Vol 4|Issue 2| 2014 | 127-131.

e-ISSN 2248 - 9169
Print ISSN 2248 - 9150

International Journal of
Experimental Pharmacology

www.ijepjournal.com

AN OVERVIEW OF THE CURRENT METHODOLOGIES USED FOR THE EVALUATION OF DRUGS HAVING WOUND HEALING ACTIVITY

M. Subalakshmi*, A. Saranya, M. Uma Maheswari, A. Jarina, S. Kavimani, R. Murali

Mother Theresa Post Graduate and Research Institute of Health Sciences, Puducherry, India.

ABSTRACT

Discoveries in the past two decades have continued to improve our understanding of the pathophysiology of wound healing and animal models have played a significant role to define the basic mechanisms of treatment of wound healing. Both *In vitro* and *In vivo* models have been developed in the past years to study the agents having wound healing activity. *In vitro* models are useful, quick, and relatively inexpensive. *In vitro* models includes scratch assay, chorio allantoic membrane assay, fibroblast assay, keratinocytes assay, electrical healing assay, collagen assay, MPO assay, hyaluronidase inhibition assay, collagenase inhibition assay and elastase inhibition assay. *In-vivo* small animals provide a multitude of model choices for various human wound conditions. *In vivo* models include incision models, excision models, dead space models, burn models. Wound healing property measurement can be categorized into physical attributes like wound contraction, epithelization and scar remodelling which can be monitored by measuring the total wound area, open wound area and noting the physical changes in scar e.g. size, shape and colour etc, mechanical attributes like tensile strength, biochemical attributes like estimation of hydroxyproline, hexosamine and hexuronic acid and histopathological attributes. This review aims to highlight some of the new and currently used experimental models that are used for the evaluation of wound healing agents.

Keywords: Wound healing, invitro, invivo, evaluation parameter, collagen.

INTRODUCTION

Wound healing agent is defined as the drug or any agent which heals the wound. Wound is defined as the disruption of the cellular and anatomic continuity of a tissue [1]. According to the Wound Healing Society, wounds are physical injuries that result in an opening or break of the skin that cause disturbance in the normal skin anatomy and function. They result in the loss of continuity of epithelium with or without the loss of underlying connective tissue [2]. Wound healing is a complex series of interrelated events that are mediated through the phases by a wide range of chemically coordinate cellular processes as well as hormonal influences [3].

Corresponding Author

M. Subalakshmi
Email id: subabpharm@gmail.com

Current estimates indicate the worldwide nearly 6 million people suffer from chronic wounds. Wounds are classified as open and closed wound on the underlying cause of wound creation and acute and chronic wounds on the basis of physiology of wound healing [4].

Both *In vitro* and *in vivo* models have been developed in the past years to study the agents having wound healing activity. The present review is an attempt to update the information on the wound healing animal models.

In Vivo Studies

The small mammals have emerged as the model of choice for researchers, which are beneficial for multiple reasons. They are inexpensive, easily obtainable; require less space, food, water & easy to maintain. Additionally, they often have multiple offspring, which develop quickly allowing experiments to proceed through multiple

generations [5]. Following types of wounds are made in laboratory animals for studying the effect of various drugs.

Excision Wound Model

Excision wound model was used for the study of rate of contraction of wound and epithelization. These types of wounds are prepared either on rats or guinea pigs. The animals were weighed individually, anaesthetized with pentobarbitone sodium (35 mg/kg, intraperitoneal). The rats were inflicted with excision wounds as described by Morton and Malone et al, 1972. The skin of the dorso lateral flank area was shaved with an electrical clipper. After wound area preparation with 70% alcohol, the skin from the predetermined shaved area was excised to its full thickness to obtain a wound area of about 500 mm. Excision wounds are inflicted on the dorsal thoracic region 1–1.5 cm away from the vertebral column on either side and 5 cm away from the ear [6]. After wound area preparation with 70% alcohol, using a sterile round seal of 2.5 cm diameter or a surgical blade or 5–8mm biopsy punch, the circular skin from the predetermined area on the depilated back of the animal is excised to its full thickness to obtain a wound area of about 200-500 mm^2 diameter and 2 mm depth.

Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open. The respective creams were topically applied on the wound area of the animals of respective groups once a day till complete epithelization; starting from the day of operation. Collagen estimation, percentage wound contraction, and period of epithelialization parameters are studied. The formulation was applied until complete wound healing.

Incision Wound Model

After wound area preparation with 70% alcohol, two longitudinal paravertebral incisions are made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on either depilated side of the vertebral column with a sterile sharp surgical blade. Each incision made is 4–6 cm in length, and after complete haemostasis, the parted skin is stitched with interrupted sutures, 0.5–1.0 cm apart using black braided silk surgical thread (no.000) and a curved needle (no. 11). The continuous threads on both wound edges are tightened for good closure of the wound. The wounds were left undressed and mopped with a cotton swab. The respective therapeutic treatment is administered either orally or topically to the animals of respective groups until 7th– 9th day starting from the day of operation. The sutures were removed on 7th day, and the skin breaking strength of the healed wound is measured on 8th–10th day. The tensile strengths, biochemical and histological study of the wound are carried out [7].

Dead Space Wound Model

In this model, the physical and mechanical changes in the granuloma tissue are studied. The subcutaneous dead space wounds are inflicted one on either side of axilla and groin on the ventral surface of each animal, by making a pouch through a small nick in the skin. The cylindrical grass piths (2.5 × 0.3 cm) or sterile cotton pellets (5–10 mg each) are introduced into the pouch. Each animal received 2 grass piths/cotton pellets in different locations [8].

The dead space wound is created by subcutaneous implantation of a sterilized, shallow, metallic ring (2.5 × 0.3 cm) known as the cylindrical pith or polypropylene tube (2.5 ×0.5 cm) on each side beneath the dorsal paravertebral lumbar skin surface and wounds are sutured [9]. The respective therapeutic treatment is administered either orally or topically to the animals of respective groups for 10 consecutive days [10].

Burn Wound Model

These wounds are also measured for the contraction. A special metal plate 2×2 cm with holder is heated to 60°C and applied to the dorsal area of the animals for 30 secs to induce partial thickness burn wound. Second-degree burns wound can be made by placing the 90°C hot plate on the selected dorsal area of the animal for 10 secs. While for full thickness burn wound, the metal plate is heated to 100°C and applied to the dorsal area for 30 secs. The animal can also be subjected to rectangular burn wounds (20 × 25mm^2) using hot (180°C) brass brick weighing 300 gm, which is pressed against the shaved skin for 10 secs in the treatment group [11].

A cylindrical metal rod (10mm diameter) is heated over the open flame for 30 secs and pressed to the shaved and disinfected surface for 20 secs on selected dorsal area of animal under light anesthesia [12] whereas a partial thickness burns wounds can also be inflicted upon animals starved overnight and under mild anaesthesia, by pouring hot molten wax at 80°C into a metal cylinder with 100–300mm^2 circular opening, placed on the back of the animal. On solidification of wax after 8–10 min the metal cylinder with wax adhered to the skin is removed, which left distinctly marked circular burn wound [13].

Animals are placed in individual cages after recovery from anaesthesia. The respective therapeutic treatment is administered either orally or topically to the animals of respective groups until the day of scab falling starting from the day of operation. The parameters studied are percentage wound contraction, hydroxyproline content and epithelialisation time.

The following parameters should be evaluated for *in vivo* evaluation of wound healing activity

Measurement of wound area

The progressive changes in wound area were monitored by a camera on predetermined days i.e., 2, 4, 8,

12, 16 and 20. Later on, wound area was measured by tracing the wound on a millimeter scale graph paper [14].

