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Measurements in Wound Healing – Effects of Topical Agents on full thickness Dermal Incised Wounds in an Animal Model

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A. Background

Inert topical agents in the form of creams, lotions and ointments are often applied to wounds to create an environment conducive to wound healing. These agents, although not pharmacologically active on the intact skin, have activity when applied to wounds where the stratum corneum barrier to penetration is absent. From measurements of simple parameters like the rate of re-epitheliasation, it is known that some topical agents enhance wound healing while others retard the healing process. There is data available on the antiseptic and anti-microbial properties of most of the topical agents in use today, but all the effects of these products on the micro-environment of wounds and their influence on the process of healing is not known.

Central to the study of wounds and wound healing is the need for accurate methods to evaluate wounds. Parameters used for measuring outcome should be unambiguous and measurements should be accurate and reproducible. At present, no universally accepted methods of assessing wounds exist. Clinical evaluation is usually subjective and not quantitative, resulting in unacceptable levels of inter- and intra-observer variation. Similarly lacking are clear histological correlates of what we consider good healing characteristics of a wound.

As our knowledge increase about the complex process of wound healing, in particular the hormones and peptide growth factors that regulate the process, possibilities arise for therapeutic intervention to enhance or improve clinical outcome[1]. At the same time, the need for objective measurements becomes more urgent, as we need to evaluate and compare treatment options.

Previous measurement systems were developed, ranging from visual scoring systems to measurement of biological and chemical wound constituents. Physical characteristics of healing wounds can also be measured by properties such as the tensile strength. The structural and ultra-structural elements of healing wounds remain difficult to measure, although immuno-histochemistry and scanning electron microscopy allows some quantification and simple morphometric measurements. With current advances in computer technology however, rapid, automatic measurements can be made from tissue sections for a variety of practical applications in the

pathology laboratory. Image analysis offers dynamic functional imaging, linking multiple data sources to provide composite quantitative systems.

Further correlation of detailed histologic examination of wounds to a detailed clinical assessment of the same wounds is important, not only to add credibility to clinical scoring schemes, but also to understand which structural features of the dermis are important for the severity of scarring (and therefore which should be the object of future therapeutic or preventative strategies)

B. Literature review

The process by which wounds heal is fundamental to all surgery. The process starts with injury to healthy tissue and ultimately results in a non-specific connective tissue scar. The adverse properties of scar tissue result both from the fact that scar tissue is weaker and more brittle than normal healthy tissue, and the fact that scar tissue tends to contract with time. This has clinical relevance in such diverse complications as incisional hernias, oesophageal strictures, peritoneal adhesions and scar contractures. Where scarring occur in areas where wounds are visible, the non-aesthetic properties of scar tissue can also be considered detrimental. As our knowledge increase about the chain of events that constitute the healing process, the opportunity arise for intervention in the early phases of the process to control the undesirable features or influence the outcome.

1. Phases of healing

1.1 Substrate phase

The first 3 days of wound healing is a period of intense biologic and chemical activity. There is early inflammation, hyperaemia of wound margins and leucocytic infiltration. Enzymes are released and there is lysis and removal of devitalised tissue. This process does not result in any recordable tensile strength or contraction, but it is essential for preparing the wound for healing.

1.2 Phase of proliferation

The proliferation phase is dominated by the growth and activity of the fibroblast-capillary system. It lasts about 3 weeks, during which collagen and ground substance are deposited in increasing amounts. Fibroblast activity increases with accelerated synthesis of collagen fibers. Collagenolysis is also increased and as a result collagen is in a state of dynamic equilibrium in the wound. During this phase tensile strength increases rapidly and contraction also occur.

1.3 Phase of maturation

Fibroblasts and macrophages disappear and the vascularity of the wound decreases. The continued synthetic and lytic activities suggest collagen remodelling, but collagen architecture is abnormal and total collagen in the wound is decreased. Maturation results in a more normal appearance, but the wound remains a somewhat weakened tissue indefinitely.

2. Elements of healing

2.1 Epithelialization

Epithelialization is a process by which surface covering of the wound is restored by a combination of cell migration and multiplication. As with all the elements that constitute healing, this process is controlled by various factors influencing the micro-environment of the wound milieu. These include tissue factors, hormones and growth factors as well as microbiological, pharmacological and biochemical variables that play a role.

2.2 Contraction

This is the physiological process by which structural elements are pulled closer towards the center of the wound, and is the result of fibroblast action on the wound edges as well as changes in the spatial organization of collagen fibers.

2.3 Connective tissue formation

Granulation tissue is the product of the fibroblast-capillary system, with the fibroblast being the key cell synthesizing both collagen and intercellular ground substance. Neovascularization, stimulated by various peptides and growth stimulating factors, is responsible for ingrowth of capillaries supplying the increased demand for oxygenation. As the mature fibrous scar develops, the fibroblasts and capillaries disappear.

2.4 Adaptation

This is the process of reformation of tissue, and generally constitutes compensatory hypertrophy or regeneration following the repair process. An example is the regeneration of liver tissue after surgical resection.

3. Factors that influence healing

3.1 Ground substance

All connective tissues contain fibres and cells embedded in a variable quantity of ground substance (mucopolysaccharides). This material is made up of large protein-polysaccharide complexes called glycos-aminoglycans. Two of these complexes are important in wound healing: chondroitin-4-sulphate and dermatan sulphate. The fibroblasts responsible for collagen formation also synthesize

the glycos-aminoglycans. The role of the ground substance in wound healing is poorly understood. Experimental studies suggest it may play a role both in the orientation and precipitation of collagen fibres, and in the strength of repair.

3.2 Oxygen

Oxygen is required for normal epithelialisation and granulation tissue formation. Fresh wounds are anoxic for the first few days (PO_2 5-10 mmHg). Actively dividing fibroblasts are confined to regions where the oxygen tension is more than 15 mmHg and this is only found within 50-70 microns of the nearest functioning capillary[2,3,4]. The delivery of oxygen to the tissues depends on factors such as blood volume and viscosity, blood pressure, temperature, vasomotor activity, and pulmonary and cardiac function. Anaemia per se seems to have little effect provided that blood volume is maintained. Wounds in highly vascular tissues heal more rapidly than those in ischaemic areas and healing is enhanced experimentally when the oxygen supply in the inspired air is increased between 35 and 70 percent. However, oxygen in excessive amount has an adverse effect. Intermittent hyperbaric oxygenation has proved beneficial where ischaemia is present but had no effect on the healing of open wounds with normal blood supply.

3.3 Collagen

Collagen is responsible for most of the strength of the wound. Unfortunately, wound collagen demonstrates physical abnormalities at several levels of examination. It's lack of birefringence (as viewed in polarized light), indicates a failure of organization at the molecular or small fibril level[5]. Fibril aggregation is defective and the fibre diameters remain less than normal[6]. The large fibre inter-relationships are also abnormal and remodeling is incomplete.

Fibroblasts can be recognized in the wound after 24 hours and collagen synthesis can be detected by the third day. At the end of a week fibroblasts dominate the cellular pattern. They are actively synthesizing collagen and mucopolisaccharides. Their highly developed endoplasmic reticulum is characteristic and indicates their protein synthesizing ability. Radioisotope studies have confirmed the intracellular synthesis of collagen. The collagen is not stored in the fibroblast but is excreted to the exterior through small channels or vesicles derived from the endoplasmic reticulum. The other proteins synthesized in the endoplasmic reticulum are transferred to the exterior via the smooth-chambered Golgi apparatus. The transmission electron microscope has proved invaluable in elucidating the fine structure of the collagen fibrils suggesting a one-quarter stagger of the adjacent

molecules on each other to give the characteristic 64nm cross-banding that is so typical. More recently it has helped to provide evidence that a three-dimentional arrangement of five molecule collagen groups is probably a more accurate concept of collagen molecule aggregation.

Physical factors, such as fibre shape and weave, play a significant role in determining the mechanical properties of skin and wound. Scanning electron micrographs of normal skin collagen reveal a well-organized network of large collagen fibres. At high magnification each large fibre can be seen to be made up of a bundle of fine cross-banded fibrils.

The fibre patterns in healing wounds, however, are quite different. In the wound, collagen fibres lie in a relatively haphazard arrangement. They are not orientated and tend not to aggregate together in small bundles as in normal skin. A time passes the collagen fibres in the wound come together to form large irregular masses without evidence of fibril substructure. Although the collagen fibre patterns clearly change as time passes, remodeling is minimal and there is little to suggest that the normal network architecture is ever restored. It is well known that foetal skin wounds heal rapidly without the scarring and inflammation that accompany adult skin wounds. In all species examined (mice, rats, rabbits, pigs, sheep, and monkeys), the prenatal wound healing process is faster and more efficient than adult repair and produces new tissue rather than scar[7]. The pattern of collagen deposition in adult and foetal wounds is of particular interest. The orderly deposition of collagen in fetal animal models has been demonstrated using histologic, immunohistochemical, biochemical, and biophysical techniques[8]. The collagen pattern in foetal wounds is reticular and indistinguishable from adjacent normal tissue, whereas the adult wound contains large, parallel collagen bundles that are orientated perpendicular to the wound surface[9].

3.3.1 Collagen content

Although fibroblast proliferation in the wound is a dominant feature of the early healing process, it is the collagen content that gives the best correlation with the development of tensile strength. However, tensile strength continues to increase long after the collagen content has retumed to normal[6]. This indicates that the quality of the collagen plays as an important role as the quantity or total content of collagen.

3.3.2 Collagen chemistry

Collagen forms 30 percent of the total protein content of most animals. Tropocollagen, the collagen molecule, is a rigid rod 300nm long and 1.5nm wide. Each molecule is composed of three

polypeptide chains wound in a left-hand helix. The molecule itself is twisted the opposite way into a right-handed superhelix. Over half of the molecule is made up of three amino acids: glycine, proline, and hydroxyproline. The bulk of the strength of mature collagen relates to the development of strong intermolecular and intramolecular covalent bonds. Aldehyde reactions form strong covalent collagen cross-links when an aldehyde group undergo an aldol condensation with an adjacent aldehyde.

3.3.3 Collagen metabolism

The individual amino acids are assembled in the endoplasmic reticulum of the fibroblast beginning at the amino-terminal end and proceeding towards the carboxy-terminal end. Hydroxyproline and hydroxylysine is not directly incorporated into the collagen molecule. Instead, a proline rich collagen precursor, protocollagen, is formed. Hydroxylation then proceeds under the influence of protocollagen hydroxylase. Requirements for this enzyme are molecular oxygen, alpha-ketoglutarate, ferrous iron and ascorbic acid. Oxidative deamination of the amino groups of lysine is controlled by the enzyme lysyl amine oxydase. Copper is required for its action and if absent or removed, aldehyde formation is blocked and collagen cross-linking cannot proceed.

3.3.4 Collagen measurement

Collagen cross-linking progresses automatically with time and the degree of bonding can be estimated by the ease with which collagen is dissolved. Newly deposited collagen is readily dissolved in neutral salt solutions and older collagen is insoluble. Saline solubility was therefore used to estimate collagen synthesis and high rates were observed for at least 120 days[5]. Solubility studies do not differentiate between the soluble new collagen and the soluble collagen produced in the final stages of collagenolysis. A more accurate measurement can be made using tritiated proline[10]. Since total collagen is stable at 60-80 days, it is apparent that collagen lysis must be occurring as well. The dynamic equilibrium may continue indefinately, for active collagenase has been isolated form human wounds as late as 30 years[11].

A method for accurately measuring collagen content was described by Stegeman and Stadler[12]. Individual wounds can be excised, rapidly frozen, and lyophilized. The dried samples can then be weighed and the hydroxyproline content measured. The amount of collagen is calculated by assuming that it contains 13.6% hydroxyproline.

Immunohistochemical staining of the different types of collagen is possible with commercially available rabbit antibody or murine monoclonal antibody products. With the growing interest in computerized quantification techniques, new ways of measuring collagen are possible. Architecture in terms of density of the fibrillar network is assumed to be reflected by the intensity of immunohistochemical staining of collagen and linearity between collagen concentration and the absorbance of the immunohistochemical staining product could be demonstrated by video-based microdensitrometry[13].

A quantitative method based on the selective capacity of two dyes, Sirius Red F3BA and Fast green, to bind to collagen and non-collagenous proteins respectively, was first implemented by Lopez-de-Leon and Rojkind[14]. They found a good relationship between the amount of collagen estimated by the dye-binding method, and the determination of hydroxyproline, suggesting that the new colorimetric method may be useful in measuring collagen in tissue. It has since been verified in studies comparing this method with morphometric techniques[15],[16].

A simpler method is described in this text, using Sirius Red F3BA staining, and quantifying percentage area by computerized area-morphometry.

4. Polypeptide growth factors

For the last two decades, studies of carcinogenesis have looked at biochemical mechanisms for the control of proliferation of malignant cells. These studies have implicated oncogenes and peptide growth factors in the transformation and expression of the cancer cell and in the induction of changes in the tumor environment, for example, neovascularization, essential for continued tumor growth.

Unrelated lines of research with growth hormone characterized somatomedins, which are polypeptide mediators of the growth-promoting effects of growth hormone. Important in postnatal growth, there is evidence that somatomedins play a significant role in fetal growth[17].

