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Comparison of Histological Changes of Recipient Wound Bed by Platelet Rich Plasma and Adipose Derived Stem Cell in Dogs

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Abstract: ***The aim of wound healing is to promote rapid wound closure and prevent excess scar formation. 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 and optimum microenvironment for successful reconstruction that can be created by employing modern methods such as Platelet Rich Plasma and Stem Cell therapy. When skin was damaged, not only epithelial cells were destroyed, but also a large quantity of collagen was also lost. Currently, there is a growing interest in the use of autologous platelet concentrates (PC), also known as platelet rich plasma (PRP) for stimulating the healing process and promoting regeneration through growth factors instead of reparation (Mazzuccoet al., 2008). Stem cells and progenitor cells offer a potential answer to this dilemma (Kim et al., 2012).Adipose derived stem cells (ADSCs) are an attractive and abundant stem cell source with therapeutic applicability in diverse fields for the repair and regeneration of acute and chronically damaged tissues. Several wound characteristics were considered that determined the level of histopatological 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 compare and evaluate the wound healing between Platelet Rich Plasma and Adipose Derived Stem Cells.***

Keywords: Wound healing- Recipient wound bed –Platelet Rich Plasma – adipose derived stem cell-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.

There is a growing interest in usage of autologous platelet concentrates (PC), also known as platelet rich plasma (PRP) that stimulate the healing process and promote regeneration instead of reparation. The author also added that platelet alpha granules contained at least seven growth factors that were directly involved in the healing process. Of those proteins, transforming growth factor beta isoform 1 (TGF-β1) was of pivotal importance for its actions on cell proliferation, angiogenesis and extra cellular matrix deposition.

The field of tissue engineering proposes to repair and regenerate damaged organs using a combination of cells, biomaterials, and cytokines. The availability of cells capable of differentiation along multiple lineage pathways has limited the progress and development of these modalities. Stem cells and progenitor cells offer a potential answer to this dilemma (Kim *et al.,* 2012).

Adipose derived stem cells (ADSCs) are an attractive and abundant stem cell source with therapeutic applicability in diverse fields for the repair and regeneration of acute and chronically damaged tissues. ADSCs display properties similar to that observed in BMSCs and, upon induction, undergo osteogenic, chondrogenic, adipogenic and neurogenic differentiation in - vitro (Kilroy *et al*., 2007).

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 platelet rich plasma was prepared by the Double centrifugation tube method as per the standard protocol of Perazzi*et al,* (2013). About 8 ml of whole blood was collected atraumatically through a single cephalic vein puncture. The blood was centrifuged for 20 minutes at 2,800 rpm to achieve separation of cell layers. The final solution, obtained by mixing different buffy coats in a sterile 15 ml Falcon tube, was centrifuged without applying a brakeat1300rpmfor 15 minutes for good separation of platelet pellets in the supernatant layer (Plate 12). The platelet poor plasma was drawn off so that the platelet rich plasma remained in the tube after resuspending the platelet pellet within the remaining volume of plasma in an

eppendorf vial (Plate 16).Immediately before being applied to the wound surface, the platelet rich plasma was activated using 1 ml of calcium gluconate 10% .The PRP was then applied to the wound treated on 0, 3rd,7th and 14thday. Based upon the granulation of the wound bed an appropriate skin flap surgery was performed.

Omental tissue (5 to 10 grams)was harvested from dogs reported to the Small Animal Operation Theatre of MVC Teaching Hospital for elective ovario-hysterectomy under written informed consent from the owners. The tissues were subjected to dissociation with 0.075% collagenase for a period of 30 to 45 minutes. The enzyme activity was neutralized by washing the cells with DMEM-HG (Dulbecco's modified Eagle Medium – High Glucose) containing 10% FBS (Fetal bovine serum). The resultant content was centrifuged at 1200g for 5 to 10 minutes to pellet the cells.(Plate 23, 24) The cells were seeded in tissue culture plates at a density of 3000 to 3500 cells per cm^2 in DMEM-HG with 10% FBS containing antibiotics.

The cells were subjected to trypsinization when they reached a confluence of 70%, using 0.025% trypsin and then plated at a density of 5000 cells per cm^2. Later, the cultures were passaged repeatedly after achieving a density of 70 to 80% (Kim *et al.,* 2013).

(Plate 26).The Endotoxin content was performed by the Limulus amebocytelysate (LAL) test and the adequacy was 2.5 EU/ml (Wagers and Wisseman, 2004). The canine adipose cells, thus obtained not exceeding the second passage was used for topical administration with appropriate substrate on the recipient wound bed.

