sequential appearance of fibronectin and collagen in experimental granulation tissue. *Laboratory investigation; a journal of technical methods and pathology* **43**(1): 47-51.

Labrie F, Luu-The V, Labrie C, Simard J (2001). DHEA and its transformation into androgens and estrogens in peripheral target tissues: intracrinology. *Frontiers in neuroendocrinology* **22**(3): 185-212.

Landa-Solis C, Gonzalez-Espinosa D, Guzman-Soriano B, Snyder M, Reyes-Teran G, Torres K, *et al.* (2005). Microcyn: a novel super-oxidized water with neutral pH and disinfectant activity. *J Hosp Infect* **61**(4): 291-299.

Lau K, Paus R, Tiede S, Day P, Bayat A (2009). Exploring the role of stem cells in cutaneous wound healing. *Experimental dermatology* **18**(11): 921-933.

LaVan FB, Hunt TK (1990). Oxygen and wound healing. *Clinics in plastic surgery* **17**(3): 463-472.

Lawrence JC (1998a). The use of iodine as an antiseptic agent. *Journal of wound care* **7**(8): 421-425.

Lawrence WT (1998b). Physiology of the acute wound. *Clinics in plastic surgery* **25**(3): 321-340.

Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecoraro RE, Rodeheaver G, *et al.* (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. *Archives of dermatology* **130**(4): 489-493.

Lee J, Singletary R, Schmader K, Anderson DJ, Bolognesi M, Kaye KS (2006). Surgical site infection in the elderly following orthopaedic surgery. Risk factors and outcomes. *The Journal of bone and joint surgery. American volume* **88**(8): 1705-1712.

Len SV, Hung YC, Erickson M, Kim C (2000). Ultraviolet spectrophotometric characterization and bactericidal properties of electrolyzed oxidizing water as influenced by amperage and pH. *Journal of food protection* **63**(11): 1534-1537.

Levonen AL, Patel RP, Brookes P, Go YM, Jo H, Parthasarathy S, *et al.* (2001). Mechanisms of cell signaling by nitric oxide and peroxynitrite: from mitochondria to MAP kinases. *Antioxidants & redox signaling* **3**(2): 215-229.

Liao LB, Chen WM, Xiao XM (2007). The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. *Journal of Food Engineering* **78**: 1326–1332.

Linda M (1991). *Nutritional biochemistry and metabolism with clinical applications*. 2nd edn. Appleton & Lange.

Lineaweaver W, McMorris S, Soucy D, Howard R (1985). Cellular and bacterial toxicities of topical antimicrobials. *Plastic and reconstructive surgery* **75**(3): 394-396.

Loden M, Andersson AC, Anderson C, Bergbrant IM, Frodin T, Ohman H, *et al.* (2002). A double-blind study comparing the effect of glycerin and urea on dry, eczematous skin in atopic patients. *Acta dermato-venereologica* **82**(1): 45-47.

Madden JW, Peacock EE, Jr. (1968). Studies on the biology of collagen during wound healing. I. Rate of collagen synthesis and deposition in cutaneous wounds of the rat. *Surgery* **64**(1): 288-294.

Maklebust J (1996). Using wound care products to promote a healing environment. *Critical care nursing clinics of North America* **8**(2): 141-158.

Margolis DJ, Knauss J, Bilker W (2002). Hormone replacement therapy and prevention of pressure ulcers and venous leg ulcers. *Lancet* **359**(9307): 675-677.

Marklund L, Behnam-Motlagh P, Henriksson R, Grankvist K (2001). Bumetanide annihilation of amphotericin B-induced apoptosis and cytotoxicity is due to its effect on cellular K⁺ flux. *The Journal of antimicrobial chemotherapy* **48**(6): 781-786.

Martínez F, s DJ (2007). Efficacy and safety of neutral pH superoxidised solution in severe diabetic foot infections. *International wound journal* **4**.