While growth factors were being sought in the context of cancer and fetal growth, wound healing studies were focused on the local wound hormones and inflammatory cells of the early response to acute injury. A temporal progression of events was documented clearly, as was the orderly sequence in which cells appeared in the wound: platelets first, followed sequentially by neutrophils, macrophages, fibroblasts, and endothelial cells. The production of cell-specific chemoattractants was postulated, and multiple cell-stimulating factors were isolated from the inflammatory cells.

However, these factors remained a hodgepodge of ill-defined activities and fractions in the absence of the technical ability to characterize them. For example, evidence for the secretion of fibroblast growth-stimulating activities by macrophages existed for many years, but their purification and biochemical characterization could not be accomplished.

Two major advances have been instrumental in propelling wound healing into its current pre-eminent position in growth factor research: first, the biochemical and analytical technology to cope with the complexities in purification introduced by the heterogeneity of the growth factors; second, recombinant DNA techniques to produce growth factors in quantities large enough for experimentation. Where previously there was only an exceedingly small amount of activity obtainable from cultured cells or from large volumes of constituent fluids, now polypeptides can be synthesized for the conduct of in vivo research. A polypeptide growth factor, as defined originally, is an agent promoting cell proliferation: its interaction with an external receptor is thought to lead to intracellular changes preparing the cell for DNA synthesis and division. It is clear that these proteins also induce the migration of cells, and thus are not only mitogens but are also chemoattractants that recruit leukocytes and fibroblasts to the injured area.

4.1 Synthesis

With a more complex description of the various growth factors and much new research under way, it is increasingly apparent that the same mediators of cell growth and stromal synthesis are involved in malignancy, fetal growth, and wound healing. The same growth factors involved in the malignant process in cancer cells are produced by cells that mediate wound inflammation and repair: the platelets, macrophages, and lymphocytes. This being the case, the wound environment offers several attractive features for the study of growth factors. It can be defined, the process is regulated, it is limited temporally, and models are plentiful.

For the wound healing researcher, the potency of growth factors is important because they appear to be fundamental to the reparative process[18]. One must recognize, however, that a narrow focus on one aspect of the inflammatory response necessarily overlooks other acute events. Concentrating on growth control leaves aside the role of vasodilatation, neutrophils, bacteria, and other synchronous factors.

4.2 Nomenclature

Growth factors have been named either for their tissue of origin (macrophage-derived growth factor, MDGF), their target cell specificity (epidermal growth factor, EGF), or their activity in tumor cell culture (transforming growth factor-β, TGF-β). This has resulted in a large number of names and confusion when factors with common names such as TGF-β have no sequence homology and are chemically distinct from transforming growth factor-α (TGF-α), which is an analog of EGF. Fortunately, however, with the ability to purify and biochemically characterize the factors more fully, there has come the realization that many of the factors are closely related and in some cases identical. Particularly with the fibroblast growth factors (FGF), previously unidentified biologic activities may be related, if not identical, for macrophage derived growth factor (MDGF), cartilage-derived growth factor (CDGF), tumor angiogenic factor (TAF), endothelial cell growth factors (ECGF), and others[19]. As this work continues, a restructuring of the nomenclature of growth factors probably will evolve.

4.3 Growth factor delivery

Growth factors can get to the wound in more than one way. Some growth factors, such as insulin-like growth factor-I (IGF-I), are transported in plasma bound to a large-molecular-weight carrier protein and thus are delivered in an endocrine fashion. Other growth factors, platelet-derived growth factor (PDGF) and TGF-β, are synthesized by one cell and used by another local cell, termed paracrine delivery. Other growth factors are produced and used by the same cell in an autocrine fashion.

Epidermal cell-derived factor (EDF) and fibroblast somatomedin (Sm-C) are examples of autocrine regulators and appear to play roles as locally produced factors for both positive and negative feedback mechanisms in the maintenance of homeostasis[20]. This role would be similar to that of a chalone, an autocrine regulator previously identified in epidermal cells.

4.4 Regulatory function in the wound

Cell migration and proliferation herald the inflammatory response and the reparative processes of wound healing. These cells are stimulated and regulated by specific peptide factors. These cells also synthesize and secrete peptide growth factors. Thus, repair of injury appears to involve a

highly orchestrated release of paracrine growth factors controlling the recruitment of new cells and the formation of new matrix. Regulation is provided by synergism and antagonism of the factors' expression and by autocrine behavior. In this scheme, the platelets are the first cellular elements in the repair of a wound. Recent studies show that in addition to being released by platelets, PDGF also is produced by macrophages, vascular endothelial cells, and certain fibroblasts. This means that PDGF so produced may act back on the cell that produced it, or upon adjacent cells as a way of amplifying or prolonging the response to factors initially released at the site of tissue injury[21]. Further increasing the complexity of these interactions is the evidence that PDGF acts in concert with TGF-β and EGF to mediate cell transformation. Synchronously, all the other cells and growth factors are playing their roles in the repair process, and as new information accumulates, it appears that the peptide growth factors act in sets or combinations.

4.5.1 Transforming Growth Factor-β

TGF-β, originally identified in conditioned media from transformed cells, was subsequently found widely distributed in tissues and in very high concentrations in platelets. It was found to be important in wound healing by stimulating total protein, collagen, and DNA content. It also stimulates expression of fibronectin and collagen by fibroblast lines in tissue culture as well as rapid, reversible formation of granulation tissue[22]. It can accelerate wound healing by increased influx of mononuclear cells and fibroblasts and by marked increases in collagen deposition at the site of application[23]. TGF-β also manifests growth inhibitory properties for specific cell-types and thus may serve as a bifunctional regulator of cellular growth and differentiation. Subsequently, isoforms of the TGF-β superfamily have been described, namely TGF-β1, TGF-β2 and TGF-β3. They are similar in function both in vivo and in vitro, but different potencies and activities exist and is believed to be a result of target cell specificity, distinct promotor regions, and differential cell surface protein binding[24].

Type β transforming growth factor is a two-chain polypeptide of 25,000 daltons first isolated from the media of transformed neoplastic cells. TGFs are defined operationally as polypeptides that stimulate anchorage-dependent cells to lose contact inhibition and to undergo anchorage-independent growth in cell culture. Despite the common name, TGF-β is chemically distinct from TGF-α, a single-chain peptide of 5700 daltons with a different primary sequence that is

homologous to that of EGF. Type α transforming growth factor and EGF both bind to the EGF receptor and have indistinguishable biologic activities in vitro[25].

Transforming growth factor-β has numerous regulatory functions and is considered a general mediator of regulation in the cell. It is of special importance for negative control of cell growth, and a "panregulin" whose function is to control the activities of several other peptide growth factors[26]. Synthesized by platelets, macrophages, and lymphocytes, as well as bone, kidney, placental, and other cells, TGF-β is a multifunctional molecule able to either stimulate or inhibit proliferation, differentiation, and processes of many cell lines, both neoplastic and non-neoplastic.

Several other peptides have been shown to be very similar, if not identical, to TGF-β. Among these are a cartilage-inducing peptide (CIF-A) isolated from bone that appears to be involved in cell differentiation for cartilage formation as the first step in endochondral bone formation[27].

Transforming growth factor-β seems to have a particularly important role in the repair process. Released by the cells of the inflammatory process, it has been shown that it is strongly anabolic, leading to fibrosis and angiogenesis. Extrinsic TGF-β can stimulate increased levels of collagen, DNA, and protein in wire-mesh wound healing chambers implanted subcutaneously in the backs of rats[28]. Subcutaneous injection in newborn mice causes the formation of granulation tissue, including the induction of angiogenesis and activation of fibroblasts to make collagen at the site of injection[29]. It has been reported that the tensile strength of rat incisional wounds treated locally with TGF-β is increased or transiently increased in healthy animals and it is speculated that this is due to earlier delivery or increased levels of growth factors than occur naturally[30].

4.5.2 Platelet-Derived Growth Factor

Growth factors present in serum and platelet extracts are considered to play important roles in inflammation and wound healing[31,32]. The platelet-derived growth factor (PDGF) and platelet factor-4 at low concentrations were first shown to be chemotactically active for human monocytes, neutrophils, smooth muscle cells, and fibroblasts and to stimulate inflammatory cells and fibroblasts.

Platelet-derived growth factor is a basic 31,000 dalton polypeptide that is stored in the alpha granules of platelets. Although the bulk of it is synthesized by and released from activated platelets, there are other cell sources including activated macrophages, vascular endothelial cells,

fibroblasts, and certain smooth muscle cells[21]. Unlike some of the other growth factors, PDGF is a potent chemoattractant as well as a mitogen. Alone, it is chemotactic for smooth muscle cells, fibroblasts, and inflammatory cells, but its mitogenic activity is dependent on the presence of other growth factors. The concerted action of PDGF with TGF-β and EGF is needed to stimulate the proliferation of mesenchymal cells, or to mediate phenotypic transformation of fibroblasts[33]. PDGF also stimulates collagenase synthesis by fibroblasts, presumably by increasing the expression of its secretory gene in fibroblasts[34].

As a chemoattractant for fibroblasts and inflammatory cells, a mitogen for these cells in the presence of other factors, and an agent for collagenase production, PDGF is a candidate for an important role in wound healing and matrix remodeling. Extrinsic PDGF in subcutaneous wound chambers in rats enhances cellularity and collagen deposition. In diabetic animals with slow healing, PDGF restores this to a normal rate[35]. The direct application of purified and recombinant PDGF to incisional wounds in rats causes a striking cellular influx early post-wounding and an increase in breaking strength beginning 5 days post-wounding and lasting for 7 weeks[36]. It has been suggested that the relative longevity of this response may be due to an alteration in matrix remodeling associated with PDGF's influence on collagenase synthesis.

4.4.3 Fibroblast Growth Factor

Fibroblast growth factor is the conventional term used to describe a number of heparin-binding growth factors from a variety of tissue and cellular sources. Regardless of source, all factors of this class appear to be potent mitogens for endothelial cells and are characterized as angiogenic agents. Assessments of their unusual heparin-binding affinities and newer techniques for structural characterization have shown that there are only two general structures; these are referred to as the acidic (acidic FGF, aFGF) and basic (basic FGF, bFGF) fibroblast growth factors and they share about a 50 per cent sequence homology[37].

4.4.4 Epidermal Growth Factor

Epidermal growth factor is a small 6000-dalton molecular weight single-chain polypeptide of 53 amino acid residues. Its existence was detected in 1962 when extracts of the submaxillary gland of the mouse were injected into newborn animals and induced precocious eyelid opening and incisor

eruption due to direct stimulation of epidermal growth and keratinization. The responsible factor was characterized; and later a polypeptide similar but not identical to mouse EGF was isolated in human urine, plasma, milk, saliva, and amniotic fluid. Numerous studies of EGF in organ culture systems and in intact animals have identified nearly exclusively the epithelial cell as the target cell. Still, many other cells are responsive to EGF including keratinocytes, fibroblasts, glia, chondrocytes, smooth muscle cells, and others[38].

Epidermal growth factor stimulates a mitogenic response with its concomitant increases in DNA, RNA, protein, and hyaluronic acid synthesis. It also appears to promote the rate of cell migration[39], and to play a role as yet unknown in the structures of the skin that do not undergo rapid proliferation- the hair follicles, eccrine sweat glands, and errector pili muscles[40]. It is postulated that the EFG receptors in these adnexae are reflecting the complex regulatory role that EGF could play in situations such as male pattern baldness.

As one of the most extensively studied of the growth factors, EGF has been evaluated in a large number of in vitro studies. Its efficacy as a potent mitogen for epidermal cells in vitro was established years ago, but until recently, experimental attempts to show enhanced epidermal regeneration in an animal or clinical model were unsuccessful. This probably reflected conditions that did not provide sufficient continuous exposure of the cells to EGF before the availability of biosynthetic re-combinant EGF. Now, acceleration of the rate of epidermal repair has been shown in split-thickness (donor site) wounds treated with topical EGF on the dorsum of pigs[41]. Additional animal studies of partial-thickness burns treated with twice daily applications of biosynthetic human EGF mixed with a topical cream have yielded similar findings[42]. Epidermal growth factor has been studied also for its effect on mesenchymal cell healing. Many cell types, including fibroblasts, possess EGF surface receptors and will proliferate in response to EGF in cell culture. With EGF in sustained release pellets embedded in sponges implanted subcutaneously in rats, an increased amount of granulation tissue, doubled DNA content, and a 50 per cent increase in collagen content were seen[43]. A similar dose-dependent response occurred in experimental rat wounds in which sponge implants were injected daily with EGF[44]. When physiologic doses of the growth factor were used, increases in both hydroxyproline (collagen) and DNA content were still significant[45]. How EGF exerts its wound healing effect on granulation tissue is unknown. Although it is mitogenic for fibroblasts in culture, there is no direct evidence that the increased cellularity seen in the in vivo models is due purely to mitogenesis. It is possible that EGF mediates its effect by way of cell

recruitment. Although EGF is not itself a chemoattractant for fibroblasts or inflammatory cells, it could be the interaction with other growth factors such as TGF-β and PDGF that is the critical factor. A prospective, randomized, double-blind clinical trial has evaluated the effect of topical EGF on the healing rate of skin graft donor sites in humans. Using topical silver sulfadiazine cream containing EGF, the average length of time to heal these partial thickness wounds was shortened significantly[46].