Measurement of wound contraction

Wound contraction, which contributes to wound closure and restoration of the functional barrier. Contractions, which contribute to wound closure, were studied on alternate days from Day 1 to Day 9, i.e. starting from the day of operation till the day of complete epithelization by tracing the raw wound on a transparent sheet. Wound contraction was calculated as percentage of the reduction in original wound area size [15].

Determination of Period of epithelisation

Falling of scab leaving no raw wound behind was taken as end point of complete epithelization and the days required for this was taken as period of epithelisation [16].

Tensile strength

Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of the repaired tissue. For this purpose the newly repaired tissue including scar was excised to measure the tensile strength. The instrument used for measurement is called a tensiometer, which was designed according to the method [17]. For the quantitation one of the edges of the wound was fixed while applying a measurable force to the other one. The load (weight) in grams required to disrupt the wound is determined after complete healing of the wound, and that was on day 20 after surgery. Tensiometer measures the breaking strength in N (Newton).

Measurement of wound index

Wound index was measured daily with an arbitrary scoring system i.e. ‘’0’’ for complete healing, 1 for incomplete but healthy healing, 2 for delayed but healthy healing, 3 for healing has not yet been started but environment is healthy, 4 for formation of pus evidence of necrosis [18].

Estimation of collagen (Hydroxy proline), Hexosamine, Hexuronic acid (HUA)

Hydroxyproline is a basic constituent of collagen. The collagen composed of amino acid (hydroxyproline) is the major component of extra-cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline hence can be used as a biochemical marker for tissue collagen and an index for collagen turnover. Hexosamine and hexuronic acid levels will decrease as collagen accumulates. This factor is therefore used to deduce the increase in collagen concentrations in the tissue [19].

Granuloma Studies

The day of the wound creation is considered as day zero. Granulation tissue forms on the dead space wound surrounding the implanted pellets/piths is harvested by careful dissection on the 10th post wounding day under light ether anaesthesia. After noting the wet weight of the piece of granuloma excised, it is dried in an oven at 60°C for 12–24 h to obtain a constant dry weight expressed as mg/100 g body weight [20].

Estimation of protein and DNA Content

The protein and DNA content of granulation tissue indicates the levels of protein synthesis and cellular proliferation. Higher protein and DNA content will indicate cellular proliferation and suggest an increase in the synthesis of collagen. According to Chithra. P et al, 1998 the collagen/DNA ratio in granulation tissue may be taken as the index of the synthesis of collagen per cell in the wound area [19].

***In vitro* Studies**

These *In vitro* assays are great for examining the effect of agents on particular cell types. *In vitro* can be useful, since they are quick, relatively inexpensive, and can be used to screen a wide variety of conditions or samples simultaneously but are incapable of replicating all the factors involved in complex processes of wound healing. In-vitro assays are useful in wound healing research for determining the possible effectiveness of various treatments, particularly antimicrobial and healing enhancing agents. Another noteworthy attribute of in-vitro testing is the ability to screen multiple agents or samples simultaneously.

Chick chorioallantoic membrane (CAM) Assay

Angiogenesis plays an important role in wound healing and newly formed blood vessels comprise 60% of the repair tissue. Neovascularization helps hypoxic wounds to attain the normoxic conditions. The wound healing agent should produce angiogenesis and promotes new vessel formation in CAM model. The increased vessel growth can facilitate both the extent and direction of fibroplasia. Improved angiogenesis, therefore, would be contributing significantly to wound healing activity of the test drug [21].

Fibroblast Assay

Fibroblasts can be grown in culture and their proliferation assessed by determination of the total protein of viable cells present by staining with an appropriate dye such as sulfo-rhodamine B (SRB) or Neutral Red or by metabolism of a coloured substance such as MTT. Fibroblasts secrete collagens and subsequently effect the remodelling of the granulation tissue into mature dermis. They also secrete growth factors, such as KGF-1, PDGF and TGF-B that stimulate proliferation/

differentiation/migration of other cells involved in the triggered into the wound area, forming the final barrier on top of the new connective tissue formed by the fibroblasts. This means that a high proliferative keratinocyte cell layer is spreading from the edges towards the wound area, finally leading to a closed cover-layer [22].

