Non-animal and Animal Wound Healing Models and Assays in Current Practice

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Abstract
Wound healing models are used as very important tools to test and study the new therapeutic approach in wound treatments. The present manuscript details different types of surrogates for the in vivo, human wound healing process based on different approaches (in vitro, in-vivo, ex-vivo, in-silico methods and animal models). The appropriate selection of a wound healing model is an important tool for wound healing research as it aids in the understanding of the wound healing mechanisms in detail. Also, this strategy enables the experimenter to focus on each stage of wound healing and aids in the evaluation of the wound healing methodologies in preventing the wounds; enhancing the healing process or in prevention of scar formation.

The thorough understanding of the appropriate wound model will aid in evaluating the pathophysiology of the wounds, better strategies in the treatment modes, formulations for enhanced wound healing and limit abnormal scar formation. The understanding of both acute and chronic wounds healing mechanism has significantly improved by adopting these models along with the advent of new wound healing products, wound healing developments and advanced wound care.

Keywords: Cutaneous wound repair, Epithelisation, Skin remodelling, Wound healing assay

Introduction
Refinement of wound healing models and assays are currently State-of-the-Art approaches for making improvements in our understanding of the overall pathogenesis of the classifiable steps in the wound healing process including the mechanistic aspects. It can also be used to detect new biomarkers such as chemokines, growth factors and cytokines which may be important determinants in the mechanisms associated with wound healing as well as scar formation. Over the years, numerous models have been used to examine, understand and dissect the various stages as well as the different types of wound healing. No single model has been proven to be adequate to study wound healing mechanisms.

Hence, this perforce requires the adoption of a combination of models, especially for chronic wound and ulcer management. The types of wounds and the phases of wound healing are critical determinants for the ideal model to be used. In this review, we will examine the current wound healing models and assays used extensively to study the mechanism of the healing process. These model systems include in vitro, in vivo, in silico, ex-vivo as well as animal models with a focus on their strengths and limitations of the respective model systems. The human in vivo/ex vivo models would perforce require appropriate approval from the Institutional Human Ethical Committees in the respective institutions.

Types of Wound Healing Model

In vitro Wound Healing Model: In vitro models are used extensively in wound healing studies to understand the underlying mechanisms for many years. It is primarily used in the process as well as the pathogenesis of scar formation. The most usually used in vitro wound models are monolayers, co-cultured cell cultures and skin explants. Assays based on in vitro models are relatively faster, cost-effective, satisfy ethical considerations and may be a realistic approach to causing reductions, if not elimination, in the use of animal models [Table 1]. Despite their extensive use, they pose obvious limitations in terms of replicating the complexities at the organ, tissue, cell and molecular levels.

2D Wound Healing Assays: In 2D wound healing assay, wounding is created by disrupting the confluent monolayer of cells, thereby creating a cell-free region. Subsequently, this model can be utilized by monitoring the healing process through cell migration into the wounding area. This monitoring involves data acquisition through a microscope or impedance-based instruments. Such 2D models are easy to develop and validate in a laboratory with minimal equipment and labour. The wound healing methodologies can involve the usage of mechanical, electrical, thermal and optical devices. Mechanical wounding is the fairly simple protocol with a wound created either through a pipette tip, toothpicks, metallic indenters or special cell scrapers.21,43,52,59,92,108

Scratch Assay: It is a widely used assay for wound healing studies since it is easy, less expensive, well-developed and widely used method for measuring cell migration in a Petri dish or 96 well plates. This monolayer of the cells is usually cultured and a scratch wound is created to study the cell migration in an attempt to close the cell-free area, thereby mimicking, at least in part, the wound healing response. The images are captured at regular intervals and used to compute the cell migration process. Scratch assay is also used to
visualize migration of cells at the individual level in the front margins of the scratch. The method is widely used in studying the interactions between cells as well as those with that of the matrix-processes relevant to the in vivo wound healing response. This assay is made possible due to advancements in live cell imaging-based technologies to monitor and measure the migration process and is correlatable with intracellular signalling events.

The scratch assay is relatively time-consuming and has an incubation period of 1-2 days and this intrinsic assay-specific feature is one of the many limitations. The procedure for creating this cell-free area can leave irregular scratches and a concomitant destruction of the noncellular scaffolding component which forms a coating on the cell culture vessel (in most cases) 28. The duration of the monitoring of the migration that needs to be done is cell type-dependent with an average of 14 hours. This assay requires a relatively large cell number (can be a challenge especially with primary cells) and consumables.

**Stamping Assay:** This assay is also a mechanical wounding method. A stamp (rubber or polymer material) is placed on a confluent monolayer of cells either manually or automatically. The placing of the stamp mold destroys the cells in a particular region leaving the cell debris behind on the stamp site and the influence of cell debris on the cell migration process is studied. This assay may serve to mimic a “real-life” in vivo scenario involving the recruitment of cells that can possibly displace and migrate amidst the dead/dying necrotic cell debris 48. In comparison with the scratch assay, an inherent advantage of this assay is that the cell culture matrix coating is relatively not affected.

