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A Review: Screening Models for Wound Healing Activity in Animals

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ABSTRACT

Wounds are the result of injuries to the skin that disrupt the other soft tissue. Healing of a wound is a complex and protracted process of tissue repair and remodeling in response to injury. A wound can be described as a defect or a break in the skin, resulting from physical or thermal damage or as a result of the presence of an underlying medical or physiological condition. Wound healing is a complex process of cellular and biochemical interactions involving various cells such as keratinocytes, fibroblasts and endothelial cells. The in vitro assays & in vivo models can be performed to evaluate wound healing activity. In vitro models are useful, quick, and relatively inexpensive. In vivo-Small animals provide a multitude of model choices for various human wound conditions. In vitro fibroblast assay, keratinocytes assay can be performed. The wound healing efficacies of various herbal extracts have been evaluated by In vivo- models excision models, incision models, dead space models, burn modes can be performed on small animals after getting approval from the Ethics committee. In vitro and in vivo assays are stepping stones to well-controlled clinical trials of herbal extracts. Wound healing property can be checked by measuring tensile strength of skin, measurement of wound area, percentage of contraction, collagen content, protein estimation, Period of epithilazation.

Key words: *In vitro* cell assays, excision models, burn wound models, incision models, dead space models

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INTRODUCTION

Aging is a universal process that began with the origination of life many years ago. Skin being the largest organ of the body deserves special attention while considering the aging process. Skin aging is due to the conjunction of intrinsic factors (chronological aging) and extrinsic factors (fundamentally photo aging). In elderly people, cells are less likely to proliferate, they have shorter life spans, are less responsive to cytokines and epithelialization of epithelial skin tissue is longer.[1]

Wound

Wound is defined as disruption of cellular, anatomical, and functional continuity of a living tissue. It may be produced by physical, chemical, thermal, microbial, or immunological insult to the tissue. When skin is torn, cut, or punctured it is termed as an open wound and when blunt force trauma causes a contusion, it is called closed wound, whereas the burn wounds are caused by fire, heat, radiation, chemicals, electricity, or sunlight.[2] In other words wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue and may also result from a contusion, hematoma, laceration or an abrasion.[3]

Wound Healing

Wound healing involves a complex interaction between epidermal and dermal cells, the extra cellular matrix, controlled angiogenesis and plasma-derived proteins all coordinated by an array of cytokines and growth factors. This dynamic process is classically divided into three overlapping phases Inflammation, Proliferation and Remodeling.[4] In other word, Healing of wounds starts from the moment of injury and can continue for varying periods of time depending on the extent of wounding and the process can be broadly categorized into three stages; inflammatory phase, proliferate phase, and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue.[5]

Wound care and maintenance involves a number of measures including dressing and administration of painkillers, use of anti-inflammatory agents, topical systemic antimicrobial agents and healing promoting drugs. To facilitate healing, dressings are applied to try to protect the wound from contamination and keep the wound surface moist to maintain the integrity of the cells present in the defect. In a dry wound environment, dividing cells at the wound edges are unable to migrate into those areas occupied by dry scab material, where healing is protracted as a result of significant tissue loss (e.g. as in deep pressure).

This systematic review of *in vitro* and *in vivo* experiments could promote closer collaboration between the research communities and encourage an iterative approach to improving the relevance of various animals' models.

IN VITRO STUDIES

Tissue repair concerns many events, both contractile and chemical, and the causing of a wound on anybody surface stimulates the wound healing in the skin, which is a complex process characterized by angiogenesis re-epithelialization, granulation tissue formation, and remodeling of extracellular matrix. These steps, accomplished primarily by dermal fibroblasts and keratinocytes, are well orchestrated by bioactive molecules including growth factors, cytokines and their receptors, and matrix molecules. Hence, the fibroblast *in vitro* model is integral to correlating the contractile events of wound healing.[6] Key to wound healing processes are the proliferation, migration, and functioning of fibroblasts and keratinocytes, thus they are the basis of 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.[7] 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

