

Histological Study of Recipient Wound Bed Healing by Ultrasound Therapy in Dogs

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Abstract: Open wound management, until the wound is considered suitable for reconstruction or until it has healed by second intention, has been the treatment of choice for centuries. Frequent and painful dressing changes over prolonged periods intensifies overall case management, and may impact treatment costs. Wound healing stimulated an optimum microenvironment for successful reconstruction that can be created by employing modern methods such as electrical stimulation, ultrasound, platelet rich plasma and stem cell therapy. Electro-physical modalities like ultrasound therapy of 0.1 w/cm² had positive effects on wound healing on proliferative stages by accelerating the inflammatory phases. Ultrasound therapy reduces the duration of the inflammatory phase of repair and enhances the release of factors which stimulated the proliferative phase of repair from macrophages and other cells. When skin was damaged, not only epithelial cells were destroyed, but also a large quantity of collagen was also lost. Wound healing was a complex dynamic process that integrated the functions of formed blood elements, the extracellular matrix (ECM), parenchymal cells, and soluble mediators. In uncomplicated wounds, the repair process followed a fairly consistent time sequence. Phases of cutaneous wound repairing is divided into three phases: inflammation (early and late), proliferation, and remodeling. Several wound characteristics were considered that determined the level of histopathological changes e.g. depth and length of healed wound, epithelial stratification, incorporation of the dermal substitute, degree of neutrophil, macrophage, fibroblast, foreign body giant (FBG) cell infiltration and extent of elastin formation. Wounded tissue stained with Masson's trichrome showed a clear view of collagen fibres deposition and reorganisation compared to Hematoxylin and Eosin staining. The present study was undertaken to evaluate the wound healing by Ultrasound therapy through histopathological evaluation.

Keywords: wound healing- Recipient wound bed –Ultrasound therapy– mason's trichrome staining- hisopathological evaluation

1. Introduction

Full-thickness skin loss occurred commonly in dogs. These wounds were often managed with dressings and bandages until healing occurred by granulation tissue formation, contraction, and epithelisation (second intention healing). The duration of open wound management contribute significantly to therapeutic challenges, patient morbidity, and increased costs of treatment. Adequate blood supply and improved oxygen tension in the wound was essential for optimal fibroplasia and collagen production.

Low intensities ultrasound therapy enhances healing whereas high intensities have pro-inflammatory effects. Ultrasound therapy initiated within the first week after injury compromised tissue repair whereas the same treatment initiated after 2 weeks was beneficial.

The therapeutic effects of ultrasound therapy consists of thermal and nonthermal components. The thermal component set at 1-1.5W/cm² had been used to improve scar outcome (Selkowitz *et al.*, 2002). The nonthermal component set at 0.3-1W/cm² produced both cavitation (formation of gas bubbles) and streaming (a steady unidirectional force), which in the laboratory caused changes in cell membrane permeability, increased cellular recruitment, collagen synthesis, tensile strength, angiogenesis, wound contraction,

fibrinolysis, and stimulated fibroblast and macrophage production (Ubbink *et al.*, 2008)

Wounded tissue stained with Masson's trichrome showed a clear view of collagen fibres deposition and reorganisation. The Abramov's histological scoring system (modified Greenhalgh's scoring system) was used for scoring epithelisation, fibrosis, angiogenesis, and collagen level; the number of macrophages under this system was modified. (Abramov *et al.*, 2007).

2. Materials and Methods

The study was carried out on dogs that were brought to Madras Veterinary College Teaching Hospital and Veterinary University Peripheral Hospital, Chennai with large wound. Low Intensity Pulsed Ultrasound (LIPUS) probe was directly applied against the dogs skin and wound bed using water soluble ultrasound gel for 15 minutes on 0, 3rd, 7th and 14th day. Mild heating to the peri wound tissue in order to stimulate circulation, higher intensity ultrasound was given. Extreme care was taken, however, to assure the tissue was capable of handling the thermal levels delivered (Selkowitz *et al.*, 2002).

Periwound tissue were treated with 1 MHz, continuous ultrasound. An ultrasound applicator 1.5 to 2 times the size of the treatment area was used.

With an aqueous coupling medium in place, the probe was placed lightly against the skin surface and moved in a slow and deliberate manner. The intensity was typically set between 1 and 1.5 watts per square centimetre. This parameter was extremely variable and depended on the animal's circulatory, sensory and mental status.

Treatment duration was slightly longer than that of pulsed ultrasound since a mild thermal effect was desired. Initial treatment was about 2-3 minutes and was increased by 30 second increments to a maximum of 5 minutes.