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<th>In vitro Models</th>
<th>Advantages</th>
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<td>Monolayer Culture</td>
<td>Simple assay to understand the cell’s response to an external stimulus</td>
<td>a) Single monolayer culture of either fibroblast or keratinocytes cannot provide adequate information on the wound environment/microenvironment13. b) All the cell mediators such as growth factors, cytokines are usually removed during cell culturing and trypsinization which, if discarded without analysis will fail to provide complete mechanistic information in terms of the temporal aspects of the process (mimicked in part by the scratch assays) the efficacy in understanding the cell’s environment through scratch assays.</td>
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<td>Co-Cultured Cell Cultures</td>
<td>a) Monolayers of different cell types are used. b) More insight on Cell-cell interactions not comprehensible/visualizable in experiments performed in monolayer culture systems.</td>
<td>a) Provides an only 2-dimensional approach to the skin healing process (when the process is 3-dimensional) b) Only 2 different types of cells interactions are studied (at a time), however, in vivo condition, multiple cells interact with each other during injury and wound healing process</td>
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<td>(Ex vivo)– Skin</td>
<td>a) Architecture of the tissue construct in 3D (relatively better mimic of cell-cell interactions) b) Subcutaneous layers and fats are usually detached and only the tissue remaining is cultured. c) Compared to the earlier two models, skin explants provide more realistic insights on the biophysical and biochemical properties governing cell interactions, along with pH, nutrient absorption better mimics of the in vivo condition 41,66.</td>
<td>a) Lacks innervation (thereby excluding the possible neurological component’s role in wound healing) - incomplete model for a more comprehensive understanding of skin repair and scar formation14. b) Inter-individual genetic differences in the wound healing response - plays an important role in treating chronic and deep wounds - not considered in this type of an in vitro model</td>
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However, the disadvantage of this assay is the irregular stamping due to the manual nature of the methodology followed for the mold creation. In recent times, this methodological limitation was overcome by the development of specific molds that can form either square or concentric circles or parallel lines on the cell monolayer\(^9\).

**Thermo Stamp Wounding Assay:** This assay is used to study the mechanisms and the outcome of the wounds created through electrocauterization. This process is often used during surgeries to remove harmful or diseased tissue or when the person comes in contact with hot objects resulting in thermo mechanical damage to the skin. In the thermo stamp wounding assay, the temperature-controlled stamps are used to create a mechanical wound by excessive heat applied on a confluent monolayer of cells to analyse the wound healing process. Also, the experimental design can be suitably modified and extended to evaluate the differential effects of thermo-mechanical stamping on cell migration and behaviour in comparison to the conventional mechanical damage caused by rubber or polymer stamping\(^8\). The major disadvantage of this assay is that the lack of adequate temperature control confined to a specific area during the stamping process may cause heat dissipation to occur and affect the nearby cells to create an irregular wounding area.

**High Throughput Migration Assay:** This assay is an extension of the traditional scratch assay, but unlike the conventional protocol for this method, the need to damage the cells by scraping the surface is avoided. The primary cells are seeded onto a 96 well plate with stoppers in the center of each well to create an artificial wound. Cell migration is monitored over time and quantified after the stopper is removed from the well. Using this assay, several parameters can be studied such as original scratch area as well as the average wound width created, wound coverage as a percentage of the total area by using various microscopic images captured during the cell migration.

Several commercially designed high throughput assays of this nature are used currently to study the \textit{in vitro} cell movement such as the OrisTM Pro cell migration assay which uses silicon-based stoppers. These stoppers act as a physical barrier by preventing the cells to adhere to the center of the well, thereby creating an annular monolayer cell formation. The cell migration is captured using confocal microscopy and quantified using fluorescent trackers based on real-time monitoring. Also, the effects of the wounding can be visualized and measured by the post-migration staining of the cells. This method enables the experimenter to precisely study the cell migration and phenotypic changes of the cells\(^38\).

**Gel Contraction Assay:** The gel contraction assay is used to study the extracellular matrix cell-mediated restructuring based on the contractility of collagen matrices as \textit{in vitro} models. In the conventional floating gel contraction assay, dermal fibroblasts are embedded into the disc-shaped collagen gel. Usually, type 1 collagen is used, since it easily polymerizes to a fibrillar meshwork which is necessary to confer key structural and correlatable functional properties to the interstitial connective tissues. Once the freshly polymerized matrix is released from the culture dish, it starts floating on the culture medium. The compressive force acting on the collagen fibers contributes to a reduction in the size of the collagen disc and this contraction occurs without the appearance of stress fibers intracellularly. The magnitude of the contraction of the disc (reduction in diameter or area measurements) indicates effective reorganization based on comparison of suitable reference values\(^10\).

Unlike the floating gel contraction assay, this variant methodology involves the continued attachment of the polymerized gel matrix-laden for the duration of the experiment. As the contraction process progresses, mechanical tension develops resulting in the appearance of cellular stress fibers. This contraction phenomenon is dependent on the mechanical loading and unloading of the fibroblast cells at the onset of the contraction and also on the growth factor used for the initiation of this process.

**ECIS Assay:** Electric cell-substrate impedance sensing wound healing assay is an alternative to the conventional method involving creation of a cell-free zone through a mechanical device and the healing process is monitored. In ECIS assay, electrical wounding is only applied on a smaller population of the cells. This cell population is in contact with the electrical electrode precisely within a diameter of 250 mm diameter.

Glaever et al\(^27\) used the electric impedance for determining cell attachment and spreading across a cell culture dish. In ECIS assay, the cells are grown on the surface of the electrodes embedded at the bottom of a cell culture dish. A small AC (I) is applied across this embedded electrode resulting in a potential (V) across the electrodes. Based on Ohm’s law (\(Z=V/I\)), the impedance (\(Z\)) is then measured by the ECIS instrument by determining the potential created across the electrodes with AC applied. The cells growing on the electrodes act as an insulator in increasing the impedance. Till the cells achieve 100% confluency, the current impeded is directly related to the number of cells, morphology and cell attachment across the ECIS electrodes. In other words, the impedance is directly proportional to the cell’s coverage.