It has also been shown that recombinant epidermal growth factor (EGF) can be used to enhance the rate of wound healing in corneal, epidermal and burn wounds as well as in the clinical setting of chronic stasis ulceration and radiogenic ulcers[47,48,49]. Topical treatment of these wounds with biosynthetic EGF showed accelerated wound healing, epithelialisation, and epidermal regeneration[42,45,46].

The Centre for Genetic Engineering and Biotechnology (CIGB) in Havana (Cuba), developed a hydrophylic topical cream with 0.1% human recombinant EGF and 1% silver-sulphadiazine. The product, HEBERMIN, was tested in a series of clinical trials in Cuba on superficial burn wounds in adults and children with good preliminary results, although Phase II randomised comparative studies are still under way. A limited study of this preparation was also performed in Moscow (Russia), showing antibiotic activity comparable to Flamazine (1% silver-sulphadiazine). There are also reports on its effectiveness in the treatment of post phlebitic leg ulceration and the chronic ulcers of radiotherapy and cytostatic extravasation in patients receiving chemotherapy.

4.4.5 Transforming Growth Factor-α

Transforming growth factor-α is a 5700-dalton single-chain peptide originally detected in the culture supernatants of transformed fibroblasts. It shares a 42 per cent homology with human EGF and a 30 per cent homology with vaccinia growth factor (VGF) and binds to the same cell-surface receptor as EGF and VGF[25]. Transforming growth factor-α stimulates the proliferation of cultured epithelial cells, fibroblasts, and endothelial cells. It is synthesized by a number of virally transformed cells, fetal tissue extracts, and human macrophages. The observation that TGF-α is one of the MGDFs, is taken as evidence for a role for the macrophage in epithelial regeneration[50]. Transforming growth factor-α also is synthesized by platelets and by human keratinocytes. The

latter is an autocrine event because TGF-α induces the proliferation of epidermal keratinocytes and thus induces its own expression. Perhaps this auto-induction is important in pathologic situations such as psoriasis in which abnormally high TGF-α expression could be contributing to an increased rate of proliferation of the keratinocytes. Similarly, squamous cell carcinomas consistently show relatively high levels of TGF-α expression[51].

Both TGF-α and its homologue, VGF, have been looked at for in vivo activity similar to that of EGF in promoting wound healing. Using a pig model with partial-thickness burns, the topical administration of TGF-α or VGF in silver sulfadiazine accelerated epidermal regeneration in comparison with control burns, and both appeared more effective than EGF[52]. Although the wound system did not allow for precise quantification, the information that TGF-α is significantly more active than EGF corroborates what has been found in cell culture.

4.4.6 Epidermal Cell Derived Factors (EDF)

A new growth factor has been found recently in the supernatant fluids of cultured epidermal cells. It is a 1 000-dalton peptide clearly distinguishable from EGF, TGF-α, or VGF, and has the singular ability to stimulate the proliferation of keratinocytes but to inhibit fibroblasts. This is a factor produced by the epidermal cells, stimulatory primarily to epidermal stem cells, and inhibitory to the proliferation of fibroblasts and the contraction of collagen matrices seeded with fibroblasts[53]. Application of EDF to partial-thickness experimental wounds in swine caused re-epithelialization twice as rapidly as in control wounds and hypocellularity of the dermis and granulation tissue. In several horses and dogs with clinical wounds and ulcers not the subject of a controlled trial, there was a similar enhancement of epithelialization and suppression of exuberant granulation tissue. This early work has led to the proposal that different growth factors may need to be selected on the basis of the type of wounds to be treated. For example, EDF would be useful in situations with proliferative granulation tissue, whereas a fibroblast-stimulating factor such as TGF-β or PDGF would be selected for an ulcer lacking connective tissue[54].

4.4.7 Others

Other polypeptides under investigation are Monocyte.Derived Growth Factor (MDGF), Insulin-like Growth Factor-I (Somatomedin-C), Interleukin-1, Interleukin-2, and Tumor Angiogenic Factor (TAF).

5. Control of wound healing

Attempts to modify the chronic fibrotic process associated with wound healing have been directed along three avenues; the reduction of collagen synthesis, the removal of collagen (collagenolysis), and modifying the intermediary metabolism of collagen[55].

Attempts to prevent synthesis using general protein inhibitors such as actinomycin or puromycin have been too non-specific to be useful. Attention has also been directed to interfering with assembly of the collagen molecule in the fibroblast. Attempts to stop hydroxylation of proline by interfering with the enzyme protocollagen hydroxylase have been unrewarding ($\alpha\alpha$-dipyridyl by chelating ferrous iron will remove an essential cofactor in this reaction but is too dangerous for human application). Some success has been obtained by presenting the fibroblast with proline analogues such as cisfluoroproline and azetidine. These are incorporated into the collagen molecule in place of proline. Since they cannot be hydroxilated, collagen formation is halted. Local X-irradiation will stop collagen synthesis by destroying fibroblasts and is used experimentally in controlling keloidal scars. The best results have been with cortisone, which inhibits fibroblast activity and collagen production as well as increasing collagen lysis. It was hoped that bacterial and tissue collagenases might be useful, but bacterial colagenases was found to be too destructive. Tissue collagenase however, splits the collagen into two fragments across all three chains approximately one-quarter of the way along from the carboxyl-terminal end. Unfortunately it cannot be produced in large enough quantities to be useful. At the moment the only practical way to increase collagen lysis is by local application of corticoids.

Since collagen turnover in scar is higher than that of normal tissue it can be selectively influenced by drugs. The main reason for scar tissue rigidity is the rapidity with which chemical cross-linking occurs in the newly deposited collagen in the wound. Treatment with β-aminopropionitrile or penicillamine could effectively block the aldehyde reactions in these linkages. The newly deposited collagen molecules are left free to orient and realign themselves in more physiological patterns.

When treatment is stopped, cross-linking proceeds rapidly. β-aminopropionitrile has proved useful both experimentally and clinically in reducing adhesions between healing tendons and their sheaths[56]. Penicillamine also seems to be useful in delaying collagen fibre cross-linking[57].

The healing process produce scar tissue that rapidly gains tensile strength and restore tissue defects by epithelial coverage and wound contraction. Unfortunately, the accelerated process produces the well-known undesirable effects of abnormal dermal architecture and excessive scar formation. This overdrive response of scarring may be beneficial in compromised situations (e.g. chronic wounds, dirt or foreign bodies), where speed of healing is advantageous and takes place at the expense of final quality in terms of scarring. It has been postulated that with contemporary hygiene and care the normal adult wound has excessive amounts of particular cytokines that drive the healing response beyond that which is required for tissue restoration[58]. An important new approach to the control of scarring in normal wound healing, complementing the practise of adding exogenous growth factors to chronic wounds in an attempt to stimulate wound healing, is to inhibit the autocatalytic and autoinducive cascades of cytokine amplification at the wound site, so reducing the eventual growth factor concentrations[59]. Neutralizing Antibody (NA) against TGF-β reduces the autoinductive effects of this factor and leads to lower numbers of blood vessels, macrophages, and monocytes at the wound site. NA treated wounds contain much less collagen and has greater fibrillar spacing, but there appears to be no difference in the tensile strength between them and control wounds. This could be the result of the orientation of the wound matrix, which is more normal in NA treated wounds. There is a more normal regenerative pattern of dermal architecture as shown by collagen fibre orientation[58].

6. Measurements in wound healing

Wound healing in human skin results in varying degrees of scar formation, ranging from fine asymptomatic scars to problematic hypertrophic and keloid scars, which may limit function and restrict further growth. Of particular interest is the measurement of scar formation in burn wounds, as the focus is changing from survival to cosmetic and functional outcome. At present, very few good objective methods of clinically assessing scars exist. Evaluation is usually subjective and not quantitative. Similarly lacking are histologic correlates of what we consider good and bad clinical scars.

Previous measurement systems were developed, ranging from visual scoring systems to dynamic functional imaging, linking multiple data sources to provide composite quantitative systems[60]. With current advances in computer technology, rapid, automatic measurements can be made from tissue sections for a variety of practical applications in the pathology laboratory[61]. Further correlation of detailed histologic examination of scars to a detailed clinical assessment of the same scars is important, not only to add credibility to clinical scoring schemes, but also to understand which structural features of the dermis are important for the severity of scarring (and therefore which should be the object of future therapeutic or preventative strategies)[62].

C. Aim

To investigate healing of dermal excised wounds in an animal model.

Specific Aims

To compare healing of wounds treated with four different topical agents.

To investigate the effect of locally applied epithelial growth factor on wound healing.

To investigate new modalities for objective measurements in wound healing.

To implement methods of computerised image analysis in the measurement of wound healing.

D. Materials and methods

The effect of four topical agents on full thickness dermal incised wounds was compared in a porcine model. The study was approved by the Animal Research Review Committee (Project No: 97/024).

Phase 1 – Feasibility study

The first phase was a feasibility study. Four test animals were used. Different wounding techniques were tested and different sampling and staining methods were evaluated. The optimal time for wound biopsy was also determined. Conclusions drawn from the feasibility study were:

The 50mm dermatome available was not able to effect a full-thickness dermal wound in the pig. It was decided to use surgically incised wounds. It was also determined that a maximum of thirty wounds of 2x2cm each could be made per animal.

For evaluating initial wound healing and re-epithelialisation, it was found that wounds should be excised on day seven, while wounds excised on day 28 would be suitable for investigating the characteristics of the scar.

Phase 2 – Data acquisition

Sixteen adult pigs of between 15 and 20 kilograms were used in the final study. The animals were stabled in individual pens, which were cleaned daily. They received regular feeds and had free access to clean water.

Each pig was subjected to two surgical procedures: initial wounding, and wound biopsy on either day 7 or day 28. All surgery was performed under general anaesthesia with adequate analgesia. Inhalation anaesthesia with Halothane was used during all procedures. Post-operative analgesia was provided on a scale that would be appropriate for humans subjected to the same procedure. Intravenous buprenorphene hydrochloride (Temgesic) was administered at a dose of 0.004mg/kg (12 hourly) for the first 48 hours post operatively, and by intramuscular injection thereafter. Animals were sacrificed immediately after the biopsy.

To eliminate factors that may contribute local tissue variables to the wound environment, we used a standardised model of surgical wounding. Other models have been used, for instance methods of thermal injury[63], but have shown to introduce variables such as infection and intense local inflammation that could compound results in a laboratory setting[64].

The four topical agents, 5% Povidone-iodine cream (Sacti-Med Biocream, Lever Industrial - Picture 1), 1% Silver-sulphadiazine ointment (Bactrazine, Smith & Nephew - Picture 2), 2% Mupirocin ointment (Bactroban, SmithKline Beecham - Picture 3), and 1% Silver-sulphadiazine plus recombinant-human epithelial growth factor (Hebermin, Heber Biotech - Picture 4) were randomly assigned to four test animals each.

1. **Povidone iodine** is a widely used external antiseptic with a broad microbicidal spectrum against bacteria, fungi, viruses, protozoa, and yeasts. In vivo studies have shown a beneficial role in wound-healing[65,66]. Hypersensitivity to iodine is a contraindication and adverse reactions of localised rash or pruritis are reported in 1-10% of patients. Contact with the eyes should be avoided and it is highly toxic if ingested. Sodium thiosulfate is the most effective chemical antidote. Systemic absorption in extensive burns causes iododerma, metabolic acidosis, and renal impairment. In normal individuals, topical application results in very little systemic absorption; with vaginal administration, however, absorption is rapid and serum concentrations of total iodine and inorganic iodide are increased significantly.