A 3.5 mm punch biopsy instrument was used to take skin specimens from the recipient wound of each animal on 3rd, 7th and 14th day with the dogs under sedation. Punch biopsy was used in dogs as a method for skin healing investigation by histological analysis. (Hamamoto *et al.*, 2009). The specimens were fixed in 10% neutral buffered formalin and processed routinely for histopathological examination. Five micrometer sections were stained with hematoxylin and eosin (H&E) and Masson's Trichrome. Although several histopathological parameters could be used to assess the progression of healing from the inflammatory to the repair stage, the progressive decrease in macrophages, fibrosis, and progressive increase in angiogenesis, epithelisation and collagen level were selected. The slides stained with Masson trichrome stain were examined using polarised light microscope and with the aid of software image analyser, measurements were made at the density of the blue colour which represent the collagen density. Collagen density was measured under the wound area compared to normal dermis.

The Abramov's histological scoring system (modified Greenhalgh's scoring system) was used for scoring epithelisation, fibrosis, angiogenesis, and collagen level; the number of macrophages under this system was modified. (Abramov *et al.*, 2007). While the Greenhalgh's scoring system compiled several histological parameters simultaneously to create a single score, the Abramov's system assessed each parameter independently and gave a score of 0-3. The collagen level was graded as: 0 - none, 1 - scant, 2 - moderate and 3 - abundant. Epithelisation was graded as either: 0 - none, 1 - partial, 2 - complete, but immature or thin, and 3 - complete and mature. Angiogenesis was graded as either: 0 - none, 1 - up to 5 vessels per highpowerfield and 2 - above 6 to 10 vessels per highpowerfield and 3 - more than 10 vessels per highpowerfield. Fibrosis was graded as
0 - none to minimal fibroblasts,
1 - few fibroblasts,
2 - more fibroblasts,
3 - predominantly fibroblasts.
The number of macrophages were scored as 0-25 = 1, 26-50 = 2 and > 51 = 3.

3. Results

The mean \pm S.E. values of collagen, epithelisation and angiogenesis of animals treated by Ultrasound therapy were presented in Table 33 and (Fig.11, 12 and 13)

The mean \pm S.E. values for collagen proliferation, epithelisation and angiogenesis was 2.00 ± 0.64 , 2.18 ± 0.61 , 2.21 ± 0.53 and 1.65 ± 0.52 , 1.73 ± 0.52 , 2.81 ± 0.62 and 1.03 ± 0.72 , 1.63 ± 0.73 and 2.34 ± 0.22 on 3rd, 7th and 14th day respectively. The dermis was devoid of epidermal covering and the wound consisted of large defects at the 3rd day. The wound area was filled with necrotic debris and crust made up of fibrin. The area was lined by proliferating granulating tissue and was made up of few myofibroblast and immature capillaries. At 3rd day animals showed moderate amount of immature collagen fibres.

The dermis of the wound area in the present study animals showed edema, vascular congestion and showed partial infiltration of neutrophils and macrophages. (Plate 1 and 2)

On 7th day, the wound area was covered by granulation tissue and fibrin crust, the defect was diminished due to partial contraction of the wound. There was moderate amount of fibres and fibroblast in the dermis region. The epidermis was hyperplastic on the margin of the wound.

The dermal region on 7th day showed moderate amount of immature collagen fibres. The inflammatory cells were few and mainly consisted of lymphocytes, plasma cells and macrophages. Neovascularisation was with few immature collagen indicated by incidence of number of capillaries was noticed (Plate 3 and 4)

In the present study on 14th day, moderate hyperplastic epidermis with reduced scar tissue due to wound contraction. In the dermis the amount of matured collagen fibres were moderate with few immature collagen fibres at the centre of the wound. Very few inflammatory mononuclear cells were present at the periphery of the recipient wound bed. The degree of neovascularisation was less on the recipient wound bed at 7th day (Plate 5 and 6).

4. Discussion

The irradiation from ultrasound applied to the edges and to the center of the lesion simultaneously due to the dimensions of the probe (22mm in diameter) stimulated the myofibroblasts, which were sensitive to ultrasound, which thereby increased the scar contraction. The treatment with pulsed low intensity ultrasound therapy begun on the first day, which interfered with the healing process from its initial stage of acute inflammation. The time for evaluation were set at 3, 7 and 14 days because most of the events that accompanied the stages of skin healing was concentrated at that time. Busse *et al.* (2002)

Ultrasound provided simultaneous cleansing and debridement of wounds. Treatment with the device involved holding an ultrasonic handset 1 cm away from the wound after applying saline solution to the handset that generated a saline mist designed to carry low levels of ultrasonic energy into the wound. This treatment promoted healing of acute, traumatic, and chronic wounds by stimulating cellular activities that contributed to healing and by cleansing the wound surface as opined by Ennis (2006).