Martínez MA, Mene'ndez SA, Toiber M, Herna'ndez HnB, Padilla LR, Quijano OF (2005). Super-oxidized water (Microcyn 60) formesh hernioplasty in grossly contaminated fields: an experimental study. 91st Annual Clinical Congress: American College of Surgeons.

Martin P (1997). Wound healing--aiming for perfect skin regeneration. *Science* **276**(5309): 75-81.

McDonald JA, Kelley DG (1980). Degradation of fibronectin by human leukocyte elastase. Release of biologically active fragments. *The Journal of biological chemistry* **255**(18): 8848-8858.

McDonnell G, Russell A (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* **12**(1): 147-179.

McKenna SM, Davies KJ (1988). The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. *The Biochemical journal* **254**(3): 685-692.

McPherson L (1993). Understanding ORP's role in the disinfection process. *Water Eng Manag* **140**: 29-31.

Mian M, Beghe F, Mian E (1992). Collagen as a pharmacological approach in wound healing. *International journal of tissue reactions* **14 Suppl**: 1-9.

Miller SJ, Burke EM, Rader MD, Coulombe PA, Lavker RM (1998). Re-epithelialization of porcine skin by the sweat apparatus. *The Journal of investigative dermatology* **110**(1): 13-19.

Miyagi M, Aoyama H, Morishita M, Iwamoto Y (1992). Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. *Journal of periodontology* **63**(1): 28-32.

Monaco JL, Lawrence WT (2003). Acute wound healing an overview. *Clinics in plastic surgery* **30**(1): 1-12.

Mori Y, Komatsu S, Hata Y (1997). Toxicity of electrolyzed strong acid aqueous solution-subacute toxicity test and effect on oral tissue in rats. *Odontology* **84**: 619-626.

Naderi J, Hung M, Pandey S (2003). Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: resistance of quiescent cells to oxidative stress. *Apoptosis : an international journal on programmed cell death* **8**(1): 91-100.

Nagamatsu Y, Chen KK, Tajima K, Kakigawa H, Kozono Y (2002). Durability of bactericidal activity in electrolyzed neutral water by storage. *Dental materials journal* **21**(2): 93-104.

Nagamatsu Y, Tajima K, Kakigawa H, Kozono Y (2001). Application of electrolyzed acid water to sterilization of denture base part 1. Examination of sterilization effects on resin plate. *Dental materials journal* **20**(2): 148-155.

Najafi R, Bernard SM (2002). Use of physiologically balanced, ionized, acidic solution in wound healing Vol. US 6,426,066 B1, A61K 31/74; A61K 33/40; A61K 33/14 edn. US: California Pacific Labs, Inc. Novato, CA (US).

Najafi R, Wang L, Bassiri M, Yang J (2008). physiologically balanced, ionized, acidic solution and methodology for use in wound healing, A61K 33/14

A61P 17/02 edn. US: NovaBay Pharmaceuticals, Inc.

Nakae H, Inaba H (2000a). Effectiveness of electrolyzed oxidized water irrigation in a burn-wound infection model. *J Trauma* **49**(3): 511-514.

Nakae H, Inaba H (2000b). Electrolyzed strong acid aqueous solution irrigation promotes wound healing in a burn wound model. *Artif Organs* **24**(7): 544-546.

Nakagawara S, Goto T, Nara M, Ozawa Y, Hotta K, Arata Y (1998). Spectroscopic characterization and the pH dependence of bactericidal activity of the aqueous chlorine solution. *Anal Sci* **14**: 691-698.

Nanney LB, Magid M, Stoscheck CM, King LE, Jr. (1984). Comparison of epidermal growth factor binding and receptor distribution in normal human epidermis and epidermal appendages. *The Journal of investigative dermatology* **83**(5): 385-393.

Nayak BS, Kanhai J, Milne DM, Swanston WH, Mayers S, Eversley M, *et al.* (2010). Investigation of the wound healing activity of *Carapa guianensis* L. (Meliaceae) bark extract in rats using excision, incision, and dead space wound models. *Journal of medicinal food* **13**(5): 1141-1146.