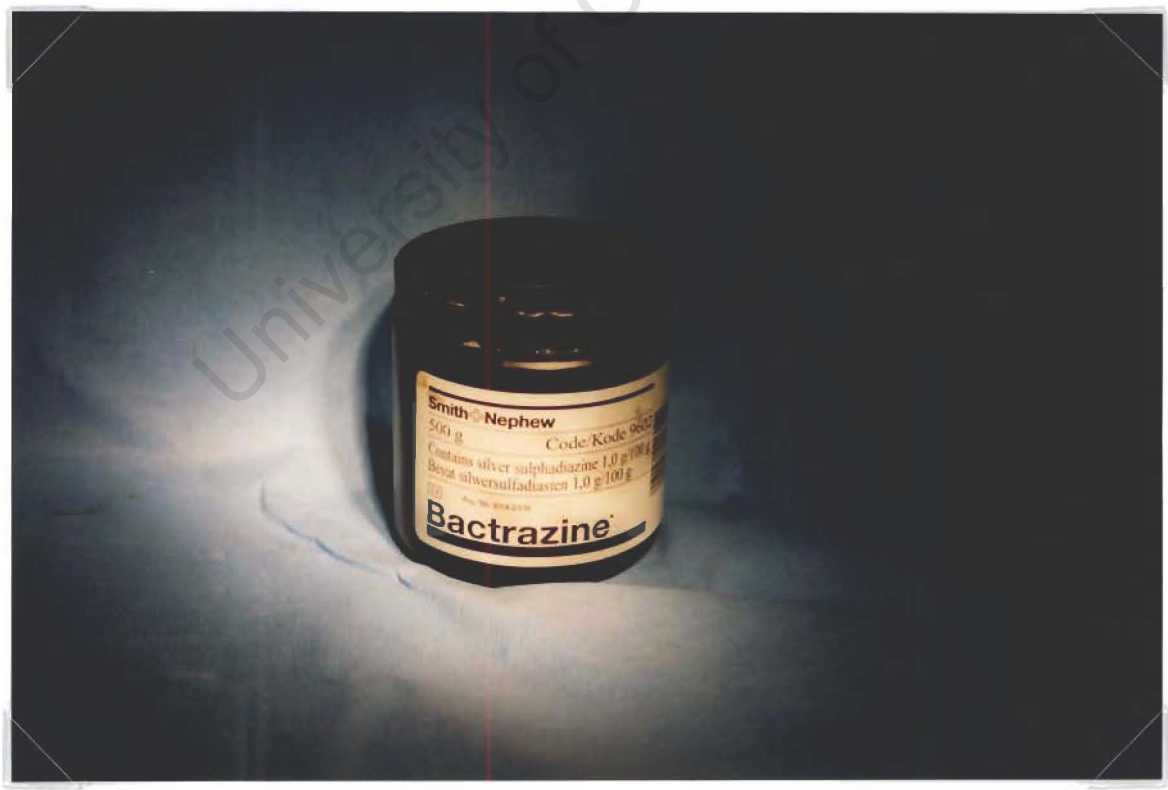
2. **Silver-sulphadiazine** reacts slowly to form silver chloride, silver protein complexes and sodium sulphadiazine. Free silver ions exert the bactericidal action, the silver ions are thought to be

reversibly adsorbed by bacterial cells by association with SH groups or histidine residues in the bacterial protein of the transport system across the cell wall. Silver sulphadiazine acts as a sustained release depot of silver and sulphadiazine at the wound surface. This slow liberation of silver does not cause the rapid and extensive depletion of chloride ion experienced when silver nitrate solutions are used and thus electrolyte disturbance is minimised. It is used as topical antibacterial for the treatment of infected leg ulcers, pressure sores and burns, caused by Gram-negative organisms such as Pseudomonas aeruginosa and Pyocyanea. It has also been effective against Candida albicans and to exert a therapeutic effect in burns which are already infected. Contra-Indications are hypersensitivity to sulphonamides, pregnancy, lactation, infants during the first months of life. It is also contra-indicated in patients with renal or hepatic dysfunction or porphyria. Appreciable amounts of sulphadiazine may be absorbed systemically to produce therapeutic blood levels, and particular attention must be paid to adequate fluid intake and acid-base balances.

3. **Mupirocin** is a topical ointment which is a preparation of 2g mupirocin in 100g of a water soluble base. It is an antibiotic that inhibits bacterial synthesis by binding to bacterial isoleucyl t-RNA synthetase. It has a low potential for sensitisation. Safety during pregnancy has not been established and adverse effects are localised to the area of application such as burning or itching. The topical ointment contains polyethylene glycol U.S.N.F. which when absorbed is excreted by the kidneys. It should therefore not be used if there is evidence of moderate or severe renal impairment.

4. **Hebermin** is a healing and antiseptic cream that contains Human Epidermal Growth Factor (EGF) and Silver Sulpphadiazine, as active products. EGF is a 53-aminoacid peptide which stimulates cellular proliferation. Human EGF is produced by recombinant DNA technology in a yeast strain genetically transformed. The strain contains the human protein gene under a high level expression system. Each 100g of Hebermin contain 1mg of human EGF and 1g Silver Sulphadiazine in a hydrophyllic base. It is contra-indicated in patients with hypersensitivity or allergy to sulphonamides or in patients with glucose 6 phosphate dehidrogenase deficiency. It should be used with caution in patients with liver or kidney disease. Adverse reactions have not been described.

Picture 1: 5% Povidone-Iodine Cream



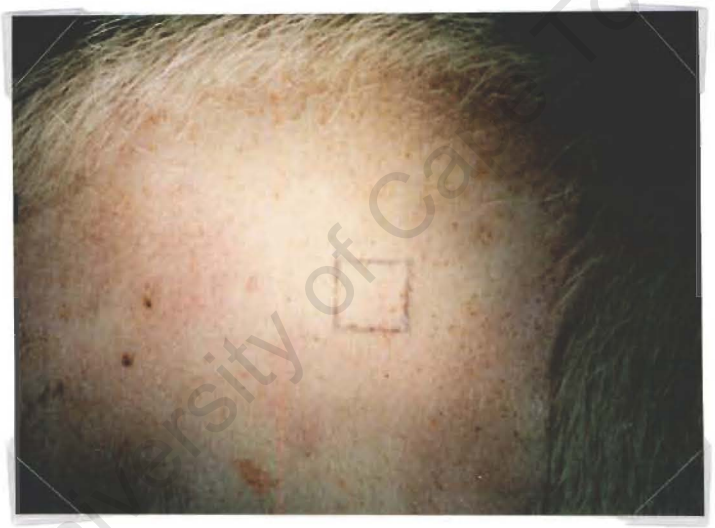
Picture 2: Silver Sulphadiazine 1g/100g

Picture 3: Mupirocin ointment

Picture 4: Hebermin (Silver-sulphadiazine plus recombinant-human EGF)

1. Wounding

Wounding was performed under general anaesthesia. After induction, the animals were intubated by endotracheal tube and connected to the ventilator. A mixture of oxygen, nitrous oxide and inhalation anaesthetic agent was administered. A random assignment was then made to one of the treatment agents by an independent assistant and a study number was marked on the ear with a marker pen. The skin was prepared by shaving off excess hair with standard animal clippers. No washing or prepping with antiseptic solution was done prior to surgery to prevent such agents affecting the outcome. A plastic template was then used to mark fifteen wounds of 2x2cm on each animal for treatment with the test agent (Picture 5).

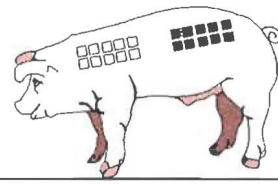
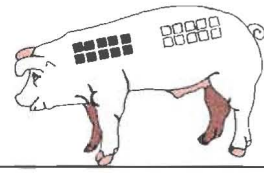
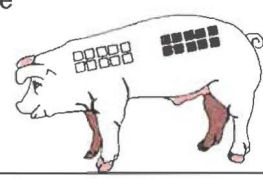
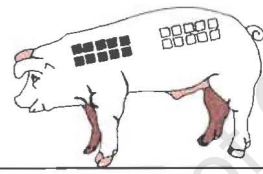
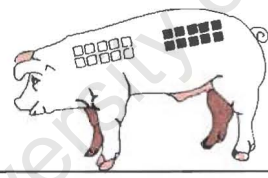
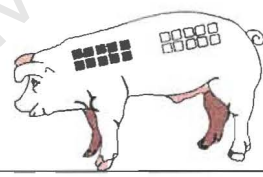
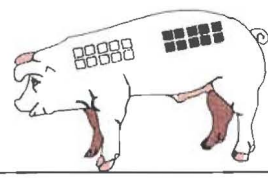
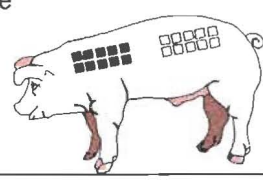


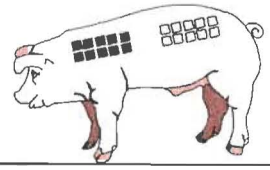
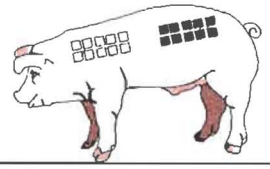
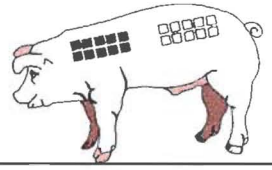
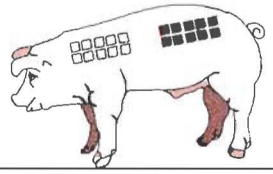
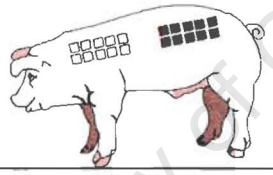
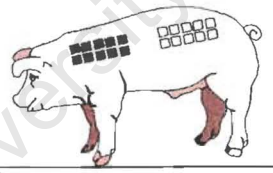
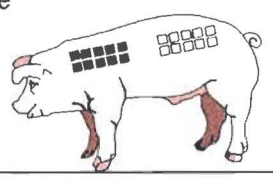
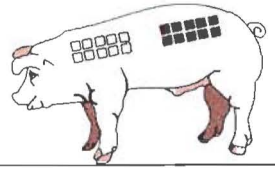
Picture 5: Wound marking

The fifteen wounds were marked at least 2cm apart to leave a bridge of normal skin. They were positioned either towards the head or the tail of the animal. Fifteen more wounds were then marked in a similar way towards the other end of the animal. These wounds were left untreated to serve as control wounds. To exclude the effect that regional placement of wounding might have on healing properties, two of the four animals assigned to a specific treatment had the test wounds towards the tail and the control wounds towards the head. The other two animals had the test wounds towards the head and the control wounds towards the tail (Table 1: Animal Allocation Table).

Table 1: Animal Allocation Table

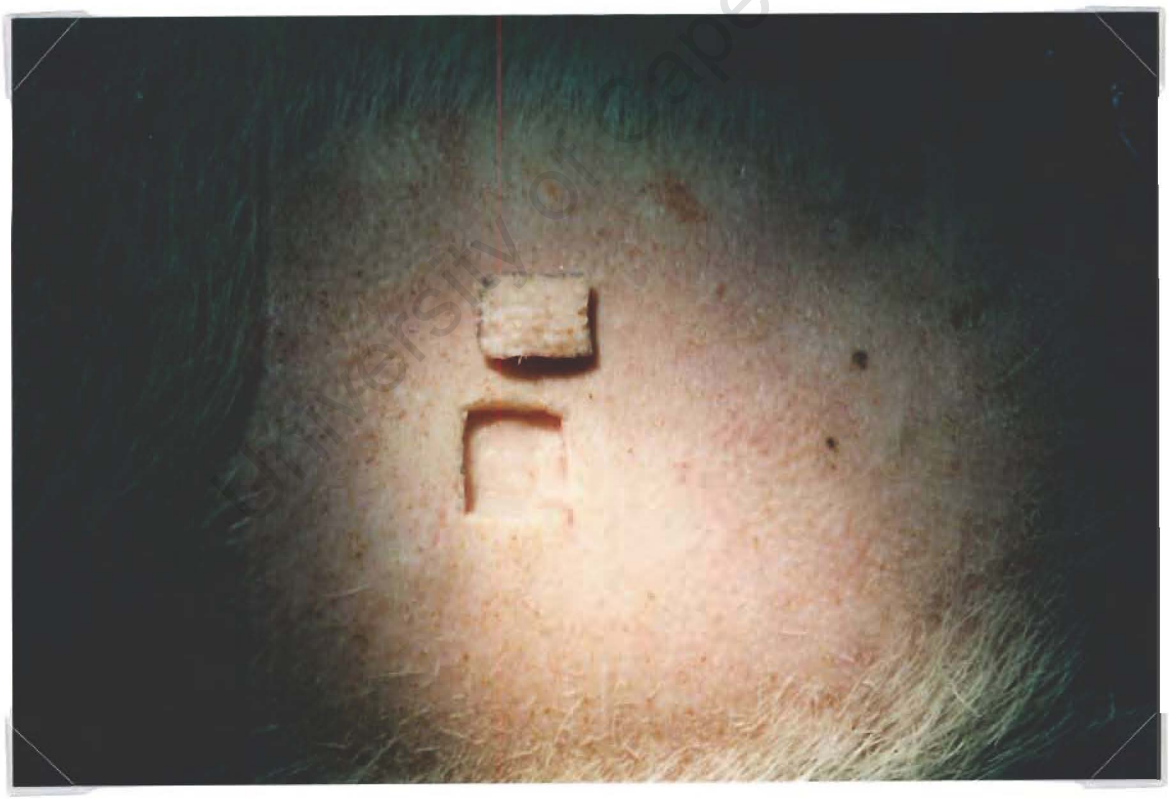
TEST AGENT CONTROL

Pig 1	Povidone iodine 	Harvesting Day 7
Pig 2	Povidone iodine 	Harvesting Day 28
Pig 3	Silver-sulphadiazine 	Harvesting Day 7
Pig 4	Mupirocin 	Harvesting Day 28
Pig 5	Povidone iodine 	Harvesting Day 28
Pig 6	Mupirocin 	Harvesting Day 7
Pig 7	Hebermin 	Harvesting Day 7
Pig 8	Silver-sulphadiazine 	Harvesting Day 7

Pig 9	Povidone iodine 	Harvesting Day 7
Pig 10	Silver-sulphadiazine 	Harvesting Day 28
Pig 11	Hebermin 	Harvesting Day 28
Pig 12	Mupirocin 	Harvesting Day 7
Pig 13	Mupirocin 	Harvesting Day 28
Pig 14	Hebermin 	Harvesting Day 7
Pig 15	Silver-sulphadiazine 	Harvesting Day 28
Pig 16	Hebermin 	Harvesting Day 28

The wounds were made by full-thickness excision of the dermis and epidermis using a scalpel blade (Picture 6). The plane under the dermis, consisting of loose connective tissue, was easy to identify.