The CAM model is used to assess the angiogenic activity of herbal extract. Nine-day-old fertilized chick eggs are selected, and a small window of 1.0 cm is made in the shell. The window is opened, and a sterile disc of methylcellulose loaded with herbal extract is placed at the junction of two large vessels on CAM. The window is resealed by tape, and the eggs are incubated at 37◦C in a well-humidified chamber for 72 h. Then, eggs are opened, and new blood vessel formation is observed in CAM treated by herbal extract which are compared with CAM containing disc without herbal extract (control) and the CAM treated with 10 µL 1000 AU/mL bFGF (Fibroblast Growth Factor) as a standard.[8] The photographic images of the CAM model are analyzed for quantitative morph metric analysis of the density of blood capillaries in terms of the number of red pixel per unit areas using Image J software and Angio Quant software.[9]

Fibro blast Bioassay

Human dermal fibroblast (HDF) cells from postauricular surgery are grown to confluence after

which they are removed from the culture flask using trypsin/EDTA after washing with phosphate buffer saline (PBS). HDFs are resuspended in 50mL of Dulbeccos' modified eagle medium (DMEM), centrifuged at 2600 rpm for 5 min, and seeded in a 96-well sterile microtitre plate at a density of 11×103 cells/well in DMEM containing 10% fetal calf serum (FCS), 0.02% fungizone, 1% penicillin, and 2% streptomycin. After 24 h, the media is removed by aspiration. Solutions are initially solubilized in water and diluted in DMEM containing 0.5% fetal bovine serum (FBS) (0.5% FBS is the maintenance level required for HDF growth) to give a final concentration of 100 µg/µL. Solutions are filtered through a 0.2 µM sterile filter prior to addition to the cells, and 1: 1 serial dilutions are prepared. Aliquot (200 µL) of herbal extract, in triplicate, is added to each well. The plates are left to incubate for 3 d. The neutral red assay is used to analyze the effects of the herbal extract on the growth of fibroblasts. Neutral red dye (1.2mL) is added to 78.8mL of Hanks' balanced salt solution (HBSS). This is incubated for 10min at 37◦C after which it is centrifuged at 2600 rpm for 5min and 100mL added to each well. The plates are incubated for 2.5 h, the media is tipped off, and the cells were washed with 100 µL of 1% formic acid followed by 100 µL of 1% acetic acid. The absorbance is recorded at 550 nm, and the values obtained for the solutions are compared with the control (0.5% FCS).[10]

The fibroblasts can also be cultured in a laboratory from fetal rat skin and subcultured through three passages before use. Cells are incubated in an atmosphere of 5% CO2, 95% air at 37◦C in a tissue culture incubator, and suspended at 0.5×105 viable cells/5mL in the growth media (DMEM). The viability of the cells is assessed by trypan blue exclusion. The suspended cells are fortified with 10% FCS and allowed to equilibrate for 3 d. Herbal extract and standard commercial drug as control is introduced into the medium separately on day 3 at a dose of 40mL in a concentration of 1 mg/mL, in phosphate buffered saline pH 7.4. The medium is changed periodically. On day 9, the medium is aspirated, and cells are washed with phosphate buffered saline. Half the quantity of cells is assayed for hydroxyproline content and the other half for DNA. Values are expressed as mg hydroxyproline/100 mg DNA[7]

Keratinocytes Assay

Keratinocytes can be isolated from the residual skin samples removed during surgery or human Fore skins that can be obtained from circumcised newborn babies. The residual skin graft is treated overnight with 0.3% solution of trypsin at 4◦C, whereas foreskins are washed extensively with multiple changes of PBS, subcutaneous tissue is removed, and the remaining samples are enzymatically dissociated in multiple changes of 0.25% trypsin and versene (50 : 50). Epidermal sheets are peeled from the dermis, minced, and dispersed in trypsin solution by repeated

pipetting. The cell suspensions are pelleted from the trypsin solution, sequentially suspended, and washed with PBS by centrifugation at 1000×g for 5min at 20◦C. Prior to cocultivation with keratinocyte, proliferation activity of fibroblasts is stopped using a solution of Mitomycin C at a concentration of 25 µg/mL for 3 h. These cells are raised in a tissue culture dish with feeder cells (J2 mouse fibroblasts). Feeder cells are seeded on a cover glass at a density of 25,000 cells/cm2 and cultured for 24 h. A suspension of keratinocytes (20,000 cells/cm2) is then added, and cells are cultivated in a keratinocyte serum-free growth medium (KGM) at 37◦C and 3.3% CO2. Cultures are maintained in a growth medium consisting of DMEM and Ham's nutrient mixture F12 at a 3 : 1 ratio. Cultures are fed every 3 days and subcultured by dispersal in 0.025% trypsin in PBS and replated at a split ratio of 1 : 3. Cultures are used between passages 2 and 3.[11]