Newman SL, Henson JE, Henson PM (1982). Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages. *The Journal of experimental medicine* **156**(2): 430-442.

Niall M, Ryan GB, O'Brien BM (1982). The effect of epidermal growth factor on wound healing in mice. *The Journal of surgical research* **33**(2): 164-169.

Nikolaev YA, Plakunov VK (2007). City of Microbes” or an Analogue of Multicellular Organisms? *Springer* **76**: 2125-138.

Nishihira J, Koyama Y, Mizue Y (1998). Identification of macrophage migration inhibitory factor (MIF) in human vascular endothelial cells and its induction by lipopolysaccharide. *Cytokine* **10**(3): 199-205.

Oike Y, Ito Y, Maekawa H, Morisada T, Kubota Y, Akao M, *et al.* (2004). Angiopoietin-related growth factor (AGF) promotes angiogenesis. *Blood* **103**(10): 3760-3765.

Osieki H (1995). *Nutrients in profile*. 2nd edn. Bioconcepts.

Pandey PK, Koushariya M, Shukla S, Das S (2011). Outcomes of superoxide solution dressings in surgical wounds: a randomized case control trial. *Int J Biol Med Res* **2**(4): 965-968.

Park H, Hung Y, Chung D (2004a). Pathogen reduction and quality of lettuce treated with electrolyzed oxidizing and acidified chlorinated water. *International journal of food microbiology* **91**: 13-18.

Park H, Hung YC, Chung D (2004b). Effects of chlorine and pH on efficacy of electrolyzed water for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *International journal of food microbiology* **91**(1): 13-18.

Parks WC (1999). Matrix metalloproteinases in repair. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **7**(6): 423-432.

Patel GK (2005). The role of nutrition in the management of lower extremity wounds. *The international journal of lower extremity wounds* **4**(1): 12-22.

Perry HM (1999). *The endocrinology of aging*. edn, vol. 45.

Pierce GF, Mustoe TA, Altmann BW, Deuel TF, Thomason A (1991). Role of platelet-derived growth factor in wound healing. *Journal of cellular biochemistry* **45**(4): 319-326.

Planz B, Wang Q, Kirley SD, Lin CW, McDougal WS (1998). Androgen responsiveness of stromal cells of the human prostate: regulation of cell proliferation and keratinocyte growth factor by androgen. *The Journal of urology* **160**(5): 1850-1855.

Pomposiello PJ, Demple B (2001). Redox-operated genetic switches: the SoxR and OxyR transcription factors. *Trends in biotechnology* **19**(3): 109-114.

Pool JG (1977). Normal hemostatic mechanisms: a review. *The American journal of medical technology* **43**(8): 776-780.

Porto da Rocha R, Lucio DP, Souza Tde L, Pereira ST, Fernandes GJ (2002). Effects of a vitamin pool (vitamins A, E, and C) on the tissue necrosis process: experimental study on rats. *Aesthetic plastic surgery* **26**(3): 197-202.

Prilutsky V, Bakhir V (1997). Electrochemically actuating water: anomalous characteristics, mechanism of biological action. *VNIIMT*.

Profyris C, Tziotzios C, Do Vale I (2012). Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics Part I. The molecular basis of scar formation. *Journal of the American Academy of Dermatology* **66**(1): 1-10; quiz 11-12.

Purna SK, Babu M (2000). Collagen based dressings--a review. *Burns : journal of the International Society for Burn Injuries* **26**(1): 54-62.

Qu X (2005). Behavior of antiseptic containing chlorine and its applications. *China Chlor-Alkal* **25**: 19-23.

Queen D, Orsted H, Sanada H, Sussman G (2004). A dressing history. *International wound journal* **1**(1): 59-77.

Ramasastry SS (2005). Acute wounds. *Clinics in plastic surgery* **32**(2): 195-208.

Rappolee DA, Mark D, Banda MJ, Werb Z (1988). Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. *Science* **241**(4866): 708-712.