At the end of the procedure, the test agent was applied to the test wounds, while the control wounds were left untreated. No occlusive dressings were applied to the wounds. Wounds were treated once a day with the topical agent assigned to them. About 10cc (50g) of ointment was required for each animal to cover the wounds with a thin film of the test agent. As the animals by preference did not lie on the side that was wounded, minimal soiling took place and the test agent appeared to stay in contact with the wound until the next dressing was done. Since it is known that Hebermin and Povidone iodine could be altered by exposure to direct sunlight, the animals were stabled indoors for the duration of the study.

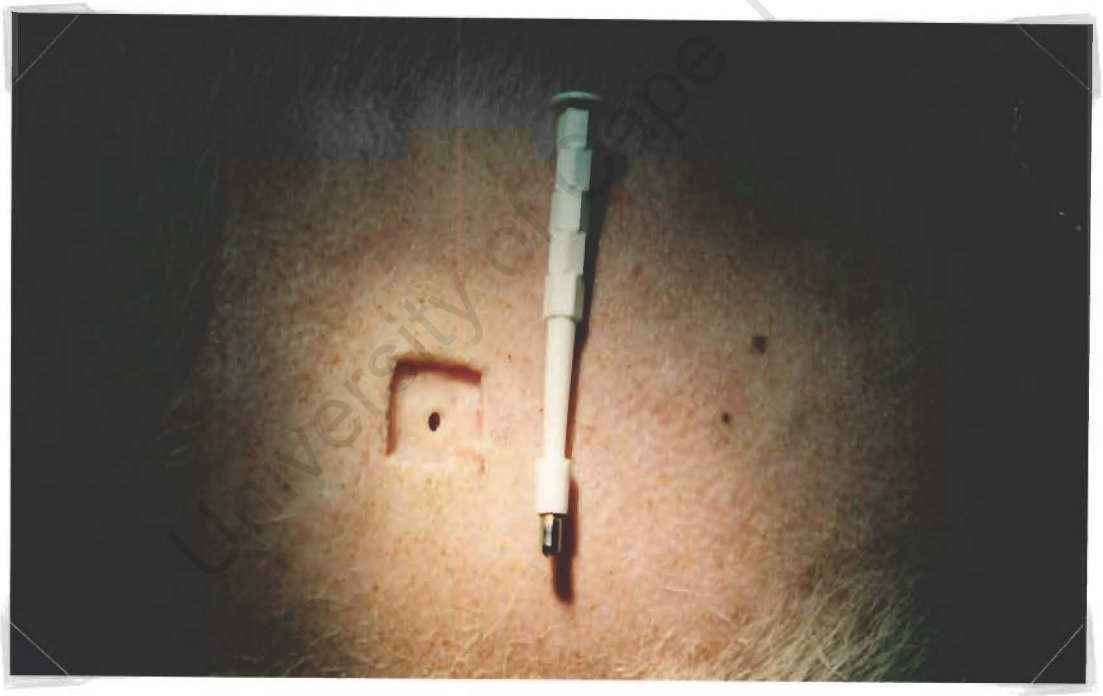


Picture 6: Wounding

2. Punch biopsy (day 3)

On day three after wounding, ten wounds from each animal were biopsied (five from the treatment wounds and five from the control wounds). A punch biopsy instrument, 3mm in diameter, was used to take a biopsy of the base of the wound (Picture 7). The wounds that were biopsied were marked with a permanent marker pen and were excluded from further analysis.

In the early phases of healing, before new structural elements have had time to appear, the wound consists mainly of an acute inflammatory exudate. As such, conventional histology is of limited use in the assessment of healing during the first 72 hours and excision biopsy was not performed on day three. However, measurement of wound constituents are important during this phase and the punch biopsy specimens were adequate for assessing collagen content by means of computerized image analysis.

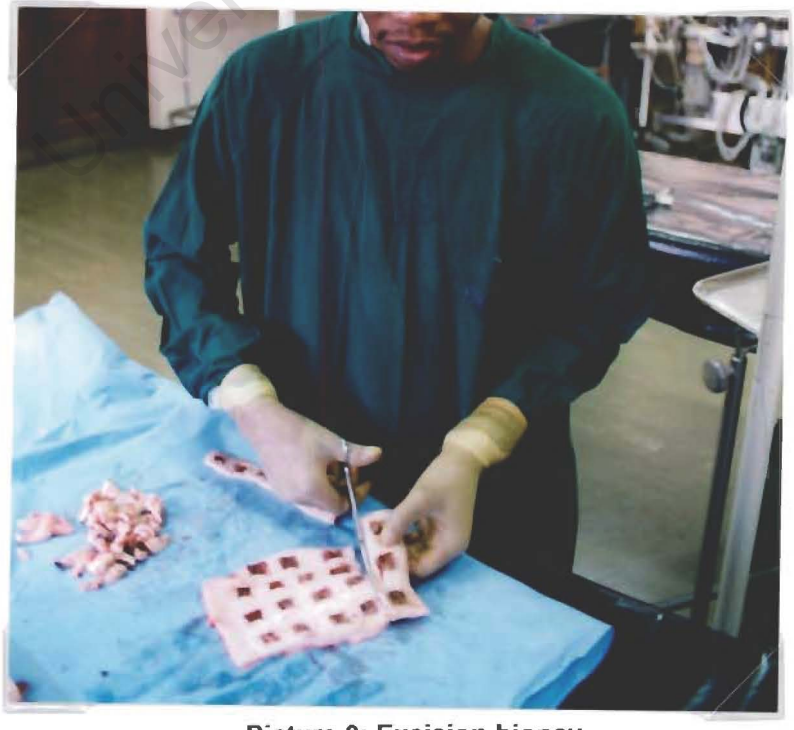


Picture 7: Punch Biopsy

3. Excision biopsy (day 7)

Two of the four animals assigned to a treatment were anaesthetised on day seven after wounding. General anaesthesia as earlier described was administered and the wounds were all surgically excised. The wounds were excised with excision margin deep to the base of the wound and with surrounding skin intact (Picture 8-9). Twenty wounds were harvested from each animal (ten treated wounds and ten control wounds). The ten wounds that had been biopsied on day three were discarded. After removing the wounds the animals were sacrificed.

Picture 8: Excision biopsy



Picture 9: Excision biopsy

4. Excision biopsy (day 28)

The remaining two animals assigned to a treatment were anaesthetised on day 28 after wounding. General anaesthesia as earlier described was administered and the wounds were all surgically excised. The wounds were excised with excision margin deep to the base of the wound and with surrounding skin intact. Twenty wounds were harvested from each animal (ten treated wounds and ten control wounds). The ten wounds that had been biopsied on day three were discarded. After removing the wounds the animals were sacrificed.

Phase 3 – Evaluation and analysis

Investigators were blinded to the treatment agent, and results were analysed only once all the data had been collected. Wounds were evaluated by three different methods: Visual Score, Histology, and Computerized Image Analysis.

1. Visual score

There is a strong correlation between the macroscopic and microscopic appearance of wounds, particularly between the clinical appearance and histologic scores of features in the epidermis and papillary dermis[67]. Visual assessment scales were previously described for wounds and scars[68,69], but these scales were not appropriate for assessment of the wide variety of wounds seen in clinical practise from other etiological causes, particularly from surgical incisions or non-burns trauma. A visual score for semi-quantitative assessment of surgical scars was developed by Beausang et al, and was found to be a sensitive instrument in scar assessment, allowing validated quantification of the severity of a wide range of scars[67].

Clinical assessment was done on day 28, using this scale which includes wound color, contour, texture, and distortion. Each of these parameters was given a score of between 1 and 4, higher values indicating poorer appearance. Whether a wound was matte or shiny was also recorded, the former scoring 1 and the latter 2. An overall assessment was also made and indicated on a visual analogue scale as a vertical mark on a 10cm line, 0 indicating an excellent wound and 10 indicating a poor wound. This score, expressed in centimeters to one decimal place, was then added to the sum of the individual parameter scores to give an overall score for each wound. Visual analogue scales have been used successfully in the past in assessing cosmetic scar outcomes[70], and takes into consideration other subjective features of a wound that affect its appearance (Clinical assessment sheet).

Clinical Assessment Sheet

Visual Analogue Scale

Excellent----------Poor

A	Color (compared to surrounding skin)	
	Perfect	1
	Slight mismatch	2
	Obvious mismatch	3
	Gross mismatch	4
B	Surface	
	Matte	1
	Shiny	2
C	Contour	
	Flush with surrounding skin	1
	Slightly proud / indented	2
	Hypertrophic	3
	Keloid	4
D	Distorsion	
	None	1
	Mild	2
	Moderate	3
	Severe	4
E	Texture	
	Normal	1
	Just palpable	2
	Firm	3
	Hard	4

2. Histology

Histologic assessment of the wounds involved quantification of the major architectural abnormalities that are known to occur in wounds. Routine H&E stains were done on biopsy wounds (day 28). Epidermis was characterised by a simple 3-point scale, depending on the degree of restoration of the rete ridges. Papillary and reticular dermis were scored in comparison to normal skin. Parameters were collagen fibre alignment, maturity, and density. This scale has been previously validated[67,71], and has been shown to be a reliable, consistent tool in histologic assessment of healing wounds in humans (Histologic Assessment Scale).

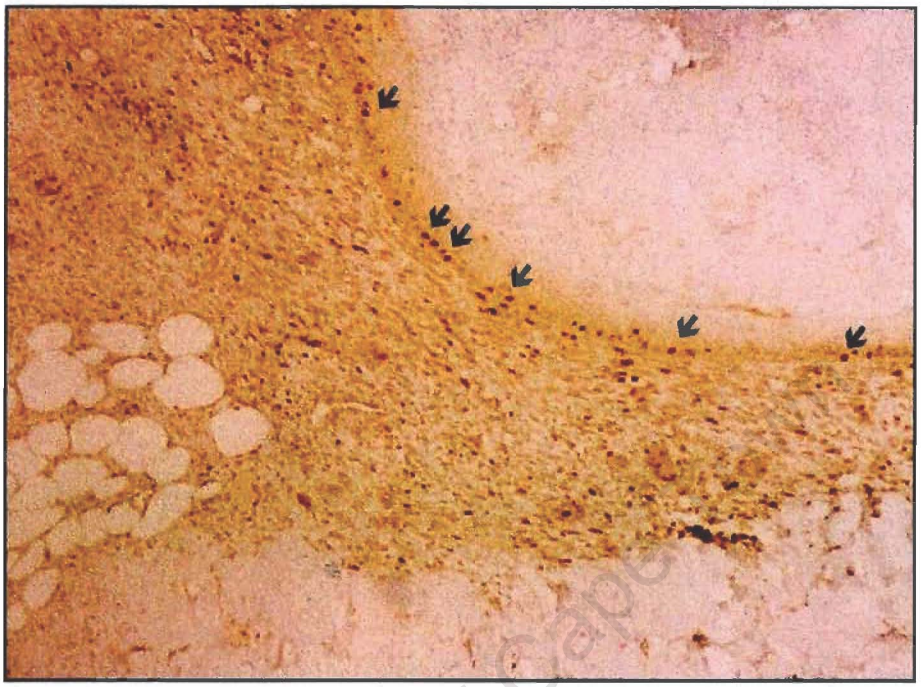
Histologic Assessment Scale

Epidermis	
Normal	0
Some restoration of rete ridges	1
No restoration of rete ridges	2
Dermis	
A. Collagen fibre orientation	
Normal basket weave pattern	0
<25% abnormal	1
26-50% abnormal	2
51-75% abnormal	3
76-100% abnormal	4
Keloid-like fibre orientation	5
B. Collagen fibre density	
Normal fibre bundle density	0
<25% abnormal	1
26-50% abnormal	2
51-75% abnormal	3
76-100% abnormal	4
Keloid-like fibres	5
C. Collagen fibre maturity	
Normal fibre maturity	0
<25% abnormal	1
26-50% abnormal	2
51-75% abnormal	3
76-100% abnormal	4
Keloid-like fibres	5

SCORE RANGE: 0-17

3. Immuno-histochemistry

Immuno-histochemistry is becoming an important tool in the measurement of healing parameters. MIB1 stains were done on Day 7 biopsy specimens (Picture 10).



Picture 10: MIB stains for mitoses (arrows).

The slides were assessed by an experienced histopathologist blinded to the treatment agent. A semi-quantitative scale was used where a score of 0-4 was given according to features of staining density, mitotic activity and epithelial growth. A score of 0 indicates slow epithelialisation and 4 indicates rapid epithelialisation.

Computerized Image Analysis, using the method described later for measuring collagen content, was also used to validate these results. Percentage surface area stained positively by MIB1 was measured, and correlated well with the results of visual scoring.

4. Computerized Image Analysis

As personal computers become more powerful, more affordable and easier to use, they have become more widely used in everyday life. Histopathology is a field where the evaluation of microscope images is central to the study of disease, not only from the point of view of diagnosis, prognostication and our understanding of disease processes, but also for medical research. Exciting possibilities exist for augmenting the skills and expertise of histopathologists with new approaches to image assessment, made possible by recent advances in the field of digital image analysis. Visual microscopic assessment of a tissue section is based on the diagnostic experience of the pathologist. It relies on mental imagery, acquired recognition skills, knowledge of histology and etiology and insight into the relevant clinical data. Being essentially a subjective evaluation, this process could be aided by subjective measurements made possible by computerized imaging systems. Quantitative data is generally more reproducible and verifiable than qualitative evaluation alone.