Keratinocytes Assay

While fibroblasts produce the initial cell mass in the first stages of wound-healing, keratinocytes take over cell production at a certain stage, leading first to the development of more or less undifferentiated cells which differentiate over the life time to highly complex barrier cells, a process which is accompanied by cell apoptosis in the final stages. Finally wound healing is successfully finished in that moment were after fibroblast formation the development of keratinocyte proliferation and differentiation has started, leading in the final stages to the occurrence of the typical epidermal barrier layers Stratum spinosum, S. Granulosum and S. corneum.

Cell culture models for studying keratinocytes physiology are cell lines (especially HaCaT, a non-tumorigenic, spontaneously immortalized cell line) or primary cells obtained from skin resectates. Proliferation of keratinocytes was enhanced by a potent wound healing agent [22].

Scratch Assay

The *In vitro* scratch assay is a straightforward and economical method to study cell migration *In vitro*. This method is based on the observation that, upon creation of a new artificial gap, so called ‘‘scratch’’, on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the ‘‘scratch’’ until new cell–cell contacts are established again. The basic steps involve creation of a ‘‘scratch’’ on monolayer cells, capture of images at the beginning and regular intervals during cell migration to close the scratch, and comparison of the images to determine the rate of cell migration [23].

Collagen lattice formation

The final stage of the wound healing process involves wound contraction and tissue remodelling. In some wounds, exaggerated contraction causes unsightly scars which, as well as having an adverse psychological effect, might also impair physical movement. An agent that slows down the contraction process might therefore lead to reduction in scarring and would be viewed as beneficial [22].

Electrical healing assay

In this approach, cells growing on small electrodes and monitored by using electric cell-substrate impedance sensing are subjected to currents, resulting in severe wound healing process. Especially keratinocytes can be electroporation and subsequent cell death. After this invasive treatment, the electrode’s impedance is again monitored to chart the migration and ultimate healing of the wound [24].

Collagen Assay

The homogenate of wound tissue can be used to measure the total acid-soluble collagen (types I–V) colorimetrically using a Sircol Collagen Assay kit. The amount of collagen protein in skin samples was adjusted to the amount of total protein using the BCA Protein Assay kit. Collagen concentrations were expressed as μg collagen per gram of total protein [25].

MPO assay

Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of neutrophils and can be used as a quantitative index of inflammatory infiltration. The tissue-associated myeloperoxidase (MPO) assay was performed to quantitate the degree of inflammatory infiltration in the wounds [25].

Hyaluronidase inhibition Assay

Hyaluronic acid, a glycosaminoglycan, is one of the chief components of the extracellular matrix. It is distributed widely in connective and epithelial tissues. Hyaluronic acid plays an essential role in wound healing since it contributes significantly to cell proliferation and migration. It grows from the base of a wound and promotes migration of fibroblasts, and endothelial cells into the wound site. Therefore, agents with anti-hyaluronidase activity could contribute wound healing [26].

Collagenase and Elastase Inhibition Assay

Extracellular matrix (ECM) is composed of proteoglycans and matrix metalloproteins such as collagen and elastin. Collagen is the major structural protein provides supportive framework to the cell, elastin maintains the skin’s elasticity and hyaluronic acid keeps the moist. All three components help the wound healing process. Therefore, inhibition of the enzymes that break down these ECM components could contribute wound healing [26].

CONCLUSION

Drug discovery and development consists of a series of processes starting with the demonstration of pharmacological effects in experimental animal models and cell lines and ending with drug safety and efficacy studies in patients. The study of wound healing agents has been an important field of research. This review on different *In-vitro* and *In-vivo* wound healing models help to wound care specialists to understand healing response.

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