Rawlings A, Harding C, Watkinson A, Banks J, Ackerman C, Sabin R (1995). The effect of glycerol and humidity on desmosome degradation in stratum corneum. *Archives of dermatological research* **287**(5): 457-464.

Ribatti D, Vacca A, Roncali L, Dammacco F (1991). Angiogenesis under normal and pathological conditions. *Haematologica* **76**(4): 311-320.

Richardson M (2004). Acute wounds: an overview of the physiological healing process. *Nursing times* **100**(4): 50-53.

Risau W (1990). Angiogenic growth factors. *Progress in growth factor research* **2**(1): 71-79.

Robson MC, Steed DL, Franz MG (2001). Wound healing: biologic features and approaches to maximize healing trajectories. *Current problems in surgery* **38**(2): 72-140.

Roh C, Lyle S (2006). Cutaneous stem cells and wound healing. *Pediatric research* **59**(4 Pt 2): 100R-103R.

Rojas AI, Phillips TJ (1999). Patients with chronic leg ulcers show diminished levels of vitamins A and E, carotenes, and zinc. *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al.]* **25**(8): 601-604.

Routley CE, Ashcroft GS (2009). Effect of estrogen and progesterone on macrophage activation during wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **17**(1): 42-50.

S E, K H (2003). Wound bed preparation: the science behind the removal of barriers to healing (part 2). *Wounds* **15**: 213-229.

Saegeman VS, De Vos R, Tebaldi ND, van der Wolf JM, Bergervoet JH, Verhaegen J, *et al.* (2007). Flow cytometric viability assessment and transmission electron microscopic

morphological study of bacteria in glycerol. *Microscopy and microanalysis : the official journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada* **13**(1): 18-29.

Sampson MN, Muir AV (2002). Not all super-oxidized waters are the same. *J Hosp Infect* **52**(3): 228-229.

Samuels P, Tan AK (1999). Fetal scarless wound healing. *The Journal of otolaryngology* **28**(5): 296-302.

Sarabahi S (2012). Recent advances in topical wound care. *Indian journal of plastic surgery : official publication of the Association of Plastic Surgeons of India* **45**(2): 379-387.

Schneikert J, Peterziel H, Defossez PA, Klocker H, de Launoit Y, Cato AC (1996). Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-modulation of matrix metalloproteinase expression. *The Journal of biological chemistry* **271**(39): 23907-23913.

Schultz GS, Sibbald RG, Falanga V, Ayello EA, Dowsett C, Harding K, *et al.* (2003). Wound bed preparation: a systematic approach to wound management. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **11 Suppl 1**: S1-28.

Schwartz, I S (1999). Wound care and wound healing. In. *Principle of surgery* 7edn. New York: McGraw Hill. pp 263-295.

Sekiya S, Ohmori K, Harii K (1997). Treatment of infectious skin defects or ulcers with electrolyzed strong acid aqueous solution. *Artif Organs* **21**(1): 31-38.

Selkon JB (2007). Wound and Ulcer Treatment with Suepr-Oxidized Water Vol. US 7,276,255 B2, A61K 33/20

A61P 17/02 edn. US: Sterilox Medical (Europe) Limited.

Sen CK, Khanna S, Babior BM, Hunt TK, Ellison EC, Roy S (2002). Oxidant-induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing. *The Journal of biological chemistry* **277**(36): 33284-33290.

Seppa H, Grotendorst G, Seppa S, Schiffmann E, Martin GR (1982). Platelet-derived growth factor is chemotactic for fibroblasts. *The Journal of cell biology* **92**(2): 584-588.

Severino J, Allen RG, Balin S, Balin A, Cristofalo VJ (2000). Is beta-galactosidase staining a marker of senescence in vitro and in vivo? *Experimental cell research* **257**(1): 162-171.

Shai A, Maibach HI (2005). *Wound Healing and Ulcers of the Skin*. edn. Springer: Germany.

Shanker G, Sorci-Thomas M, Adams MR (1995). Estrogen modulates the inducible expression of platelet-derived growth factor mRNA by monocyte/macrophages. *Life sciences* **56**(7): 499-507.