4.1 Components

In computerized image analysis, the basic workstation is the Video Photometer. It comprises four major components: an optical light microscope including the relay optics to the video system, a video camera plus image frame buffer, a computer system, and the software to analyze the captured image[72]. The microscope provides the images and projects it onto the video detector. The video system scans the image and sends the video signal to the image frame buffer. There, the video signal is sampled, digitized and stored. The computer system processes the image, and extracts information. Although integrated workstations are commercially available, a basic system can readily be assembled by combining the different components in a custom application. Any microscope can be converted into a video photometer by mounting a video camera and connecting it to a computer fitted with a video capturing card and running appropriate software.

4.1.1 Microscope

Some special considerations apply to the choice of objectives, condensers, and light source to ensure minimal spherical aberration and that the images in the microscope and video monitor are parfocal. A motorized coordinate stage is preferred for accurate recording and relocation precision. The light source should be stabilized so that voltage fluctuations are kept below millivolt level, as radiance is highly sensitive to even small changes in supply voltage. Unfortunately, the problem of

stray light (light originating from objects in the field of view close to the picture element being measured), cannot always be corrected for as it is 'object dependent'. Higher staining densities can provide control over stray light photometric errors[73], but care should be taken to ensure the condenser is well centered and measurements should be limited to the central portion of the microscope image.

4.1.2 Camera

The second component, the image-acquisition device, is usually a charge- coupled device (CCD) camera or a video camera, which sends out analog signals, like regular household camcorders. However, improvements have enabled these devices to now transmit digital signals instead. Cameras being employed range from simple video to proprietary interface devices for customized capabilities. They differ in a number of features: camera resolution; color capability; geometric correction; and the number of data bits per pixel of the image. The trend is towards higher-resolution video cameras combined with some kind of frame grabber. The video connection will soon be built directly into computers so that frame grabbers will become more specialized. As the electronics shrink, more of the electronics will be involved in the camera head and less in separate controller boxes. The greatest progress today lies in better-cooled CCD cameras. Cooling lowers the thermal "noise" in the signal, providing a cleaner and sharper image.

Other imaging devices include hand-held scanners, desktop or flatbed scanners, digital cameras, and various specialized instruments. The component that transmits the image from the camera to the computer is usually a frame grabber board, but many new digital cameras now interface directly with the computer. Scanners also directly transmit their signals.

4.1.3 Computer

The third component is the computer, and current imaging systems require at least a Pentium-level PC. Operating systems are application-specific at the moment, but the trend over the next few years appears to be that personal computers (PCs) and UNIX workstations will develop into more powerful, easier-to-use network-ready systems whose capabilities will be nearly indistinguishable from each other.

4.1.4 Software

The final component is software, enabling users to make measurements of the sample and export the data into a useful form, such as a report or a graphic. The software also controls the camera or scanner acquiring the image and does any image enhancement necessary to correct for problems in image acquisition. The capability to perform statistical analyses can also be built into the program. In many applications, the user can eventually automate the process so that it can be accomplished, more or less, at the touch of a button rather than by a laborious series of manual steps each time.

The cost of software need not be prohibitive for setting up a system, with most packages retailing for around $1500 - $2000 (e.g. KhorosPro 2000[74], IPLab[75]). Although specialized packages can cost up to $40 000 or more (Optimas[76], Visilog[77]), there are also excellent software products available that are distributed free of charge, e.g. NIH Image, made available by the National Institutes of Health[78]. Although this program runs on the Macintosh operating system only, there are versions available for UNIX (ImageJ[79]), or Windows (Scion Image[80]). A similar program, OSIRIS[81], is also available for free.

Some software packages are not available as a stand-alone application, but as part of an imaging system including hardware and development tools.

The trend in software will be toward ease of use and multiple applications in an integrated package. Top-end systems integrate the imaging hardware and software as a workstation unit, while other companies specialize in software solutions that can be transported onto a variety of computing environments.

4.2 The process of Digital Image Analysis

There are four stages to consider in the process of digital image analysis:

4.2.1 Image acquisition

A camera captures analogue data, such as a tissue section on a microscope slide, and the information is transformed to discrete bits of digital information. In image processing, the smallest of such data units, or picture elements, are pixels. At the minimum spatial resolution required to identify nuclear chromatin patterns, that is, approximately two to four sampling points per micron along a scan line, a 2cm x 2cm area on a slide results in 1.6 billion pixels. Fortunately, image data

acquisition has seen spectacular advances recently, and the developments in processing technology over the past 30 years are somewhat awe-inspiring. In 1966 the first scanning stage offered for the Zeiss UMSP1 allowed recording of 15 pixels per second. Today, video technology allows data recording at rates of several megapixels per second. High-performance systems are commercially available that more than adequately serves the needs of the histopathology laboratory.

4.2.2 Image processing

Once the image is captured, image processing takes place where the image is enhanced or optimised in a format suitable for analysis. It may be necessary to remove artifacts, to rotate or darken the picture, to remove noise, to reduce the color depth to only two or three colors or even to separate color channels for individual analysis. Complex mathematical algorithms exist that perform functions such as detecting edges around structures or separating a composite field into different cells.

4.2.3 Image analysis

The third step, image analysis, is the actual measurement of features of interest in the image. The process has evolved from simple measurements of points, lines and areas requiring manual identification or tracing of features, to automated processing guided by image-smart systems. The challenge of quantitation in histopathology is to describe, in numeric format, elements that are traditionally defined in descriptive or linguistic form. 'Nuclear area,' for example, is readily measured as so many square microns or pixels in a digitized image. But sometimes it is necessary to define a concept, such as 'undifferentiated', by a whole set of measured entities describing tissue architectural and nuclear features, for instance, degree of randomness of nuclear placement and nuclear area and chromatin pattern.

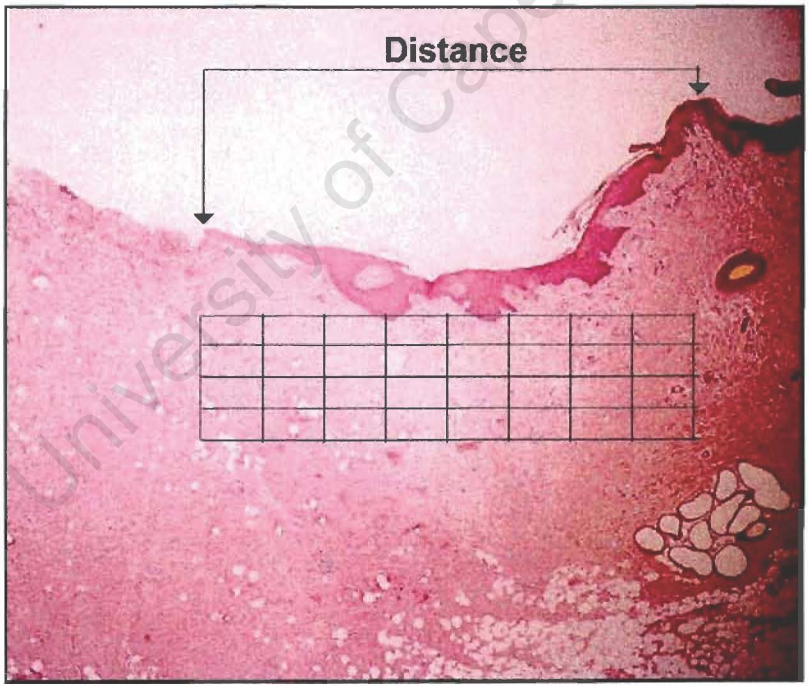
The computer measures elements of a histological image that introduce a whole new language to the field. Instead of merely observing that a cell is '*not round*', the computer can find and trace the outline and then calculate exactly *how round* the cell really is (ratio of perimeter length squared by the area). So the term 'measured area circularity' should now become part of the pathologists vocabulary.

4.2.4 Image interpretation

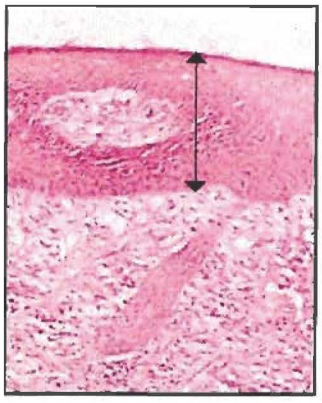
The last stage in the process is interpreting the data offered by the digitized image. The problem facing information science is not whether these characteristics can be measured, but rather whether calculating a value such as the 'binary weighted center-of-mass' can be biologically interpreted and eventually found to be clinically relevant. We are only in the early research stages in endowing powerful information-processing systems with the capability to 'understand'. Understanding, in diagnostic histopathology, involves much more than the information offered by the histologic section and its microscopic image. It requires all the information that might enter into a human diagnostic decision, the full definition of the mutual dependencies among all involved findings. We are not at a point in the development of machine intelligence and automated reasoning systems at which information technology can offer an understanding of a complex visual field. There is no doubt, however, that quantitation in histopathology will involve automated reasoning in combining evidence. Work is in progress utilizing inference networks, training algorithms[82], fuzzy set theory and neural network learning[83], that introduce non-numeric data handling and cognition to the interpretation of images[84,85].

4.3 Rate of healing

Computerized Image Analysis was used to measure the rate of re-epitheliasation. There are many methods in use for determining the rate of epithelial ingrowth from the wound edge towards the center of a wound. These methods range from visual inspection and calliper measurements to morphometric measurements performed on a traced image of the wound outline. Methods have been described of determining the weight of paper cut to the size of the wound margins and of direct measurements on histology specimens using a microscope with a calibrated graticule. Our method makes use of measurements of distance in histologic specimens. The excision biopsies of wounds on day 7 were used to prepare slides with routine H&E staining, which was then digitized and analyzed using computerized imaging software. The outcome parameters used were maximum distance of epitheliasation from the wound edge (Picture 11), and maximum thickness of the new epithelial layer sampled at the wound edge (Picture 12).



Picture 11: Distance of epithelial growth



Picture 12: Thickness of epithelium

4.4 Collagen content

Computerized Image Analysis was also used to determine collagen content of wounds biopsied on day 3, day 7 and day 28. In previous studies it was shown that this method (calculating total collagen content by measuring percentage surface area of collagen on Sirius Red stained biopsy samples), correlate well with direct measurement of hydroxyproline[14,15, 16].

4.5 Methodology

To outline the basic methodology and workflow process, the example of measurement of percentage surface area of collagen in the histological sections of wounds on day 28 is described in detail.

4.5.1 Specimen preparation/staining

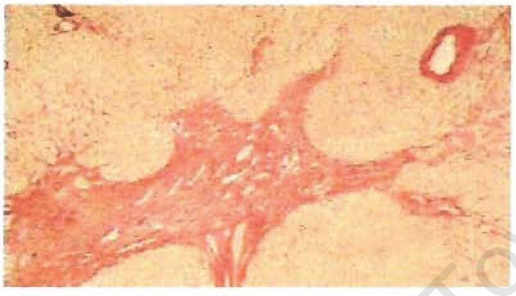
Morphometry on histopathological sections is usually performed on tissue fixed in a 10% formal saline solution. The tissue is then processed into paraffin wax and sectioned. However, resin-embedding can be used to enable the cutting of uniform thin sections, but this is costly and labour intensive. The best measurement results are obtained from the thinnest possible sections, usually 2 –3 µm in thickness. The stain used is dependent on the element being measured e.g. Perls' method for iron, Sirius Red for collagen, or osmium tetroxide for fat. Irrespective of whether the study is retrospective or prospective it is important for a single person to cut and stain the sections, preferably in a single batch, or otherwise in carefully selected batches. For thresholding (in area morphometry), high contrast between elements of interest and the background is essential.

4.5.2 Calibration of video equipment

The precision and reproducibility of measurements obtained with a video-based system is important. When setting up a system, there should be a stable electrical voltage, the video camera should respond linearly to changes in light, and factors influencing variation in ambient light should be compensated for. Before starting a session, strict quality assurance protocols should be followed. Equipment should be switched on and allowed at least 10 minutes to stabilize at the thermal equilibrium of the operating environment. Scaling can be done with a calibrated graticule placed in different positions of the video screen and rotated to different angles. The optical density of a Fuelgen stained slide is measured and compared with previous measurements of the same slide. A coefficient of variation smaller than 5% is preferred when measuring normal cells, although measurements from tissue sections can exhibit larger coefficients of variance[86]. Another way to calibrate optical density is to place an empty slide onto the microscope stage and after the microscope and condenser is focused and field stop adjusted with the light source set to the required level, the video signal is brought to near saturation. The light source is then adjusted so that an empty field gives a pixel value of 255. More precise calibration can be performed with neutral density filters and correction masks, but it should not be necessary to repeat it during a capturing session provided that the condenser, aperture stop and light source settings are kept constant. Most video cameras have automatic gain control (circuits automatically adjusting the gain and dynamic range to changing light conditions), and for obvious reasons this should be disabled on cameras used for videophotometry.

4.5.3 Capture of images

The selection of fields for capture is important. Where the whole specimen is not measured, protocols for random sampling should be followed to avoid biased selection of fields. Fields for capture should be from the center of the image. The format in which images are saved is also important, and image file formats using 'lossy' compression (e.g. JPEG), should be avoided. An example of a captured field is shown in Picture 13. The collagen, stained red, is clearly visible.