For in vitro assay human keratinocytes, either second or third passage (P2 or P3) is trypsinized, seeded into 32 mm, tissue culture dishes at densities from 5 × 105 to 8 × 105 cells/dish in KGM, and fed every 2 d until they reached 100% confluence. The medium is then replaced with RPMI 1640/10% FCS. After 4 h a scratch is made with a micropipette tip, and cells are washed with PBS in order to remove loosened debris. RPMI 1640/2% FCS medium with or without the herbal extract (1mg/mL to 300 mg/mL) is added to sets of 2 dishes per dose. Each dish is orientated on the bottom of a six-well plate lid and fixed using glue. For each scratch, four consecutive fields are selected using the following criteria: relatively little cell debris within the scratch, even scratch, with straight edges, both edges visible under a single field using the X 10 objective, fields not too close to either end of the scratch, where distortions uncharacteristic of the main part of the scratch could be seen to occur during the "healing" process. The coordinates on the vernier scales of the inverted microscope stage are noted for every field. The condition of each field is recorded on video at various intervals over periods up to 72 h, as long as there was still a denuded area. Photographs of each field are also taken at the same time points. The average percentage of the initial area still denuded is calculated for each treatment set for the total of 8 fields per set.[12]

Effect of Herbal Drugs on Evaluation Parameters of *In vitro* models

The herbal extracts containing compounds with angiogenesis modulating properties showed strong angiogenic activity in CAM treated with herbal extract, by increasing the size and number of blood vessels as compared to control (positive control with sterile disc without herbal extract or negative control with normal saline solution). The changes in the distribution and density of CAM vessels next to the implant are evaluated by means of a stereomicroscope at regular intervals following the graft procedure, and the percentage increase of blood vessels could be

calculated by the following formula:

$$\text{ge increase of blood vessels} = \frac{\text{Vessel number of CAM treated by herbal extract} - \text{Vessel number of CAM treated by normal saline}}{\text{Vessel number of CAM treated by normal saline}} * 100$$

The herbal extract promotes fibroblast proliferation and motility; this activity was also greater than that of a positive control in which fibroblasts were allowed to grow in 10% FCS. The use of 10% FCS to indicate HDF growth stimulation is an established comparative technique and provides a suitable positive control.

The activity of herbal extract neither alters motility and proliferation of primary keratinocytes nor show any increase or decrease in EGFR (epidermal growth factor receptor) phosphorylation. It only stimulated the downstream effector in ERK (extracellular signal regulated kinase) activation when compared with control.

***IN VIVO* STUDIES**

The small mammals have emerged as the model of choice for reseachers, 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.[13] Small animals usually have accelerated modes of healing in comparison to humans, thus experiment duration lasts for days, in contrast to weeks or months in human experiments. Some small mammals can easily be altered genetically and provide a wound model capable of approximating defective human conditions such as diabetes, immunological deficiencies, and obesity.[14]

Small animals provide a multitude of model choices for various human wound conditions. Some models have been developed to investigate the mechanistic particulars of certain aspects of healing. The major in-vivo methods are histological techniques, use of tracers, analysis of body fluids and tissues and elicitation of biological response. Tissue changes in skin following the application of various substances to the cuetaneous surface can yield information about specific tissues affected, so that not only absorption is revealed but also the route of penetration. For studying the wound in the laboratory, mainly two types of wounds are produced experimentally. These are excised or open wounds and incised or sutured wounds. The assessment of healing is made by studying the regenerating tissue by different parameters.[15]

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

All the surgical interventions are carried out under sterile conditions under general anaesthesia. The predetermined area for wound infliction at the back of the animal is prepared for surgery by removing hairs with depilatory cream/shaving machine/razor. The animal is anaesthetized with anaesthetic ether/chloroform by open mask method or intraperitoneally with anaesthetic drug (35 mg pentobarbitone sodium/25mg thiopentone sodium/10mg ketamine/40mg thiopental/60 mg pentobarbital sodium/0.3mg chloralhydrate solution per kg body weight of an animal) and placed on the operation table in its natural position. The animal can also be anaesthetized using a combination of anaesthesia (90 mg ketamine + 10mg xylazine/10mg xylazine HCl + 50 mg ketamine HCl/50 mg ketamine HCl + 5mg diazepam/25 mg Ketamine HCl + 5mg Diazepam per kg body weight of an animal). The induction of localized anesthesia can be done by subcutaneous injection of a lodocain solution (2 mL, 2%) or lignocain HCl (1 mL, 2%) at and around the area under investigation to render area painless.