In addition to the cell coverage, change in cell morphology also changes the impedance. ECIS assay is primarily used by recording the time-resolved impedance measurements. ECIS methods have several advantages over conventional 2D assays: there is precision in measurement and wounding, it is less time consuming, it involves automation and real-time data acquisition and is highly reproducible with matrix preservation. Human errors are eliminated through automation thereby making it highly reproducible and
quantitative compared to other assays. Changes of detachment of confluent cell layer, changes in adhesion, morphology or density in response to the impedance as well as heat dissipation affecting the nearby cells are the major disadvantages of the ECIS assay.

**Optical Wounding:** The wound is created by focussing a laser beam on the confluent cell monolayer. Creation of the wound through a laser beam provides an excellent opportunity to study the dynamics of cells migration and response under sterile conditions. This assay is highly reproducible and is amenable to be performed in the HTS format. Laser-enabled analysis and processing equipment (Leap™) used in optical wounding assay were developed by Zordan and colleagues. This equipment enables the users to collect data at regular time intervals through bright-field microscopy and analysis with in-built, customized algorithms for final data analysis.

**In vivo Wound Healing Models:** The most important advantage of in vivo models is that they more closely resemble the commonly encountered injuries found in the patient and hence, are relatively more useful for a more comprehensive understanding of the skin’s pathophysiological features and the subsequent wound healing. This in vivo model studies the endpoints such as extent as well as the temporal aspects pertaining to the healing process and scar formation. The other unique feature of this model is that it enables the experimenter to study the in vivo efficacy of different topical agents or therapies in a better manner, thereby enabling the validation of results obtained from standardized, widely accepted in vitro model systems.

Also, reproducibility of the assay and the results allows for experiments to be conducted in parallel whereby similar wounds can be created and different healing parameters were assessed quantitatively. In addition, validation of in vitro assays based on correlatable results from the aforesaid in vivo experiments obviates the need for the excessive use of laboratory animals. This in vivo model has an inherent disadvantage in that wound healing occurs through the edge and requires contraction to occur. There are several in vivo wound models used to investigate the skin’s complexity and different stages of wound healing as well as those for acute and chronic wounds.

**Full Thickness Wound Model:** The full thickness wound model is the well-known and established technique to investigate the skin tissue’s complexity and wound healing process without the influence of systemic factors. The major advantage of using a full thickness skin model is that it involves all dermal skin components and epithelisation occurs from the wound margins. It also allows analysing the histological organization of numerous connective tissues, angiogenesis; wound contraction, closure. The full thickness wound model can be created by using many devices such as punch biopsies, scalpel, laser or a dermatome to inflict a lesion which allows for the precise control over the size and depth of the wound. The full thickness wound model is precisely used to determine the effect of new ingredients or a formulation on the reparative process.

The topical effects of a growth factor produced by recombinant DNA are re-epithelisation, wound contraction and wound closure with scar formation. Researchers report that interleukin 10, streptolysin O and substitutes for the dermis have been evaluated using this model. In recent times, the full thickness wound model is used in determining the modifying effects of infra-red radiation on the healing process along with the evaluation of numerous new test compounds (bismuth subgallate/borneol) in comparison with certain reference molecules such as bacitracin in the forearm biopsy model. Despite promising results, the lack of standardization and the consequent inter-experimental variability remain the major limitations of this model. This model can be used in pre-clinical research by replacing the animal models.

**Partial Thickness Model:** The partial thickness model is used primarily for the analysis of the epidermis and superficial injury to the dermis. The dermal blood vessels are not damaged in this model. Despite the simple nature of this methodology, it is relatively time consuming.

**Tape Stripping:** One of the simple methods to create a partial thickness wound is by the removing the stratum corneum with an adhesive tape. With only the removal of stratum corneum layers and epidermal layers left intact, it is still sufficient to cause a superficial damage with a minor injury. This model can also be used to study, transepidermal water loss, thereby activating the epidermal layer repair process by a compensatory epidermal proliferation and hyperplasia. Tape strips with adhesiveness need to control for the variability in the pressure exerted during the tape adhesion process onto the skin, velocity as well as the direction of tape removal direction are one of the important factors to be considered during this study. The transepidermal water loss measurement device is used to measure water loss and to study the mechanism(s) of epidermal layer skin damage as well as alterations in its permeability characteristics.

**Blister Model:** The blister model is used for studying the epidermal regeneration process as well as the transepidermal water loss. The wound is created by suction by various types of mechanical devices. Alternatively, chemicals or biological vesicants can be used to produce a split in the basal membrane by removing the epidermal portion with the dermal layer being intact. The suction blister model is most commonly used for producing standardised small epidermal bulges. Once the normal human skin forms blisters after a stipulated time, blister roofs can be removed using a scalpel for the creation of superficial wounds of similar dimensions. The suction blister model is used to analyse the
kinetics of the healing, especially movement, proliferation and differentiation of keratinocytes in acute wound healing and for studying the regeneration of the epidermis in the long-term.\textsuperscript{44,64,89}

In addition to studying the trans-epidermal water loss, it can be used a potential research tool for evaluating the therapeutic potential of agents developed specifically for skin disorders, chronic ulcers and impaired wound healing\textsuperscript{55}. The major disadvantage of this model is that different types of deeper dermal wound types cannot be studied.

**Abrasive Wound Model:** Abrasive wound model is created with an extra wound depth compared to the earlier method discussed with respect to the use of suction for blister creation. In this model, only the epidermal cell layers are removed by inflicting superficial and standardized abrasions with a surgical brush. Upon abrading the skin, punctate bleeding and uniform glistening are observed. The positive aspect of this model is that it mimics a commonly encountered wound type (very close to the real-life condition) and is extensively used as a testing tool for determining the efficacy of topical antimicrobial agents as well as wound dressings\textsuperscript{104}.