Picture 13: Captured image

4.5.4 Image analysis

Using the software tools, in this case Optimas 6.1[76], the region of interest is defined and a threshold is applied that selects the feature to be measured. It is important that the same threshold values are used for all the samples. Picture 14 shows the same image after the threshold is applied: all the positively stained tissue is selected and appears as yellow areas (known as the foreground). Now the computer can measure the total size of the foreground elements either in pixel size or in units of area measurement, and express it as a percentage of the total area of the image (Picture 15).

Picture 14: Threshold

Picture 15:Binarised image

4.5.5 Data analysis

The raw data is then exported to the appropriate software for statistical analysis. In Optimas, the measurements can be exported in numeric format straight into a Microsoft Excel spreadsheet, but any appropriate statistical package can be used for analysis or presentation. Some image analysis programs have built-in tools for data analysis.

E. Results

Results were analysed once all data had been acquired. Statistical analysis was done using student's t-test, chi-square, ANOVA, and correlation statistics where appropriate. P-values of <0.05 were interpreted as statistically significant.

1. Visual score

Results are given in APPENDIX Table 1: Visual Score wounds (Day 28). All treatment agents had good results compared with their untreated control wounds ($p<0.01$). The best result was achieved with Povidone Iodine (136.2), followed by Silver Sulphadiazine (144.2), Mupirocin (148), and Hebermin (150.1), although differences between treatment groups were not significant ($p=0.11$).

2. Histology

The results of the histologic assessment scale is given in APPENDIX Table 2: Histologic Assessment Scale. All treatment agents had good results compared with their untreated control wounds ($p<0.01$). The best results were achieved with Povidone Iodine (74), followed by Mupirocin (77), Silver Sulphadiazine (79), and Hebermin (79). Differences between treatment groups were not significant ($p=0.72$).

3. Immuno-histochemistry

Day 7 biopsy specimens were stained with MIB1, showing increased mitotic activity in the upper papillary dermis, with proliferation of fibroblasts and epithelial cell growth. The result of visual scoring is given in APPENDIX Table 3: MIB1 Score wounds.

All agents tested appear to stimulate regeneration in the granulation tissue as compared to untreated control wounds ($p<0.01$). Hebermin, containing Epidermal Growth Factor, had the most pronounced effect, scoring 7, followed by Mupirocin (10), Silver Sulphadiazine (10), and Povidone Iodine (12). This effect approached statistical significance ($p=0.06$).

Computerized Image Analysis was used to validate these findings, the result of percentage surface area stained is given in APPENDIX Table 4: MIB1 stain Surface Area (Day 7). The mean value for control wounds was 2.81%, with Povidone Iodine 3.14%, Silver Sulphadiazine 3.58%, Mupirocin 3.38% and Hebermin 4.10%.

4. Computerised Image Analysis

4.1 Rate of healing

4.1.1 Distance of epithelial growth

The distance of re-epithelialisation was measured from the wound edge along the wound surface to the end of new epidermis tissue. Results are given in APPENDIX Table 5: Distance of epithelial growth from wound edge (Day 7). There was a significant difference in the distance between treatment groups ($p<0.01$), and all groups showed a higher rate of epithelialisation when compared to untreated control wounds ($p<0.01$). The distance of epithelialisation correlates with the surface area covered by the new epidermal layer, with Hebermin showing the fastest healing rate (distance = 1902.78 μm), followed by Povidone Iodine (1723.22 μm), Silver Sulphadiazine (1401.22 μm), and Mupirocin (1179.36 μm).

4.1.2 Thickness of epithelial growth

The thickness of the new epidermal layer was measured at the wound edge, believed to be the germinal centre from where new epidermal cells originate before migration along the wound surface. Results are given in APPENDIX Table 6: Thickness of epithelial layer at wound edge (Day 7). A thicker layer of epidermis was measured in wounds from treatment groups Hebermin and Mupirocin when compared with Silver Sulphadiazine and Povidone Iodine ($p<0.01$). These two groups were the only groups significantly different from the untreated control wounds ($p<0.01$). Mupirocin had the thickest epidermal layer (130.08 μm), followed by Hebermin (120.56 μm), Control (103.14 μm), Povidone Iodine (101.68 μm), and Silver Sulphadiazine (96.19 μm).

4.2 Collagen content

Collagen content was measured by Computerized Image Analysis as described before. Measurements were done on biopsy wounds from day 3, day 7 and day 28. The results are given in APPENDIX Table 7: Collagen content by Computerized Image Analysis.

In biopsies from day 3, collagen formed 2.7% of the granulation tissue. This increased to 41.4% on day 7 and 68% by day 28. Collagen content was similar for all wounds on day 3, with Mupirocin 2.63%, Povidone Iodine 2.87%, Silver Sulphadiazine 2.91% and Hebermin 3.05% ($p=0.8$). There was also no difference between treated and untreated wounds ($p=0.062$).

By day 7, collagen content increased in the treated group to average 42.51%, while in the untreated wounds there was significantly less collagen (40.39%). P<0.01. The difference in collagen content between treated and untreated wounds was even more pronounced at day 28, with average collagen content 70.27% and 65.73% respectively (p<0.01). Between treatment groups, Hebermin-treated wounds had the most collagen by day 7 (43.27%), followed by Silver Sulphadiazine (42.46%), Mupirocin (42.41%), and Povidone Iodine (41.90%). However, there was no statistical significance between groups (p=0.85). By day 28 Hebermin wounds again had the most collagen (72.30%), followed by Silver Suphadiazine (70.55%), Mupirocin (70.29%), and Povidone Iodine (67.94%). There was no statistical significance between groups (p=0.27).

F. Discussion

Adult human wound healing invariably leads to scarring, which may give rise to both functional and cosmetic defects. The former may restrict further growth and frequently requires secondary treatment to improve symptoms. Cosmetic defects can be both physically and psychologically disturbing for patients and have become increasingly important in some societies. This increases patients' demands on their physicians, many expecting invisible healing.

In the continuing battle to improve healing, several treatment modalities have been described. These include surgery, radiation[87], cryotherapy[88], non-invasive methods such as pressure therapy[69,89,90] and topical silicone gel[91], and various drug treatments including steroid injections[92], interferon[93], and retinoids[94]. At present, there is no universally accepted objective method of measuring these treatments' effects, and we are dependent on semi-quantitative subjective clinical means. Various investigators have attempted to determine what clinical criteria are important in assessing wounds, both from the functional and cosmetic viewpoints. These parameters include pigmentation, redness, texture, contour, and distorsion[68,69,95,96]. Pain, pruritis, and scar contracture have also been used by others as assessment features[97,98].

In this study, the effects of Epidermal Growth Factor was seen in the increased rate of epithelialisation, indicating its potential as a topical agent where rapid healing is required. Mitoses are stimulated in wounds treated with EGF, as seen in the higher score in the MIB1 stained slides. EGF also had an effect on collagen production, with higher collagen content in wounds treated with Hebermin at day 7 and day 28. This would suggest that wound tensile strength and scar thickness would increase with the use of EGF topical agents. Unfortunately there appears to be a trade-off between speed of healing and cosmetic appearance, with treatment agents that accelerate healing also stimulating collagen deposits. Collagen fibers are immature and do not have time for normal cross-linking when fibroblasts are over-stimulated. The effect of rapid collagen deposition on local texture orientation of the intercellular matrix could explain the undesired effects such as hypertrophic scar formation and contractures.

Most methods used to evaluate wound healing are subjective and inaccurate. As realisation grows that clinical assessment alone represents a subjective impression that can at best be considered semi-quantitative, histopathology becomes important as an instrument for more objective measurements in wound healing. With the evolution of electron microscopy, molecular biology and immuno-histochemistry, evaluation could be advanced beyond cellular level to include quantification of sub-cellular components that play a role in the complex chain of events that constitute healing. Even so, methods to independently and reproducibly interpret clinical data are limited mostly to methods of biochemical analysis, and the structured interpretation of visual data has only recently been explored. The computerized conversion of images into data for analysis, quantification, and presentation has promised to become a widely used - even necessary - tool in the life sciences laboratory.

G. Conclusion

It is important to determine the best topical agent for treatment of full thickness dermal wounds. This information has direct clinical application, for instance in the treatment of burn wounds.
The wounds treated with different test agents differed in the rate of epithelialisation, clinical healing properties, and indices of wound maturity. All were shown to improve wound healing as compared to untreated control wounds. The wounds healed faster, histologic appearance resembled normal architecture sooner, clinical appearance was improved, mitotic activity was stimulated, and more collagen was deposited when the wounds were treated with the test agents.
Povidone iodine and Silver sulphadiazine showed the best results in terms of clinical appearance as well as histologic indices of good healing characteristics, although the difference as compared to the other agents was not statistically significant. Mupirocin, and especially Hebermin, was shown to increase the rate of healing as seen in accelerated epithelial growth, stimulation of mitoses and collagen production. The stimulation of healing is most apparent when the effects of Hebermin is compared with Silver Sulphadiazine, where the only difference between the two agents is the effect of epithelial growth factor.
The appropriate clinical application for EGF seems to be in wounds where increased rate of healing and tensile strength is required, as in extensive burns, donor areas of split skin grafts, non-healing ulcers and tissue pathology such as in radiation ulcers and where extravasation of

chemotherapeutic agents occur. Agents like Hebermin that show the most potential for increasing the rate of healing could therefore be important in the treatment of complicated or large wounds, or where the natural healing ability of a patient is compromised.

The different methods used in this study to assess healing showed good correlation, and in combination could be useful to establish valid outcome parameters for evaluation protocols. Computerised Image Analysis could provide a quantitative assessment of wound healing that is more comprehensive than simple morphometric measurements, and less subjective than conventional histopathologic scoring systems. Recent advances in computer hardware and software are lowering, if not removing, the barriers to wider acceptance of image-analysis systems by life scientists.

H. Recommendations

This study should be extended to include other modalities of local wound management such as topical application of other polypeptide growth factors, polyethylene film, new wound irrigation or suction systems or even more recent advances in biologic dressings. More studies are also required to develop more accurate measurement tools and in combination with scanning electron microscopy, computerized image analysis should extend to measurements of sub-cellular structures. In vivo measurement systems should be developed to extract measurements from wounds without the need for biopsies. It is important that validation of laboratory data and knowledge gained from animal studies should follow with clinical studies in humans.
The ultimate goal is to develop comprehensive tools for measuring wound healing, which would include clinical, histological, histochemical, biochemical and morphometric parameters.

APPENDICES

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Table 1. Visual Score wounds (Day 28) .

		Visual Analogue Score	Color	Surface	Contour	Distorsion	Texture	Total	Total
Mupirocin	1	3.5	3	2	2	3	3	16.5	
	2	2.1	3	2	2	3	3	15.1	
	3	3.7	2	2	2	3	4	16.7	
	4	3	3	2	1	3	3	15	
	5	3.2	2	2	2	3	2	14.2	
	6	2.2	3	2	2	2	2	13.2	
	7	2.5	3	1	2	4	3	15.5	
	8	2.6	3	2	2	3	2	14.6	
	9	1.9	2	2	2	3	2	12.9	
	10	3.3	4	1	2	3	1	14.3	148
Mupirocin control	1	3.7	2	2	2	4	3	16.7	
	2	4.5	2	2	1	4	3	16.5	
	3	2.8	3	2	2	3	3	15.8	
	4	5	3	2	2	3	3	18	
	5	3.6	3	2	2	3	3	16.6	
	6	3.8	3	2	3	4	4	19.8	
	7	4.7	3	2	2	3	3	17.7	
	8	3.8	4	1	2	4	2	16.8	
	9	2.4	2	2	2	3	3	14.4	
	10	4.6	4	2	2	3	3	18.6	170.9
Povidone Iodine	1	2.7	2	2	2	3	3	14.7	
	2	2.5	2	1	1	3	3	12.5	
	3	1.9	2	2	2	2	2	11.9	
	4	3.3	2	2	2	4	3	16.3	
	5	2.6	3	1	2	3	3	14.6	
	6	1.7	2	1	2	3	3	12.7	
	7	1	3	2	1	3	1	11	
	8	2.3	3	2	2	3	3	15.3	
	9	2.1	2	2	2	4	2	14.1	
	10	2.1	2	2	2	3	2	13.1	136.2
Povidone Iodine control	1	3.8	3	1	2	3	3	15.8	
	2	3.3	2	2	2	3	3	15.3	
	3	4.3	2	2	1	2	3	14.3	
	4	3	2	2	3	4	3	17	
	5	3.7	3	2	2	4	3	17.7	
	6	2.1	4	2	3	4	3	18.1	
	7	4.3	3	2	2	4	3	18.3	
	8	4.5	2	2	2	3	3	16.5	
	9	3.6	3	1	2	4	3	16.6	
	10	4.1	2	2	2	3	3	16.1	165.7
Hebermin	1	3.3	3	1	2	2	3	14.3	
	2	3.4	3	2	1	3	3	15.4	
	3	2.9	3	2	2	3	2	14.9	
	4	4	3	2	1	3	3	16	
	5	2.2	2	1	2	4	4	15.2	
	6	3.6	3	2	2	2	2	14.6	
	7	3.6	2	2	2	2	2	13.6	
	8	2.5	3	2	2	3	3	15.5	
	9	3.5	3	2	2	3	3	16.5	
	10	2.1	2	2	2	3	3	14.1	150.1
Hebermin control	1	4.2	3	1	1	2	2	13.2	
	2	3	3	1	3	4	4	18	
	3	3.6	4	2	3	4	4	20.6	
	4	3.5	3	2	2	3	4	17.5	
	5	5	2	2	2	4	3	18	
	6	4.4	2	2	2	3	4	17.4	
	7	2.2	3	2	2	3	3	15.2	
	8	3.9	3	2	2	3	3	16.9	
	9	3.6	4	1	1	3	4	16.6	
	10	4.5	4	2	2	3	3	18.5	171.9
Silver Sulphadiazine	1	2.5	3	2	2	3	3	15.5	
	2	2.2	3	2	1	2	2	12.2	
	3	3.6	2	2	1	3	3	14.6	
	4	4	3	2	2	3	3	17	
	5	1.5	2	2	2	3	3	13.5	
	6	2.3	3	2	2	2	3	14.3	
	7	2.8	2	1	2	3	2	12.8	
	8	3.3	2	2	2	3	3	15.3	
	9	1.9	3	2	2	3	2	13.9	
	10	3.1	3	2	2	3	2	15.1	144.2
Silver Sulphadiazine control	1	3.8	4	2	2	3	3	17.8	
	2	4.2	4	2	2	2	2	16.2	
	3	3.3	3	2	3	4	2	17.3	
	4	3	2	2	2	3	3	15	
	5	2.6	3	1	2	4	4	16.6	
	6	3.8	3	2	1	3	4	16.8	
	7	3.7	2	2	2	3	4	16.7	
	8	4.1	3	1	2	3	3	16.1	
	9	2.8	4	1	2	3	3	15.8	
	10	3.6	3	2	2	3	3	16.6	164.9