Shimizu Y, Sugawara H (1996). Virucidal and bactericidal effects of electrolyzed oxidizing water: comparison of disinfectant effect with electrolyzed oxidizing water and hypochlorous acid. *Jpn J Oral Biol* **38**: 464-571.

Sibbald RG, Woo K, Ayello EA (2006). Increased bacterial burden and infection: the story of NERDS and STONES. *Advances in skin & wound care* **19**(8): 447-461; quiz 461-443.

Skover GR (1991). Cellular and biochemical dynamics of wound repair. Wound environment in collagen regeneration. *Clinics in podiatric medicine and surgery* **8**(4): 723-756.

Son ED, Lee JY, Lee S, Kim MS, Lee BG, Chang IS, *et al.* (2005). Topical application of 17beta-estradiol increases extracellular matrix protein synthesis by stimulating tgfbeta signaling in aged human skin in vivo. *The Journal of investigative dermatology* **124**(6): 1149-1161.

Sorg H, Krueger C, Vollmar B (2007). Intravital insights in skin wound healing using the mouse dorsal skin fold chamber. *Journal of anatomy* **211**(6): 810-818.

Stashak TS (2008). Management practices that influence wound infection and healing.

Stiernberg J, Redin WR, Warner WS, Carney DH (1993). The role of thrombin and thrombin receptor activating peptide (TRAP-508) in initiation of tissue repair. *Thrombosis and haemostasis* **70**(1): 158-162.

Strodtbeck F (2001). Physiology of wound healing. *Newborn Infant Nurs Rev* **1**: 43-52.

Suslow TV oxidation reduction potential (ORP) for water disinfection. monitoring, control and documentation, University of Calofornia, 2004.

Sylvia CJ (2003). The role of neutrophil apoptosis in influencing tissue repair. *Journal of wound care* **12**(1): 13-16.

Tanaka H, Hirakata Y, Kaku M, Yashida R, Takemura H (1996). Antimicrobial activity of duper oxidized water. *J Hosp Infect* **34**: 43-49.

Tanaka K, Asanuma M, Ogawa N (2004). Molecular basis of anti-apoptotic effect of immunophilin ligands on hydrogen peroxide-induced apoptosis in human glioma cells. *Neurochemical research* **29**(8): 1529-1536.

Tesch GH (2007). Role of macrophages in complications of type 2 diabetes. *Clinical and experimental pharmacology & physiology* **34**(10): 1016-1019.

Thomas GW, Rael LT, Bar-Or R, Shimonkevitz R, Mains CW, Slone DS, *et al.* (2009). Mechanisms of delayed wound healing by commonly used antiseptics. *J Trauma* **66**(1): 82-90; discussion 90-81.

Thomson PD (2000). Immunology, microbiology, and the recalcitrant wound. *Ostomy/wound management* **46**(1A Suppl): 77S-82S; quiz 83S-84S.

Thorn RM, Lee SW, Robinson GM, Greenman J, Reynolds DM (2012). Electrochemically activated solutions: evidence for antimicrobial efficacy and applications in healthcare environments. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **31**(5): 641-653.

Tonnesen MG, Feng X, Clark RA (2000). Angiogenesis in wound healing. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **5**(1): 40-46.

Trengove NJ, Stacey MC, McGeachie DF, Mata S (1996). Qualitative bacteriology and leg ulcer healing. *Journal of wound care* **5**(6): 277-280.

Tziotzios C, Profyris C, Sterling J (2012). Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics Part II. Strategies to reduce scar formation after dermatologic procedures. *Journal of the American Academy of Dermatology* **66**(1): 13-24; quiz 25-16.

van Baare J, Buitenwerf J, Hoekstra MJ, du Pont JS (1994). Virucidal effect of glycerol as used in donor skin preservation. *Burns : journal of the International Society for Burn Injuries* **20 Suppl 1**: S77-80.

van den Beld AW, de Jong FH, Grobbee DE, Pols HA, Lamberts SW (2000). Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. *The Journal of clinical endocrinology and metabolism* **85**(9): 3276-3282.