**Ex vivo Wound Healing Model:** \textit{Ex vivo} models are considered as a reliable and most functional model for epidermal healing. Such models have an edge over other models by using fresh skin biopsies in culture, since they closely mimic normal skin\textsuperscript{77}. Skin biopsies in culture are considered to be a best source for testing the individual, novel ingredients and new formulations.\textsuperscript{16,61,68,74} \textit{Ex vivo} models have primarily been used for studying the alterations in re-epithelisation as a function of time.\textsuperscript{32,78} The rationale for this approach also stems from the fact that these models have exhibited a comparatively similar gene expression pattern and epithelisation phase as observed in the case of acute human wound healing response.

Apart from the modelling of physiological, pathophysiological aspects of wound healing, pathological skin models using organ culture are used for studying cutaneous wound healing process, keloid scars, hypertrophic scars and stretch marks\textsuperscript{55}. Hodgkinson et al\textsuperscript{36} reported the use of \textit{ex vivo} donut-shaped wound model to investigate human cutaneous repair and effects of photodynamic therapy on skin scars and striae distensae.

Human \textit{ex vivo} models have been employed to study and monitor the temporal aspects of the re-epithelisation process using infrared and confocal spectral Raman imaging analysis. The study demonstrated spectral differences in the images and documented the characteristic spatial distribution of keratin as well as keratinocyte behaviour during epithelisation in the wound healing. The advantage of the study is that the epithelisation process at different time points can be assessed histologically. The other important advantage is the possibility of obtaining skin biopsies from the wound area from a single patient or a donor after ethical approval obtained at the Institutional level.

Inability to model perfusion characteristics seen \textit{in vivo} is a significant disadvantage of this method\textsuperscript{87}. An \textit{ex vivo} model by a wound healing organ culture model (WHOC) was employed to study the therapeutic response of photodynamic therapy (PDT) on cutaneous wound healing repair in human skin. Wound geometry, choice of growth media, certain key cellular and matrix biomarkers were important parameters that were considered in WHOC models. In order to analyse extracellular matrix remodelling, cellular activity and wound repair protein markers levels, the effects of PDT were evaluated using cells that were embedded in collagen and cultured in DMEM medium.

The results provided evidence for PDT increasing re-epithelisation, extra cellular matrix construction and optimal remodelling. This study proved that the \textit{ex vivo} wound healing model as being the best in its class for analysing scars on the skin and anti-stretch marks in humans who exhibit dermal fibrosis as well\textsuperscript{77}.

In another study, \textit{ex vivo} models are also used to evaluate hypertrophic and keloid scars. Keloid explant models are used to analyse the keloid-derived mesenchymal like stem cells (multipotent in nature) and the results of their experiments implicated them as being the ancestors of this skin-related pathology. This type of keloid-specific explant models was used to understand the pathophysiological basis of tissue deterioration, in this skin disorder\textsuperscript{22}.

**In Silico Model:** The complexity, multiplicity of mechanisms and several variables influencing the in wound healing process in the different stages warranted the need to develop cost-effective, relatively rapid \textit{in silico} modelling strategies that can aid in mechanistic research as well as in drug testing. Specifically, the \textit{in silico} computational model approach can be employed to understand complex tissue regeneration, cell growth, repair, migration to collagen deposition, scar formation to angiogenesis and aid in the design of scaffolds and tissue substrates using tissue-realistic computational models. The algorithms used therein a framework for model development and/or refinement and/or upgradation can facilitate the construction recapitulation of wound-healing relevant events in time and space.

It is possible to develop and validate \textit{in silico} models that can aid in the selection of effective scaffold-tissue combination by modelling inter-individual differences in wound healing and regenerative processes. Refinements of such models can help in improving their predictability in selecting the right model system and the appropriate experimental design to make significant reductions in the costs incurred.

In the past, \textit{in silico} models were primarily used to study the evolution of inflammation over time in the wound healing response based on histo-pathological findings. Due to
complexities in the etiology as well as the inherent challenges in modelling blood flow characteristics in the different aberrant wound healing-based pathologies, there is a need for realistic, agent-based, multiphase, mechanistic, hybrid-based computational models and multi scale simulation approaches of wound healing. In silico models may offer a non-invasive approach between animal and human subject-based. This approach can enhance the success rates of a clinical trial by aiding in the selection of an appropriate animal model as well as the right experimental design for providing significant pre-clinical data. This data will provide solid evidence for obtaining regulatory clearance for the conducting the clinical trial.

**Differential Equation based In silico Model:** The differential equation-based approach is the most standard, classical method that is employed in studying and modelling the complex wound healing process at every phase. It is used in investigating the effects of commercially developed skin substitutes, interpatient variability, vacuum assisted wound closure (VACs) and hyperbaric oxygen therapies for diabetic foot ulcers. There are currently two equation-based models centred on ordinary differential as well as the partial differential equations. The ordinary differential equation-based models (ODE) are a basic wound healing approach to formulate strategizes to capture the sequential events in the time-dependent wound closure. This is done by an equation considering the ratio of the wound area to the perimeter as a function of time along with fitting the constants in the equation to the observed data.

DE models are mostly linear or exponential-based functions involving two parameters and lack in detailing the initial delay in the wound healing process. ODE models involving four parameters were designed by Cukjati et al. and assessed their significance in 226 chronic wounds by analysing the models both qualitatively and quantitatively using five sets of criteria. Their studies concluded that the three parameter-delayed exponential models are the apt for the wound healing process.