A 3.5 mm punch biopsy instrument was used to take skin specimens from the recipient wound of each animal on 3rd, 7thand 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 highpower field and 2 -above 6 to 10 vessels per highpower field and 3 - more than 10 vessels per highpower field. Fibrosis was graded as 0 - none to minimal fibroblasts, 1 - few fibroblasts, 2-morefibroblasts, 3-predominantly fibroblasts. The number of macrophages were scored as 0-25 = 1, 26-50 = 2 and > 51 = 3.

3. Results

The mean ± S.E. values of collagen, epithelisation and angiogenesis of animals treated by platelet rich plasma and adipose derived stem cell therapywere presented in Table 1 and 2 (Fig.1, 2 and 3)

The mean ± S.E. values for collagen proliferation, epithelisation and angiogenesis was 2.28 ± 0.83, 2.38 ± 0.72, 2.40 ± 0.62 and 1.80 ± 0.78, 2.47 ± 0.62, 2.99± 0.00 and 2.50 ± 0.91, 2.23 ± 0.63 and 1.57 ± 0.12 respectively on 3rd, 7th and 14th day for animals treated with platelet rich plasma and the mean ± S.E. values for collagen proliferation, epithelisation and angiogenesis was 2.20 ± 0.63, 2.30 ± 0.51, 2.35 ± 0.71 and 1.72 ± 0.80, 2.40 ± 0.63, 2.97± 0.03 and 2.47 ± 0.71, 1.88 ± 0.59 and 1.50 ± 0.22 respectively on 3, 7 and 14 days for animals treated with adipose derived stem cell.

On 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 numerous myofibroblast and immature capillaries. At 3rd day animals treated by adipose derived stem cells showed moderate amount of immature collagen fibres but not better than animals treated with Platelet rich plasma.

On 7th day, partial contraction of the wound bed which was covered with granulation tissue and fibrin crust. There was abundant amount of fibres and fibroblast in the dermis region in animals treated with platelet rich plasma when compared with other group of animals. The epidermis was hyperplastic on the margin of the wound.

The dermal region on 7th day showed thin incomplete, immature amount of collagen fibres. The inflammatory cells were few and mainly consisted of lymphocytes, plasma cells and macrophages. Moderate neovascularisation with immature collagen were observed in animals treated with adipose derived stem cells when compared to platelet derived stem cell group.

In the present study on 14th day, the scar surface got reduced due to wound contraction and hyperplasia of the epidermis rapidly in the dermis the amount of mature collagen fibres increased and were complete 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 greater on the recipient wound bed at 7th day in group of animals treated with PRP.

4. Discussion

Platelet growth factors of the platelet rich plasma are peptides that promoted cell proliferation, differentiation, chemotaxis, and induced the migrations of various cells preferably macrophages and myofibroblast. Raul *et al.* (2007) showed that a single application of PRP used in incisional wounds amplified the inflammatory response with an increased wound influx of neutrophils and macrophages as observed in the present study also.

Damage of the skin inevitably induces local inflammation and recruits an inflammatory cell which acts as a source of cytokines that affect the wound healing process. Previous studies by Beldon (2010) demonstrated that adipose derived stem cells download pro-inflammatory cytokines and upregulated anti-inflammatory cytokines.

On 7th day granulation tissue with fibrin crusts, fibroblast and neovascularistion was observed in all the cases. However increased amount of immature collagen fibres were observed in Masson trichrome staining in platelet rich plasma group followed by ADSC group 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 Hall*et al.* (2010) also.

Platelet rich plasma and adipose derived stem cells stimulated the synthesis and maturation of collagen, thus resulting in an increased tensile strength of the healed skin. Tensile strength has commonly been associated with the organization, content, and physical properties of the collagen fibril network and was one of the necessary parameters for determining the pharmacological effects of potential wound healing agents as opined by Pol *et al*. (2000). Angiogenesis and Fibroplasia was higher in platelet rich plasma cell treated animals when compared with other groupon the recipient wound bed which indicated platelet rich plasma and adipose derived stem cells accelerated the inflammatory reaction and initiated the healing process in the early phases of healing of recipient wound bed more rapidly

Neovascularisation was progressively higher in PRP treated animals in comparison with the ADSC treated animals on the recipient wound bed. During the treatment with PRP all wounds reacted with a clear increase in redness due to augmented blood supply. Therefore it was proposed that platelet rich plasma on skin lesion enhanced capillary growth increased overall blood supply to the injured area, a feature regarded as a milestone for successful wound repair (Sartin *et al*., 1998).