Vanwijck R (2001). [Surgical biology of wound healing]. *Bulletin et memoires de l'Academie royale de medecine de Belgique* **156**(3-4): 175-184; discussion 185.

Venkitanarayanan KS, Ezeike GO, Hung YC, Doyle MP (1999). Efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes*. *Applied and environmental microbiology* **65**(9): 4276-4279.

Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts AB, *et al.* (1987). Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. *Proceedings of the National Academy of Sciences of the United States of America* **84**(16): 5788-5792.

Wallace E (1994). Feeding the wound: nutrition and wound care. *British journal of nursing* **3**(13): 662-667.

Wang J, Zhu S, Xu C (2002). *Biochemistry. 3rd ed. Beijing: Higher Education Press.* 3rd edn. Beijing: Higher Education Press.

Warriner R, Burrell R (2005). Infection and the chronic wound: a focus on silver. *Advances in skin & wound care* **18 Suppl 1**: 2-12.

Werner S, Grose R (2003). Regulation of wound healing by growth factors and cytokines. *Physiological reviews* **83**(3): 835-870.

Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT (1992). Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proceedings of the National Academy of Sciences of the United States of America* **89**(15): 6896-6900.

White RJ, Cutting K, Kingsley A (2006). Topical antimicrobials in the control of wound bioburden. *Ostomy/wound management* **52**(8): 26-58.

Williams CL, Stancel GM, Hardman JG, Limbird LE, Molinoff PB, Rudden RW (1996). *Goodman and Gilman's the Pharmacological Basis for Therapeutics*. edn. NY: McGraw-Hill: New York.

Wilson JR, Mills JG, Prather ID, Dimitrijevič SD (2005). A toxicity index of skin and wound cleansers used on in vitro fibroblasts and keratinocytes. *Advances in skin & wound care* **18**(7): 373-378.

Witte MB, Barbul A (1997). General principles of wound healing. *The Surgical clinics of North America* **77**(3): 509-528.

Wolf FI, Torsello A, Covacci V, Fasanella S, Montanari M, Boninsegna A, *et al.* (2002). Oxidative DNA damage as a marker of aging in WI-38 human fibroblasts. *Experimental gerontology* **37**(5): 647-656.

Wolff K, Goldsmith LA, Katz SI, Gilchrest BA, Paller AS, Leffell DJ (eds) (2008). *Fitzpatrick's Dermatology in General Medicine*. Mc Graw Hill: United State.

Xavier JB, Foster KR (2007). Cooperation and conflict in microbial biofilms. *Proceedings of the National Academy of Sciences of the United States of America* **104**(3): 876-881.

Xiong K, Liu HJ, Liu R, Li LT (2010). Differences in fungicidal efficiency against *Aspergillus flavus* for neutralized and acidic electrolyzed oxidizing waters. *International journal of food microbiology* **137**(1): 67-75.

Xue G (1985). *Practical disinfection. 1st ed. Beijing: People's Military Medical Press.* edn.

Yahagi N, Kono M, Kitahara M, Ohmura A, Sumita O, Hashimoto T, *et al.* (2000). Effect of electrolyzed water on wound healing. *Artif Organs* **24**(12): 984-987.

Yahagi N, Sumita O (2002). Solution for promoting growth of tissue cells at wound sites and production process therefor, A61K 33/00; A61K 33/14; C25C 1/02 edn. US.

Yonemori S, Takimoto Y, Min K, Jitsugiri Y, Simohira T, Miyake H (1997). Analysis of hydroxyl radical generated in electrolyzed strong acid aqueous solution by electron spin resonance spectroscopy. *Nippon Kagaku Kaishi* **7**: 497-501.

Yosipovitch G, Xiong GL, Haus E, Sackett-Lundeen L, Ashkenazi I, Maibach HI (1998). Time-dependent variations of the skin barrier function in humans: transepidermal water loss, stratum corneum hydration, skin surface pH, and skin temperature. *The Journal of investigative dermatology* **110**(1): 20-23.