The animals are allowed to recover, housed individually in their cages, and monitored for respiration, colour, and temperature. They are maintained under standard husbandry conditions and on a uniform diet and managed throughout the experimental period. Animals are closely observed for any infection; those who show signs of infection are separated and excluded from the study. They are periodically weighed before and after the experiments.[8]

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.[16] 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.[17] 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 200–500mm2 diameter and 2mm 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 parameters studied were wound closure and epithelization time. The formulation was applied until complete wound healing. The percentage of wound closure and the period of epithelization were calculated. The period of epithelization was calculated as the number of days required for wound healing.

Incision Models[18]

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.

Musculoperitoneal Wounds

To prepare this wound, animals are prepared in the same fashion as described earlier but their abdomen is opened completely incision measuring between 2-5c.m according to the size of the animals are made. The wound is caused in one layer by interrupted linen stitches. Tensile strength and busting abdomen, by chemical and histological studies are done on this wounds tissue, after sacrifice the animals on desired post operative days.

Dead Space Wound Method

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 (5–10mg each) are introduced into the pouch. Each animal received 2 grass piths/cotton pellets in different locations.[19] 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.[20] The respective

therapeutic treatment is administered either orally or topically to the animals of respective groups for 10 consecutive days.[21]

Burn Wound Model

This wounds are also measured for the contraction. A special metal plate 2×2 cmwith holder is heated to 60◦C and applied to the dorsal area of the animals for 30 s 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 s. While for full thickness burn wound, the metal plate is heated to 100◦C and applied to the dorsal area for 30 s. The animal can also be subjected to rectangular burn wounds (20 × 25mm2) using hot (180◦C) brass brick weighing 300 g, which is pressed against the shaved skin for 10 s in the treatment group.[22] A cylindrical metal rod (10mm diameter) is heated over the open flame for 30 s and pressed to the shaved and disinfected surface for 20 s on selected dorsal area of animal under light anaesthesia.[23] 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 100–300mm2 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.[24] 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

Effect of herbal drugs on Evaluation of Wound healing *in vivo* models

Measurement of wound area:[25] 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. [26]

$$\% \text{ Wound Contraction} = \frac{\text{Healed Area}}{\text{Total Area}} \times 100$$

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. It was calculate by following formula.[27]

$$\text{Percentage wound contraction} = \frac{\text{Initial area of wound} - \text{Nth dayarea of wound}}{\text{Initial area of wound}} * 100$$

Determination of Period of epithelization[28]

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 epithelization.

Tensile Strength

One of the most crucial phases in dermal wound healing is the progressive increase in biomechanical strength of the tissue; the mechanical properties of the skin are mainly attributed to the function of the dermis in relation to the structure of collagen and elastic fiber networks. Breaking strength of the healed wound is measured as the minimum force required to break the incision apart. Skin breaking strength gives an indication of the tensile strength of wound tissues and represents the degree of wound healing.[18] Tensile strength has commonly been associated with the organization, content, and physical properties of the collagen fibril network. Its resistance to breaking under tension; it indicates how much, the repaired tissue resists breaking under tension and may indicate in part the quality of the repaired tissue.[18] The sutures were removed on the 7th–9th post wounding day, and the tensile strength was measured on the 8th–10th day. The mean tensile strength on the two paravertebral incisions on both sides of the animal is taken as measures of the tensile strength of the wound for an individual animal. [29]

$$\text{Tensile strength} = \frac{\text{breaking strength (g)}}{\text{Cross-sectional area of skin (mm22)}}$$

Measurement of wound Index[30]

Wound index was measured daily with an arbitrary scoring system.