The platelet concentration in most of the cases were relatively four times the baseline intravascular platelet value which was considered as the minimal concentration for accelerating epithelisation, angiogenesis, growth, vascular fibroblast proliferation and extracellular collagen matrix synthesis and deposition as also observed by Arguelles *et al.* (2006).

Similarly adipose derived stem cells also promoted fibroblastic proliferation resulting in intensified granulation tissue formation. The accumulation of large collagen fibres likely increased the wound strength as opined by Kim *et al.* (2013) also. Another hypothesis was that adipose derived stem cells exerted paracrine effects on wound healing such as promoting fibroblast proliferation and exaggerating angiogenesis as opined by Lee *et al.* (2011). MSC released significant amounts of growth factors such as epidermal growth factor and transforming growth factor in a hypoxic environment in vitro Chen *et al.* (2009) which could also be the reason for the beneficial effects on wound in group of animals treated with adipose derived stem cell.

References

[1] Abramov, Y., Golden, B. and Sullivan, M. 2007. Histologic characterization of vaginal vs. abdominal surgical wound healing in a rabbit model. *Wound Repair Regen*. **15**(1):80–86.
[2] Anna Perazzi., Roberto, B., Tiziana M., Michele. S., Daniela, P., Francesco, C. And Iacopetti, I. 2013. Description of a double centrifugation tube method for concentrating canine platelets. *BMC Veterinary Research*. **9**:146.
[3] Arguelles, D., Carmona, J.U., Pastor, J., Iborra, A., Viñals, L., Martínez, P., Bach, E. and Prades, M. 2006. Evaluation of single and double centrifugation tube methods for concentrating equine platelets. *Res Vet Sci*.**81**:237–245.
[4] Beldon, P. 2010. Basic Science of wound healing. *Surgery*. **28** (9):409–412.
[5] Chen, C.W., Montelatici, E., Crisan, M., Corselli, M., Huard, J., Lazzari, L. and Peault, B. 2009. Perivascular multi-lineage progenitor cells in human organs: regenerative units, cytokine sources or both? Cytokine. *Growth Factor. Rev.***20**: 429-434.
[6] Cho, J.W., Kim, S.A. and Lee, K.S. 2012. Platelet-rich plasma induces increased expression of G1 cell cycle regulators, type I collagen, and matrix metalloproteinase-1 human skin fibroblasts. *Int J Mol Med*, **29**:32–36.
[7] Hall, M.N, Rosenkruntz, W.S., Hong, J.H. and Griffin, C.E. 2010. Evaluation of the potential use of Adipose Derived Mesenchymal Stromal Cells in the treatment of Cannine Atopic Dermatitis: A Pilot Study. *Veterinary Therapeutics*. **11:**E1-E14.
[8] Hamamoto, T., Yabuki, A., Yamato, O., Fujiki, M., Misumi, K. and Matsumoto, M.2009. Immunohistochemical analysis of cyclooxygenase-2 induction during wound healing in dog skin. *Res.Vet. Sci*. **87**, 349-354.
[9] Kilroy, G.E., Foster, S.J., Wu, X., Ruiz, J., Sherwood, S., Heifetz, A., Ludlow, J.W. and Gimble, J.M. 2007. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J. Cell. Physio.***212**: 702-709.
[10] Kim, S., Chang, K.A., Kim, J., Park, H.G., Ra, J.C., Kim, H.S. and Suh, Y.H. 2012. The preventive and therapeutic effects of intravenous human adipose-derived stem cells in Alzheimer's disease mice. *PLoSOne*.**7**(9):e45757.
[11] Lee, K.B., Choi, J., Cho, S.B., Chung, J.Y., Moon, E.S., Kim, N.S. and Han, H.J. 2011. Topical embryonic stem

cells enhance wound healing in diabetic rats. *J. Orthop. Res.***29**:97-102.
[12] Pol, I.E., Mastwijk, H.C., Bartels, P.V. and Smid, E.J. 2000. Pulsed-electric field treatment enhances the bactericidal action of nisin against Bacillus cereus. *Appl Environ Microbiol*. **66** (1): 428–30.
[13] Raul, F., Jorge, U. and Cleuza, M.F. 2007. Comparison of the effect of calcium gluconate and batroxobin on the release of transforming growth factor beta 1 in canine platelet concentrates.*BMC Veterinary Research*. **8**:212.
[14] Sartin, E. M. S., Swaim, S. F. and Scardino. A. 1998. Evaluation of treatment with a pulsed electromagnetic field on wound healing, clinicopathologic variables, and central nervous system activity of dogs. *Am. J. Vet. Res.***59**: 1177-1181
[15] Wagers, A.J. and Weissman, I.L. 2004. Plasticity of Adult Stem Cells. *Cell*. **116**: 639-648.