Zeng X, Tang W, Ye G, Ouyang T, Tian L, Ni Y, *et al.* (2010). Studies on disinfection mechanism of electrolyzed oxidizing water on *E. coli* and *Staphylococcus aureus*. *Journal of food science* **75**(5): M253-260.

Zhang QX, Magovern CJ, Mack CA, Budenbender KT, Ko W, Rosengart TK (1997). Vascular endothelial growth factor is the major angiogenic factor in omentum: mechanism of the omentum-mediated angiogenesis. *The Journal of surgical research* **67**(2): 147-154.

Zhao H, Zhang X (2004). The applications of electrolyzed oxidizing water. *J Chinese Pharm* **39**: 632-634.

Zheng M, Aslund F, Storz G (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**(5357): 1718-1721.

Zinkevich V, Beech IB, Tapper R, Bogdarina I (2000). The effect of super-oxidized water on *Escherichia coli*. *J Hosp Infect* **46**(2): 153-156.

Zulkowski K (2013). Skin bacteria: implications for wound care. *Advances in skin & wound care* **26**(5): 231-236; quiz 237-238.

چکیده

محلول سوپر اکسیداز که از الکترولیز کردن نمک سدیم کلرید تشکیل شده، محلولی است غیر سمی که اثر ضد عفونی کننده دارد و برای از بین بردن طیف گسترده ای از میکروارگانیسم ها بکار می رود. در ضمن اینکه این محلول به بافت میزبان آسیب نمی رساند و مقاومت باکتریایی را تسهیل نمی کند. همچنین تشکیل بافت گرانولاسیون را بهبود می بخشد. این محلول میزان نیاز به دبریدمان را کاهش می دهد و درد کمتری در طول دوران درمان گزارش شده است. هدف از این مطالعه بررسی تاثیر محلول سوپر اکسیداز و ژل آن بر روی ترمیم زخم است. در این مطالعه از زخم باز پوستی در موش با نژاد Swiss Webster استفاده شده است. موش ها به پنج گروه به شرح زیر تقسیم شده اند: گروه کنترل منفی که هیچ درمانی دریافت نمی کند، گروه کنترل مثبت که از پمادی موجود در بازار برای بهبود زخم استفاده می شود (کرم سبامد)، گروه محلول سوپر اکسیداز، گروه ژل سوپر اکسیداز و گروه پایه ژل. مشاهده زخم ها تا تکثیر کامل اپیتلیوم با چشم غیر مسلح ادامه داشته است و هر سه روز از زخم ها برای بررسی در زیر میکروسکوپ نمونه گیری می شده است. راندمان درمان برای هر حیوان در مقایسه با سه روز قبل آن نشان می دهد که ترمیم در گروه سوپر اکسیداز و گروه کنترل مثبت به صورت معناداری در فاصله زمانی روز شش تا نه تسریع یافته است. البته این موضوع در گروه ژل سوپر اکسیداز سه روز دیر تر اتفاق می افتد. براساس مشاهدات میکروسکوپی، تشکیل کامل اپیتلیوم در گروه محلول سوپر اکسیداز و گروه ژل سوپر اکسیداز سه روز زودتر از سایر گروه ها اتفاق افتاد. می توان اینطور نتیجه گرفت که محلول سوپر اکسیداز و ژل آن زخم را از طریق تسریع تشکیل اپیتلیوم بهبود می بخشد. با وجود اینکه نتایج بررسی اثر جنسیت بر ترمیم زخم هیچ تفاوت معناداری را بین موش ماده و نر نشان نمی دهد، اما میزان تاثیر درمان های مختلف بر روی ماده و نر متفاوت است.

لغات کلیدی: محلول سوپر اکسیداز، زخم باز پوستی، ترمیم زخم، پتانسیل اکسیداسیون و احیا، تشکیل اپیتلیوم