The mechanistic ODE models are usually designed to represent the densities of cell populations such as fibroblasts and macrophages, concentration of inflammatory mediators, other growth factors /cytokines namely platelet derived growth factor (PDGF), hyaluronan and collagen. It is also used in as a surrogate to recapitulate the in vivo tissue re-modelling with extra cellular matrix components (ECM) being represented as a vector field with this variable pointed along the direction of collagen deposition. These vectors point along the density of ECM at each point influenced by the direction of fibroblast growth and direction of new collagen deposition.

**Partial Differential Equation (PDE) based In silico Model:** It is used primarily in predicting the shape of the wound and focuses on the final stages of wound healing process, especially involving the epithelial layer repair and scar tissue remodelling. The major difference in PDE with ODE model is wound healing process and its time dependence with spatial variability. PDE models are further classified into four different models such as reaction-diffusion model, continuum mechanical model, cell signalling, angiogenesis and chemotaxis model. Each model is used in studying various cell types, cell migration, cell proliferation, wound contraction, new capillary formation and chemical migration.

**Reaction-Diffusion Model:** It is a simplest PDE model constructed for concentration of the cell and wound closure with a single equation. The wound closure is interpreted as a cells moving in the form of a wave. Fisher–KPP equation is a reaction–diffusion equation with a linear diffusion term to describe motility of the cell and a logistic term that can be used to describe its proliferation behaviour. The Reaction–diffusion model focuses on the reparative process in the epithelial layer which primarily involves epithelial cell migration as well as its proliferation into the wound.

**Continuum Mechanical Model:** The continuum mechanical model is largely used to study the third stage of wound healing wherein there is a synchronized movement of epithelial cells, thereby covering the wound without any hole’s formation in the wound sheet. The single epithelial cell migration is studied extensively with cells moving in a cyclical fashion involving lamellipodium formation, nucleus translocation in the direction and detachment of the trailing edge. The process is regulated by complex cell signalling and networking.

In contrast to the widespread single cell migration studies done, there is a lack of understanding of the mechanisms associated with the synchronized collective migration of cells in wound closure. Numerous continuum mechanical wound closure models such as leader cell steered migration differential adhesion hypothesis and cooperative traction force mechanisms have been used to study the cell migration, proliferation.

**Cell Signalling Model:** Cell signalling model is used to study both the mechanical as well as the biochemical factors governing cell migration processes upon injury as well as their migration speed and direction. The cell signalling model is also used to study the re-modelling of scar tissue including collagen deposition, fibre orientation and wound contraction.

**Angiogenesis Model:** It is used in studying the process of angiogenesis in the growing tissue in both wound healing as well as in tumour growth. The model is used extensively to study the capillary ingrowth process which is essential in wound healing. This helps in the maintenance of adequate blood supply for sustaining the high metabolic activity levels perfuse required for the optimal modelling of the processes involved in the restoration of the integrity of the wound. The angiogenesis model developed by Pettet et al. during wound
healing focused on capillary tips, fibroblasts, macrophage derived chemical attractants, oxygen and ECM. The model distinguished the differences in the process of angiogenesis in wound healing and tumour growth by demonstrating the dependence of macrophage activity on variations in the local oxygen concentrations.

**Chemotaxis Model:** Chemical gradients that dictate migration of the cells play an important role in various processes including cell migration as an inflammatory response, tumour growth, wound healing, new vessel formation as well as embryonic development. The PDE model designed to study the release of growth factors (e.g., VGEF for the growth of the vasculature) by inflammatory cells by Schugart et al.\textsuperscript{81} is a classic chemotaxis model with a circular wound considered in the theoretical study with the growth factor-induced fibroblast-mediated collagen production along with the other ECM components.

The results of this model suggested two important aspects of wound healing factors contributing to a delay in wound closure. The model outlined the fact that the hypoxic microenvironment can never sustain vascular growth and will delay the entire wound healing process unlike hyperoxia which helps in quicker angiogenesis and healing.

**Agent based in silico Model:** Agent based in silico models focus on three vital processes such as cell-cell adhesion, diffusion and proliferation. Several agent-based in silico models have been studied extensively such as cell adhesion speed as being dependent on adhesion receptor/ligand binding\textsuperscript{19}. The model predicted different cell behaviour which varied in accordance with their bonding strength and rate of proliferation. In another model, cell migrations and their rate of division were less in crowded areas, implying the importance of cell density. Agent-based in silico model describes the role of growth factors, organisation of keratinocytes, presence of fibroblasts and the long-term skin epithelial regeneration by stem cells. In addition, in assessing the cell migration patterns and cellular stimulus, the model is also used to analyse debridement of the wound and the subsequent topical administration of growth factors

**Deterministic Computational in silico Model:** Wound morphology changes have been studied using the deterministic computational approach. The level set method is used to study the wound boundaries and finite element methods are used for analysing wound contraction in the two-dimensional wound models.

**Mechanical in silico Model:** Mechanical in silico models were designed using finite element methods which simulated the skin incision, excision and closure. The finite element method based mechanical models are usually patient-specific and are shown to provide critical information with respect to the optimal excision shape that can possibly reduce scarring. It is currently used in analysing VAC therapy.