At 3rd day, wound consisted of large defect within the dermis and was devoid of epidermal covering in all the cases of different groups. The wound area was filled with necrotic debris and crust made up of fibrin. The area was lined by proliferating granulating tissue made up of numerous myofibroblast and immature capillaries however the proliferation of granulation tissue with myofibroblast was less in ultrasound animals. The dermis of the wound area in the present study showed edema, vascular congestion and showed increased infiltration of neutrophils and macrophages.

It has been reported that electro-physical modalities like ultrasound reduces the duration of the inflammatory phase of repair and enhances the release of factors which stimulated the proliferative phase of repair from macrophages and other cells (Hosgood, 2006) as observed in the present study. There was also evidence that ultrasound could modify plasma membrane permeability to ions such as calcium, and that this might act as a stimulus to cell activity. Thus, repeated stimulation of these cells accounts for the observed acceleration of the resolution of inflammation and progress through the subsequent phases of repair.

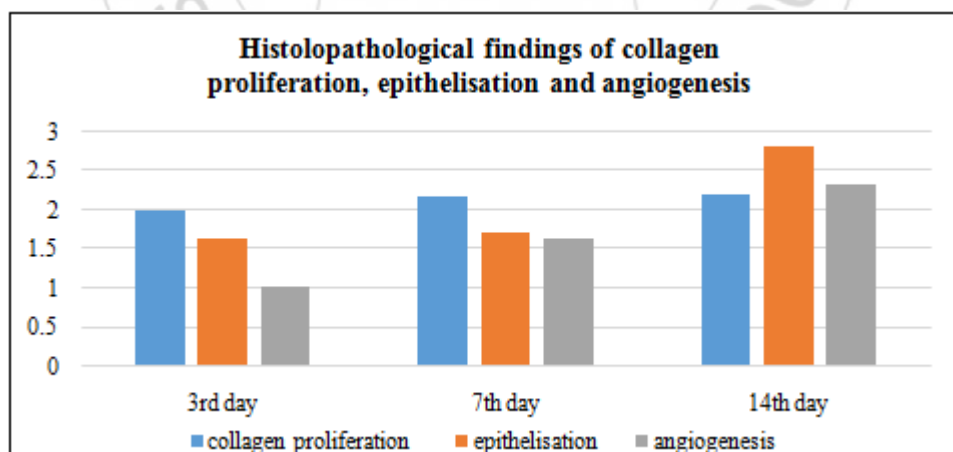
On 7th day granulation tissue with fibrin crusts, fibroblast and neovascularisation was observed in all the cases. However increased amount of immature collagen fibres were observed in Masson trichrome staining as per Abramov's histological scoring system (modified Greenhalgh's scoring system). Dermis cellularity increased mainly due to fibroblasts proliferation and new matrix deposition as opined by Hallet *et al.* (2010).

During the treatment with ultrasound therapy all wounds reacted with a clear increase in redness due to augmented blood supply. Therefore it was proposed that ultrasound

therapy of skin lesion enhanced capillary growth increased overall blood supply to the injured area, a feature regarded as a milestone for successful wound repair.

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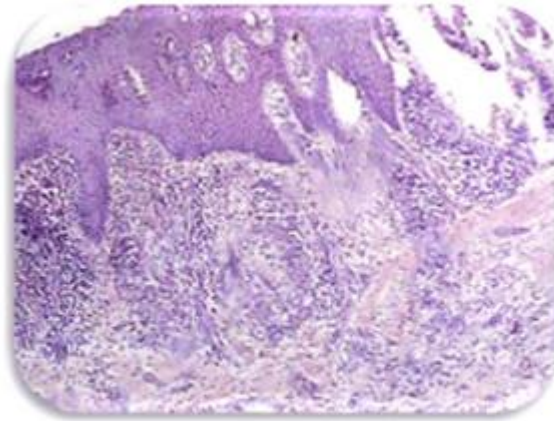


Plate 1 - 3rd Day
Wound Bed- Moderate Infiltration of Neutrophils and macrophages
H&E Stain = 10 μ m