Table 2. Histologic Assessment Scale (Day 28)

		Epidermis	Collagen orientation	Collagen density	Collagen maturity	Total	Total
Mupirocin	1	0	2	1	3	6	
	2	1	1	2	3	7	
	3	2	4	1	3	10	
	4	0	2	2	3	7	
	5	1	2	2	3	8	
	6	1	2	2	3	8	
	7	0	2	2	4	8	
	8	0	1	2	4	7	
	9	1	1	3	3	8	
	10	1	2	2	3	8	77
Mupirocin control	1	1	1	2	3	7	
	2	1	2	3	3	9	
	3	0	2	2	3	7	
	4	1	2	2	4	9	
	5	1	1	1	3	6	
	6	1	2	2	3	8	
	7	1	4	2	3	10	
	8	2	2	2	3	9	
	9	2	2	2	3	9	
	10	1	3	3	3	10	84
Povidone Iodine	1	1	3	2	3	9	
	2	0	1	3	4	8	
	3	0	2	2	3	7	
	4	1	1	2	3	7	
	5	0	2	1	3	6	
	6	1	1	1	3	6	
	7	0	2	2	3	7	
	8	1	2	3	3	9	
	9	0	2	2	3	7	
	10	1	2	2	3	8	74
Povidone Iodine control	1	2	1	2	3	8	
	2	0	1	2	3	6	
	3	0	2	1	3	6	
	4	1	2	3	3	9	
	5	1	1	2	3	7	
	6	1	3	2	3	9	
	7	1	3	2	4	10	
	8	1	2	2	4	9	
	9	1	2	2	3	8	
	10	1	2	2	3	8	80
Silver Sulphadiazine	1	1	2	2	3	8	
	2	1	2	2	3	8	
	3	2	2	1	4	9	
	4	1	1	2	4	8	
	5	0	1	2	3	6	
	6	1	1	3	3	8	
	7	2	3	1	3	9	
	8	0	2	1	3	6	
	9	2	2	2	3	9	
	10	1	2	2	3	8	79
er Sulphadiazine control	1	1	2	2	3	8	
	2	1	4	2	3	10	
	3	0	2	2	3	7	
	4	2	1	3	3	9	
	5	1	2	2	4	9	
	6	0	1	3	3	7	
	7	1	2	2	3	8	
	8	1	2	2	3	8	
	9	2	3	4	3	12	
	10	1	3	2	3	9	87
Hebermin	1	1	2	2	4	9	
	2	1	2	2	3	8	
	3	1	1	3	3	8	
	4	0	2	3	4	9	
	5	0	2	2	3	7	
	6	0	1	2	3	6	
	7	1	1	1	3	6	
	8	2	1	2	3	8	
	9	0	2	4	4	10	
	10	1	1	3	3	8	79
Hebermin control	1	1	2	1	3	7	
	2	2	1	2	4	9	
	3	1	4	3	3	11	
	4	2	1	3	3	9	
	5	0	2	2	3	7	
	6	0	2	2	3	7	
	7	1	1	2	4	8	
	8	0	3	1	4	8	
	9	2	2	2	3	9	
	10	1	3	2	3	9	84

Table 3. MIB1 Score wounds (Day 7)

		Visual Score (0-4)	Total
Mupirocin	1	1	
	2	1	
	3	1	
	4	1	
	5	1	
	6	1	
	7	1	
	8	1	
	9	1	
	10	1	10
Povidone Iodine	1	1	
	2	1	
	3	1	
	4	1	
	5	1	
	6	1	
	7	2	
	8	1	
	9	1	
	10	2	12
Silver Sulphadizine	1	1	
	2	0	
	3	1	
	4	1	
	5	1	
	6	1	
	7	2	
	8	1	
	9	1	
	10	1	10
Hebermin	1	1	
	2	0	
	3	1	
	4	0	
	5	0	
	6	1	
	7	1	
	8	1	
	9	1	
	10	1	7
Control	1	3	
	2	3	
	3	3	
	4	2	
	5	0	
	6	2	
	7	2	
	8	2	
	9	2	
	10	2	21

Table 4. MIB1 stain Surface Area (Day 7)

		Percentage surface area	Mean
Mupirocin	1	2.29	
	2	3.78	
	3	3.22	
	4	4.96	
	5	4.76	
	6	3.40	
	7	2.58	
	8	2.52	
	9	3.37	
	10	2.96	3.38
Povidone Iodine	1	4.76	
	2	2.11	
	3	2.00	
	4	2.34	
	5	4.24	
	6	3.48	
	7	2.40	
	8	3.89	
	9	1.88	
	10	4.26	3.14
Silver Sulphadiazine	1	2.61	
	2	3.11	
	3	4.06	
	4	3.90	
	5	3.57	
	6	4.71	
	7	3.25	
	8	4.98	
	9	2.66	
	10	2.91	3.58
Hebermin	1	5.10	
	2	3.39	
	3	3.33	
	4	3.22	
	5	3.00	
	6	4.66	
	7	3.73	
	8	4.76	
	9	4.97	
	10	4.82	4.10
Control	1	2.32	
	2	3.20	
	3	2.10	
	4	2.87	
	5	2.04	
	6	3.28	
	7	2.60	
	8	1.80	
	9	3.05	
	10	4.83	2.81

Table 5. Distance of epithelial growth from wound edge (Day 7)

Distance (μm)				
Control	Silver Sulphadiazine	Povidone Iodine	Mupirocin	Hebermin
1321.20	1386.70	1487.60	852.78	2087.90
948.72	1622.70	2433.10	1094.70	2114.10
1198.80	1471.00	2738.50	1320.50	2327.60
620.41	1608.20	1804.20	1319.20	2241.90
1136.80	1599.80	976.25	1067.60	1041.10
849.21	660.50	405.00	873.58	1766.90
676.91	950.86	1477.00	1213.30	1419.80
500.20	1647.70	1359.30	1124.10	2780.50
987.66	1445.30	1955.20	1687.30	1479.70
919.04	1803.70	2085.10	1302.90	1741.40
995.96	1867.90	2181.60	1802.60	2309.50
1639.10	1370.60	2096.30	1011.30	1645.50
585.26	1644.40	2458.00	903.02	971.28
784.43	1114.40	2039.60	971.58	2688.70
743.85	1559.70	1814.60	776.85	2292.30
1405.90	1497.30	1270.20	1415.10	2020.90
795.64	1292.20	1438.70	759.02	1573.70
646.19	1255.50	1506.20	1733.00	1335.60
1203.10	824.77	1214.80	N/A	2314.50
945.18	1401.22	1723.22	1179.36	1902.78

Table 6. Thickness of epithelial layer at wound edge (Day 7)

Thickness (μm)				
Control	Silver Sulphadiazine	Povidone Iodine	Mupirocin	Hebermin
98.08	86.28	137.93	124.73	142.01
53.26	62.08	151.16	116.05	88.72
79.68	131.77	104.34	61.75	105.43
203.20	113.47	76.55	61.21	73.10
103.96	124.16	110.44	134.79	132.91
125.37	41.50	111.34	132.77	167.71
84.06	74.80	63.93	135.16	162.78
130.19	94.80	86.57	223.81	135.72
106.67	88.65	88.93	188.15	114.46
78.73	39.17	120.77	102.83	90.89
100.40	125.32	80.86	117.07	49.77
105.43	63.93	39.17	102.83	143.99
105.43	92.81	80.00	107.32	127.86
79.29	100.91	80.86	85.40	127.04
87.79	120.99	143.28	106.91	92.83
78.49	83.69	81.86	173.34	104.29
90.26	120.61	221.12	173.89	120.61
146.49	138.47	97.56	193.36	99.54
102.80	124.16	55.16	N/A	210.91
103.14	96.19	101.68	130.08	120.56

Table 7. Collagen content by Computerized Image Analysis.

		Punch biopsy day 3	Mean	Excision biopsy day 7	Mean	Excision biopsy day 28	Mean
Mupirocin	1	2.48		41.97		73.14	
	2	2.95		43.60		66.24	
	3	2.34		40.82		73.62	
	4	3.75		39.32		74.16	
	5	2.31	2.63	43.43	42.41	63.79	70.29
	6	1.29		45.65		67.68	
	7	3.64		40.76		74.56	
	8	2.46		44.47		72.00	
	9	2.83		42.63		63.76	
	10	2.23		41.42		73.95	
Mupirocin control	1	3.85		42.21		68.66	
	2	3.01		36.98		71.01	
	3	2.62		40.95		63.92	
	4	1.67		39.56		66.62	
	5	2.87	2.60	35.58	39.91	69.82	66.90
	6	2.63		41.22		61.95	
	7	2.69		39.02		68.05	
	8	1.88		40.39		71.86	
	9	3.54		38.76		62.81	
	10	1.26		44.40		64.30	
Povidone Iodine	1	3.29		41.13		64.73	
	2	3.32		38.81		71.30	
	3	2.17		42.13		69.83	
	4	3.37		44.62		63.56	
	5	3.35	2.87	44.63	41.90	64.85	67.94
	6	3.89		45.31		71.70	
	7	1.69		39.14		68.10	
	8	2.89		44.38		71.19	
	9	2.40		40.03		71.14	
	10	2.32		38.81		62.94	
Povidone Iodine control	1	3.22		39.35		65.12	
	2	2.81		45.48		65.72	
	3	3.81		40.03		65.25	
	4	1.05		39.47		61.86	
	5	2.54	2.31	39.66	40.69	66.54	65.77
	6	2.32		42.50		61.19	
	7	1.23		40.53		66.82	
	8	2.09		44.17		71.81	
	9	1.08		37.58		71.95	
	10	2.93		38.10		61.42	
Silver Sulphadiazine	1	3.97		45.47		69.97	
	2	3.37		39.69		71.12	
	3	1.05		45.18		61.79	
	4	2.70		38.26		71.33	
	5	2.37	2.91	40.76	42.46	69.81	70.55
	6	3.99		43.38		73.46	
	7	3.34		44.77		74.66	
	8	2.83		42.50		73.78	
	9	3.67		40.17		66.19	
	10	1.86		44.44		73.36	
Silver Sulphadiazine control	1	1.58		44.68		65.12	
	2	1.82		39.45		68.13	
	3	3.71		41.23		62.51	
	4	1.26		38.42		66.78	
	5	3.31	2.34	44.41	40.04	61.36	65.29
	6	1.42		40.75		64.02	
	7	3.67		37.28		63.66	
	8	1.66		37.15		74.06	
	9	2.43		37.46		60.78	
	10	2.56		39.53		66.52	
Hebermin	1	3.38		45.53		73.96	
	2	3.41		48.10		75.04	
	3	2.95		41.01		68.95	
	4	3.52		38.53		76.28	
	5	2.45	3.05	45.35	43.27	67.23	72.30
	6	1.61		44.55		73.77	
	7	3.25		42.94		71.78	
	8	2.57		38.18		70.61	
	9	3.48		44.67		67.63	
	10	3.87		43.84		77.71	
Hebermin control	1	1.76		45.21		72.62	
	2	2.24		37.64		69.34	
	3	3.94		41.55		61.77	
	4	1.09		43.26		63.42	
	5	2.36	2.59	41.13	40.93	63.97	66.13
	6	2.45		39.00		61.28	
	7	3.72		38.07		63.43	
	8	2.11		40.01		64.19	
	9	2.46		37.57		64.58	
	10	3.82		45.84		65.15	