**Multiscale Hybrid in silico Model:** This multiscale hybrid approach is the current need to design a mathematical model which can represent various scales (in space) from the cellular to the organ levels. The holistic multiscale multifield continuum models with high resolution system are currently used for modelling the dynamics of hypertrophic scar tissue after wound healing. The major disadvantages of in silico models reported are that they lack biophysical aspects of human skin and are theoretical units proven in either in vitro or in vivo models.\textsuperscript{50,94}

**Animal Models:** Animal models provide an excellent platform and tool for studying wound healing mechanisms and also for testing various new therapeutic agents. Even though animal skin does not resemble human skin, various animals have been used to study the healing process in terms of its complexities.\textsuperscript{75,93} Amongst the various animals, rodents remain the model of choice for wound healing studies even with skin morphology completely being different from humans with thin epidermis, absence of apocrine and eccrine glands like humans, loose skin adherence, dense hair. Most importantly, vitamin C is produced endogenously in rodents compared to humans, which plays an important role in the different phases of wound healing.\textsuperscript{20,30,82}

Amidst the difference in the skin morphology and complexity, rodents remain the best animal model due to low cost, easy availability, reproducibility in comparison with other animals such as rabbits and porcine animal models. Rabbit and porcine animal models have their advantages but due to their high breeding cost and difficulties in conducting large scale experimental studies, these animal models are not considered an option for wound healing experiments and as models for pharmacological testing’s prior to the initiation of human trials.\textsuperscript{31,82}

**Excision Wound Model:** It is a commonly used wound model. The circular wounds are made which are about 2 cm on the dorsal side of the animal under aseptic conditions. The area of the created wound is measured immediately to denote the initial wound area reading. The alterations in wound area and wound closure percentage are calculated on alternate post wounding days to study the efficacy of the drugs being used to study the wound healing mechanism.\textsuperscript{40,69,96} This type of model, especially using rodents for studying acute wound healing, is considered less efficient.

Further, the wound healing mechanism in terms of the closure of such wounds is different in rodents compared to that seen in humans with contractions of the muscle in the former in contrast to the re-epithelialisation process in the latter case. The change in the conventional excision wound model was made to overcome the problems in the difference in these mechanisms in rodents compared to humans by creating a splinted version of the injury. This splinted excision model assessed the acute wound healing process as...
the blood vessel remained intact during the experiment, thereby aiding studies involving assessing the role of the vasculature in the wound healing process.

**Incision Wound Model:** The wound is created after giving anaesthesia to the animal. The incision wound is made parallel to the midline on the dorsal side with an incision length of 6 cm passing through the epidermis, dermis and subcutaneous tissue down to the muscle. Incision wound models are the second most commonly used models especially for scar formation studies.

**Burn Wound Model:** Burn models are established after anesthetizing the animal by pouring hot molten wax at 80°C into a cylinder with 300 mm² circular openings placed on the dorsal side. The cylinder is removed after the solidification of the wax leaving the circular burn model with the partial-thickness. Other methods used to create the burn wounds are by using steam to create superficial or deep full-thickness burns. Although these models are extensively studied in rats and mice, the skin morphology of the rodents leaves this model with a major disadvantage because of rapid healing due to subcutaneous tissue contraction, thinner skin structure and denser hair structure compared to that observed in humans.

**Dead Space Wound Model:** The models in rodents are usually created by implanting a polypropylene tube with a length of 2.5 cm and diameter of 0.3 cm below the dorsal paravertebral lumbar skin. Dead space wound models are primarily used for studying the physical changes in the granulation tissue which helps to understand the wound environment due to the increases in the interstitial fluid during the wound healing process.

**Infected Model:** Infected models are established by making an incisional/excisional wound followed by the inoculation of microorganisms or foreign bodies. The infected models are used extensively in studying the antibacterial effect of new drugs. The presence of biofilm on the wound mirrors the local milieu around the injured site with numerous bacterial species. The infected model is very beneficial in understanding chronic wound healing in diabetes due to the infection commonly observed in late diabetic wound.

**Tape stripping Model:** Tape stripping models are primarily developed only in rodent using an adhesive tape on the stratum corneum (dorsal side) to disintegrate the skin barrier by successive stripping of the epidermis. The trans-epidermal water loss (TEWL) is measured using an evaporimeter for the disintegration. Tape stripping models are used to study skin barrier functions by evaluating the re-epithelisation process. The effectiveness of adhesive wound dressing on the skin barrier is also studied. The model is considered as being very simple wound with moderate pain to the animal. The disadvantage of this model is that it specifically addresses only superficial wounds.

**Skin aging Model:** Skin aging models are developed only in rodents by exposing mice to ultraviolet rays for 12 weeks with wavelengths ranging from 290-320 to induce aging to the skin. This model system in mice is used mostly for anti-aging drug testing. The major disadvantage of the model is the skin morphology of rodents in comparison to humans. The other drawbacks are the high mortality rate due to continuous exposure to radiation.

**Xeno-grafts:** This model is prepared by placing a full-thickness human xeno-graft on the full-thickness wound created on the nude mice (dorsal side). The dorsal full thickness of the mice is removed to place the human xeno-graft. Xeno-grafts can be used for studying hypertrophic scars and human keloids. It can also be used for testing the drug penetration characteristics of potential therapeutic drugs in pre-clinical studies before proceeding for human trials.

**Partial Thickness Excision Wound Model:** It is used to study the local wound environmental factors and efficacy of topical agents. Partial-thickness excision wounds are created either with the handheld or electrical dermatome. This model can be used in rodents as well as in humans. Since the partial thickness wound is created, the surface area of the injured site can be calculated precisely. Collagen development and matrix measurement cannot be studied, since only a partial thickness wound is created.

**The Hairless Mouse Model:** The hairless mouse models have been used in various studies: modelling burn as well as reperfusion injuries. The full-thickness wound is created on the dorsal side of the ears. One of the advantages of this model is that it allows studying the efficiency of diverse topical agents on epithelisation and vascularization.

**Radiation-induced Wound Model:** Radiation of 20 Gy is administered followed by a punch biopsy resulting in ulcer formation. This model is used extensively for studying skin ulcers formed due to cancers. The high mortality rate of the rodents is the disadvantage of this model in larger experimental studies.