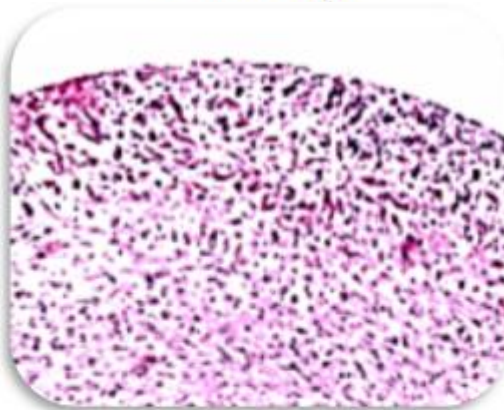


Plate 2 - 3rd Day
Wound Bed- Absence of epithelisation with fibrin and immature capillaries
Masson's Trichrome Stain = 50 μ m

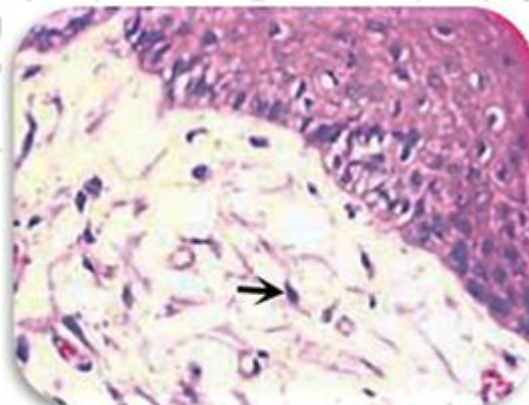


Plate 3: 7th Day
Wound Bed - Mild angiogenesis (arrow) in dermis
H&E Stain = 10 μ m

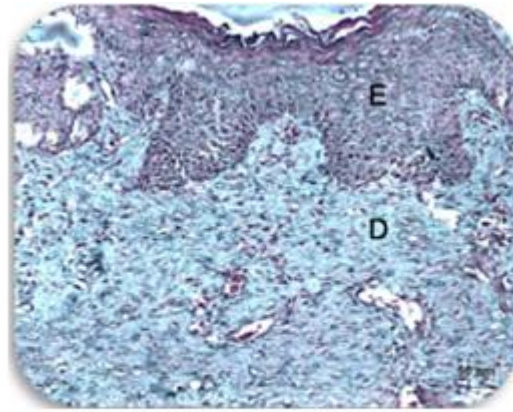


Plate 4: 7th Day
 Wound Bed - Dermis (D) showing mild collagen levels, less Hyperplasia of Epidermis (E)
 Masson's Trichrome Stain = 50 μ m

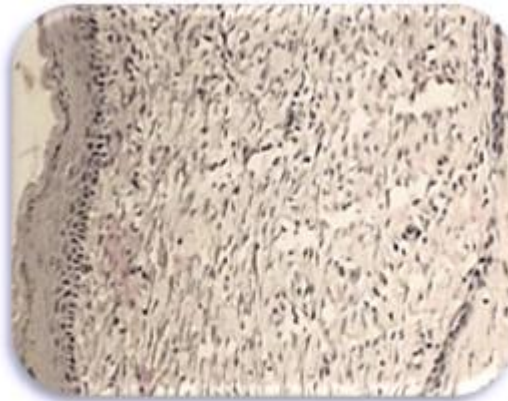


Plate 5: 14th Day
 Wound Bed - Moderate thickening and hyperplasia of epidermis and dermis
 H&E Stain = 50 μ m

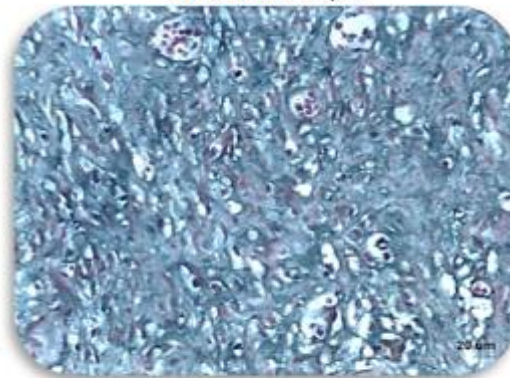


Plate 6: 14th Day
 Wound Bed - Moderate Neovascularisation with Matured collagen fibres
 Masson's Trichrome Stain = 50 μ m

Table 1: Histopathological findings among Groups collagen, epithelisation and angiogenesis of recipient wound bed

Parameters	Treatment Days (Recipient Wound Bed)		
	Mean \pm S.E.		
	3 rd Day	7 th Day	14 th Day
Collagen	2.00 \pm 0.64	2.18 \pm 0.61	2.21 \pm 0.53
Epithelisation	1.65 \pm 0.52	1.73 \pm 0.52	2.81 \pm 0.62
Angiogenesis	1.03 \pm 0.72	1.63 \pm 0.74	2.34 \pm 0.22