Table 1: An arbitory scoring system for measurement of wound healing index

Gross Change	Wound Index
Complete healing of wound	0
Incomplete but healthy healing	1
Delayed but healthy healing	2
Healing has not yet been started but environment is healthy	3
Formation of Pus-Evidence of necrosis	4
Total	10

Collagen Estimation (Hydroxyproline Content)[31]

Wound tissues were analyzed for hydroxyproline content, which is 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. For preparation of protein hydrolysate, 50 mg of tissue sample in 1.0 ml of 6.0N HCl was weighed and sealed in screw-capped glass tube. The tubes were autoclaved at 15 1.056 kilograms per square centimetre for 3 h. The hydrolysate was neutralized to pH 7.0 and brought to the appropriate volume (filtered if necessary). Test tubes marked as sample, standard and blank were taken. One milliliter of test sample was added to test tubes marked as sample, 1.0 ml of DM water to test tubes marked as blank and 1.0 ml standard solutions to test tubes marked as standard. One milliliter of 0.01M copper sulphate solution was added to all the test tubes followed by the addition of 1.0 ml of 2.5N sodium hydroxide and 1.0 ml of 6% hydrogen peroxide. The solutions were occasionally mixed for 5 min and then kept for 5 min in a water bath at 80^0C. Tubes were chilled in ice-cold water bath and 4.0 ml of 3.0N sulphuric acid was added with agitation. Two milliliters of p-(dimethylamino)benzaldehyde was then added and heated in water bath at temperature 70^0C for 15 min. The absorbance wasmeasured at 540nm using Synergy HT Multi-Detection Microplate Reader (MDMR). The concentration of the sample was calculated as:

$$\text{Concentration of the sample} = \frac{\text{OD of the sample}}{\text{OD of the standard}} * \text{Concentration of standard}$$

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. The granuloma tissues are trimmed to obtain the rectangular strip measuring about 15mm in length and 8mm width to determine its breaking/tensile strength by continuous water flow technique. The dry granulation tissue is used for the estimation of hexuronic acid, hexosamines, hydroxyproline content, which can be assayed calorimetrically/spectrophotometrically, and a piece of wet granuloma is preserved in 10% formaldehyde for histological studies to evaluate the effect of the herbal extract on collagen formation. Granulation tissue is collected in phosphate-buffered saline (maintained at −64◦C) for

the estimation of antioxidant enzymes like superoxide dismutase (SOD), catalase, reduced glutathione (GSH), and tissues lipid peroxidation .[2]

Hexosamine Estimation

The granulation tissue is obtained from wound area on 11th post wounding day is dried in an oven at 60◦C and hydrolyzed with 2NHCl at 100◦C for 2 h. The hydrolyzed solution is filtered and pH of the filtrate is adjusted to 6-7. This acid hydrolysate solution is subjected to deamination and nondeamination of hexosamine to determine the amount of hexosamine. For deamination, 0.5mL of a 5% solution of sodium nitrite and 0.5mL of a 33% solution of acetic acid is added to 0.5mL of acid hydrolysate solution. The tubes are shaken and left for 10 min for complete deamination. The excess nitrous acid is removed by adding 0.5mL of a 12.5% solution of ammonium sulfamate and by shaking the mixture for 30 min. For indole reaction, 2mL of 5% HCl and 0.2mL of a 1% solution of indole in alcohol is added to 2mL of the deaminated hexosamines. The tubes are immersed for 5min in a boiling water bath. An intense orange colour and a slight turbidity is seen. To remove turbidity, 2mL of alcohol is added, and tubes are shaken. For nondeamination of hexosamine, 1.5mL of a mixture of equal volumes of solutions of 5% sodium nitrite, 33% acetic acid and 12.5% ammonium sulfamate are added to 0.5mL of acid hydrolysate solution. This serves as the control without deamination. The indole reaction is carried out on this mixture as described above. The absorbances of the solutions are determined spectrophotometrically at 492 and 520 nm. The absorbance value for the non deaminated solutions is subtracted from the corresponding absorbance values for the deaminated unknown and standard solutions of glucosamine hydrochlorides. The increase in the difference in absorbance after the deamination procedure is considered as the measure of the amount of hexosamine. The hexosamine value in µg is computed from the standard curve.[32]

Estimation of total protein[33]

Protein concentration was estimated according to the method of Lowry et al and Kandhare et al using BSA (bovine serum albumin) as a standard.

Biochemical analysis of wound tissue:

The animals were anaesthetize on 4th, 8th and 12th day after treatment. Then, the granulation tissue was removed from each wound and tissue was divided into two parts for following analysis:

♦Estimation of mucoplysacharides and collagen

♦Estimation of DNA

CONCLUSION

This review on different *In-vitro In-vivo* wound healing models help to wound care specialists to understand healing response. It will be used to develop new herbal formulation on wound care area.

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