**Ischemic Skin Wound Model:** Couples of full-thickness excisional wounds are created on the dorsal skin flap. Panniculus carnosus muscle is removed from the wound bed. The flap has a narrower dimension ensuring that the wound located at the centre of the flap is ischemic. A silicone sheet is placed underneath the flap to prevent wound contraction and revascularization. This model is used recently in studies such as the effects of hyperbaric oxygen on ischemic wound healings to ischemic wound healing.

**Skinfold chamber Model:** The skin folder chamber models with the use of dorsal skin sandwiched between two complementary plates, have been used extensively to...
understand the micro vascularization over a period of time. They are also used to study wound healing, vascularization of transplants and compatibility of biomaterial implants. Focusing on a smaller and lighter dorsal skinfold chamber will reduce the discomfort of the animals and will help us to understand the micro vascularization circulation through microscopic and real-time imaging.

Biofilm – Infected Wound Model: Biofilm-Infected wound models are usually used to study chronic wounds wherein the rate and extent of the reparative process are limited due to the colonization of infectious microbes and their creation of a pro-inflammatory cytokine milieu. In this model, after a wound is created, a suspension of either Pseudomonas aerugionsa or Streptococcus aureus is applied on the wound surface. Occlusive dressings are made, so there is no cross-contamination to provide a conducive environment for bacterial growth. Rabbit’s ear is generally used for studying the effectiveness of wound care regimens in the presence of P. aerugionsa biofilms.

![Wound Healing Models Diagram]

Figure 1: Diagram summarizing the five main categories of Wound Healing Models

<table>
<thead>
<tr>
<th>TYPES</th>
<th>IN VITRO</th>
<th>IN VIVO</th>
<th>EX VIVO</th>
<th>IN SILICO</th>
<th>ANIMAL MODEL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. Co-culture 2. Pathological Skin 2. Agent Based 2. Excision</td>
<td></td>
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<td></td>
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<td></td>
<td>3. Organotypic 3. Human Skin Bioequivalent 3. Hybrid</td>
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<td>1. Patients 4. Computational</td>
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<td>2. Volunteers 5. Deterministic</td>
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<td>1. Incision</td>
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<td></td>
<td></td>
<td></td>
<td>2. Excision</td>
</tr>
<tr>
<td>Wound Healing Assays</td>
<td>1. Migration 1. Proliferation 1. Epidermal healing</td>
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</tr>
<tr>
<td></td>
<td>2. Invasion 2. Angiogenesis 2. Contraction</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3. Proliferation 3. Apoptotic 3. Dermal repair</td>
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<td></td>
<td></td>
<td>1. Invasive 5. Fibrotic</td>
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<td></td>
<td></td>
<td>2. Non-Invasive</td>
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<td>N/A</td>
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<td></td>
<td>1. Proliferation</td>
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<td>2. Angiogenesis</td>
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<td>3. Epidermal healing</td>
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<td>4. Contraction</td>
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<td>5. Dermal repair</td>
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<td>6. Angiogenesis</td>
</tr>
</tbody>
</table>
Table 3
Certain Recently Established Wound Healing Models and their significance in Wound Healing Approach

<table>
<thead>
<tr>
<th>S.N</th>
<th>Type of Wound being Modelled</th>
<th>Name of the Model</th>
<th>Salient Features</th>
<th>Year of Establishment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Re- Epithelialization</td>
<td>Human Ex-vivo Skin Model</td>
<td>Wound created in centre of the skin (that was discarded) and treated with collagen, polyethylene glycol (PEG) with platelet free plasma (PFP) and fibrin. Epithelialization was observed faster in the explants (abdominal skin with underlying adipose tissue) treated with hydrogel PEG-PFP compared to other groups such as collagen and PEG-fibrin alone at 6 days to 14 days. The Human ex vivo model established can be used to promote wound closure and potentially used as a screening tool to replace animal testing (^{15}).</td>
<td>2018</td>
</tr>
<tr>
<td>2.</td>
<td>Re-Epithelialization and Homeostasis</td>
<td>3D- In vitro Model through Keratinocytes migration</td>
<td>3D epidermal migration model in a keratinocyte migration situation to study vertical skin regeneration plus the re-epithelialization as observed in 2(^{nd}) phase of wound healing precisely study the 3D behaviour of keratinocytes in a regeneration process. The 3D migration model will help to understand the keratinocytes migration under different biochemical environments plus also further will help to elucidate efficacy of new compound screening to study mechanisms involved in skin regeneration process (^{65}).</td>
<td>2017</td>
</tr>
<tr>
<td>3.</td>
<td>Re-Epithelialization</td>
<td>Co-culture and In vitro Model through Fibrin provisional matrix</td>
<td>Fibrin provisional matrix containing factor VIII, fibronectin, thrombin, macrophages as bio-ink was injected into the wounded bio printed human skin equivalent. The fibrin provisional matrix ensured the migration of keratinocytes in the in-vitro tissue culture resulting in wound closure through re-epithelialization signalled by fibrin provisional matrix. In-vitro wound closure was observed after the migration of keratinocytes over fibrin provisional matrix at 3 days (^{72}).</td>
<td>2020</td>
</tr>
<tr>
<td>4.</td>
<td>Haemostasis</td>
<td>In vitro bleeding model -using microfluidics recapitulating in vivo mechanical injury of the microvasculature</td>
<td>To study how biophysical and biochemical variables in the wound environment behave and assist in haemostasis. The microsystem in combination with fluorescent dyes can be used to study and measure entire haemostatic response (measured quantitatively using microscopy (^{59}).</td>
<td>2018</td>
</tr>
<tr>
<td>5.</td>
<td>Chronic Wound Closure and Remodelling</td>
<td>In silico Logistic regression; classification tree model to forecast the probability of wound healing within 12 weeks</td>
<td>The model was created and established by validating the medical records of over 620356 chronic wounds and the probability prediction using random samples of 70 % of the wounds. Using the model, 59 % of the wounds healed within 12 weeks. The model predicted the healing with an area under the stipulated curve. The model can be used as a quality measure, performance, based treatments with reasonable accuracy (^{88}).</td>
<td>2020</td>
</tr>
</tbody>
</table>

**Mouse-tail full-thickness Model:** It is primarily used for studying delayed wound healing. The wound is created by making a rectangular full-thickness excision on the dorsum of the tail. With the minimum contraction of the tail, this model is used in studying the delayed wound healing process for up to 21 days. The main drawback is the difference in the anatomical structure of rodent and human skin.

**Conclusion**
A comprehensive review on the wound healing models...
including non-animal models such as in vitro, in vivo, ex vivo, in silico, animal model available and currently practiced, is presented. The choice of an appropriate wound healing model and assay is extremely important in wound healing research to determine the outcome in understanding the wound healing cascades, growth factor’s role and various mechanisms involved in healing and scar formation [Table 2]. The thorough understanding of the appropriate wound model will aid in evaluating the pathophysiology of the wounds, better strategies in the treatment modes, formulations for enhanced healing of the wound and will limit abnormal formation of the scar. The understanding of acute and chronic wounds healing mechanism has significantly increased by adopting these models along with the advent of new wound healing products, wound healing developments and advanced wound care.

The numbers of parameters like equipment available, number of experiments to be carried out and data analysis tools are to be considered before designing an appropriate wound model. The recent developments in the image analysis with dataset tools have enabled studies involving large datasets containing micrographs limiting the variability in analysing the images. The reproducibility of the results along with the automation is one of the major developments in the recent times which enabled studies involving various parameters in detail. It has considerably deepened our understanding of the wound healing mechanism. This improved understanding can become an important support tool in possibly devising treatment strategies through the testing of various substances that aid or help in wound healing [Table 3] in the high throughput mode.

In vitro models provide the best quantitative examination of a physiological process with prime focus on specific cell types. The model is very useful in studying a role of specific cell type including its communication pattern, cell signalling cascades and its pathophysiological changes during a wound healing process. Despite the obvious limitations, the in vitro models have been in use for studying healing and scar formations for many decades enabling them to be a go model for wound healing studies.

The conventional use of 2D models as a primary model can be used to understand the wound therapeutic process. Once promising results are obtained, 3D models could be used to deepen our understanding of the healing process, as it is cost effective and more importantly can replace the use of animal models of wound healing, since they are better surrogates in terms of recapitulating important events in the wound healing process. However, the disadvantage of the in vitro model is the difficulty in correlating the results of the in vitro findings to the in vivo condition and circumvent the pitfalls to bridge the gap and better simulation wounds normally encountered in the real-life scenario.

Human in vivo models offer a quite unique feature as the model is very identical to the natural wound healing process. They include model incision, excision, burn injuries and those that attempt to replicate the dead space. They are the presently used models for evaluating the effectiveness or healing potential of drugs/biopharmaceuticals by estimating certain key variables like tensile strength, contraction area and index of the wound, epithelization of the cells and the estimation of collagen formation (provided appropriate ethical clearance has been obtained).

In recent times, wound debridement, pressure therapy, use of growth factors and topical applications (with certain nanoparticles) are some of the advancements made using the in vivo model of wound healing. The problems associated with the lack of standardization, reproducibility, evaluation and design conformity remains the major disadvantages of the in vivo models. The other concern is the limitations in human cell culture models, the number of cell types and diverse communications cascades involved in the wound healing process. These challenges require harmonization of protocols and the inter-laboratory validation of assays as well as considerable technical expertise.

Ex vivo models are used presently to study epithelization, extracellular matrix reconstruction, as well as remodeling. They also offer a unique feature to study the specific effect on cells and its surrounding tissue in a shorter time frame. The standardization and design conformity are the limitations of this model.

In silico models offer a non-invasive approach that is intermediate between animal and human models. It is currently used as a tool to screen, identify and determine the effects of a drug or a formulation, thereby helping in reducing time and other valuable resources spent in selecting the appropriate model for validating the in silico findings pertaining to selection of a drug candidate. This approach, in turn, will aid in selecting the best drug/formulation or safety and efficacy studies as part of a clinical trial. The model is theoretical. Using ordinary differential equations, many computational and mathematical models have been used to improve the elementary approach in understanding the wound healing process. With more models being validated and experimented, it can reduce the overall cost, time and invasive testing if needed. The standardization like other two models remain the challenge though.

Animal models have been used for decades in studying the multifarious cellular, biochemical process of wound repair and in evaluating the safety and efficacy of new therapeutic agents. They still provide necessary information that can be related with human wound healing especially those that are chronic in nature. Rodents are the conventional model systems chosen for wound healing studies due to their cost-effectiveness, easy of handling and better reproducibility of the results. Differences in skin morphology, lack of apocrine and eccrine glands unlike in humans, loose skin adherence, dense hair are the limitations of the animal models.
In conclusion, the model chosen for the wound healing research should be as close as possible to that which mimics the regenerative/reparative processes in human skin including those at the cellular and molecular levels. This perforce requires us to dissect and duplicate the homeostatic responses associated with the different stages of acute wound healing as well as those that occur after injury. In addition, we need to more accurately model the pathophysiological as well as pathological states associated with chronic wound healing for precise evaluation of the current and future generation of wound healing products.

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