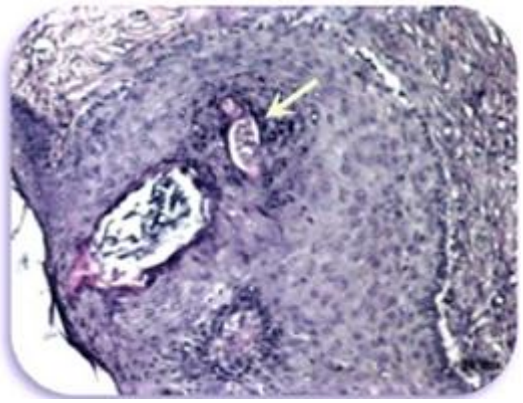

Plate 1: Group I– 3rd Day
Wound Bed - Moderate Angiogenesis (arrow)
H&E Stain = 50µm

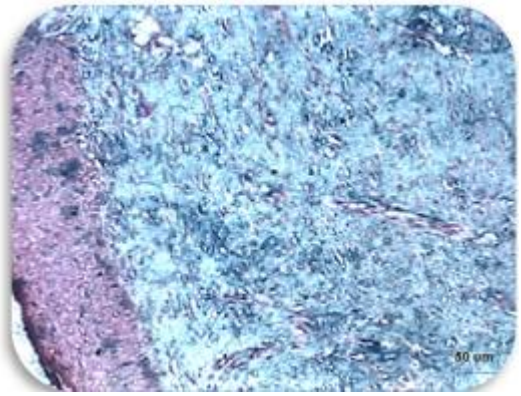

Plate 2: Group I-3rd Day
Wound bed - Granulating tissue with moderate immature collagen fibres with Neovascularisation
Masson's Trichrome Stain = 50µm

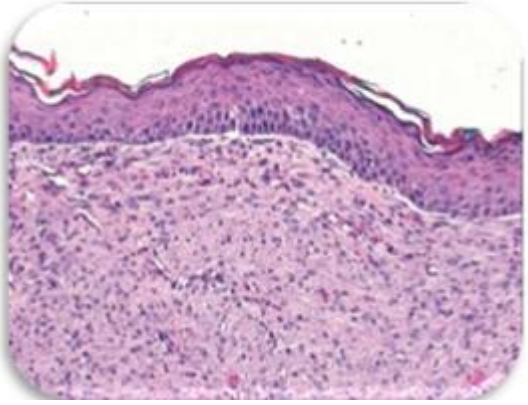

Plate 3: Group I– 7th Day
Wound Bed - Moderate Epithelisation and Neutrophilic Infiltration
H&E Stain = 50µm

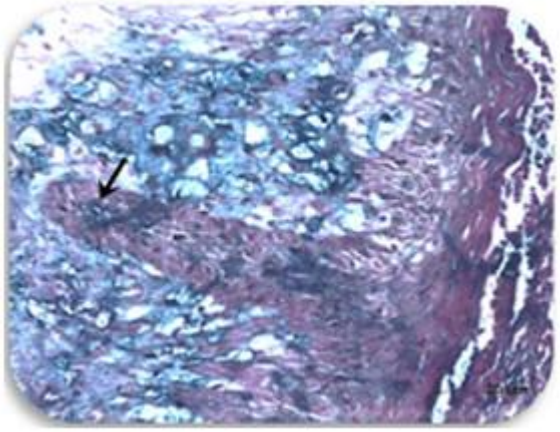

Plate 4: Group I- 7th Day
Wound Bed -Moderate Pulpy immature collagen fibres with Epidermal Peg
Masson's Trichrome Stain = 20µm

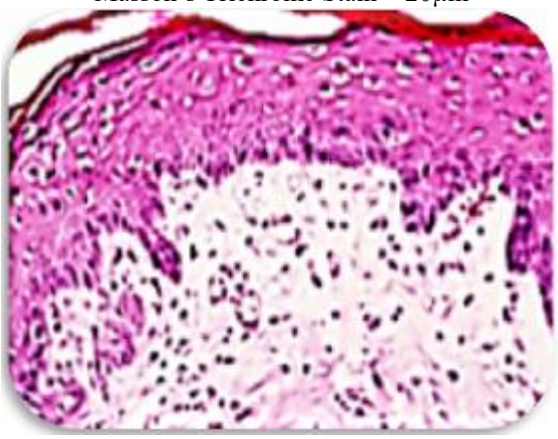

Plate 5: Group I -14th Day
Wound Bed -Hyperplastic, Complete epithelisation
H&E Stain = 100µm

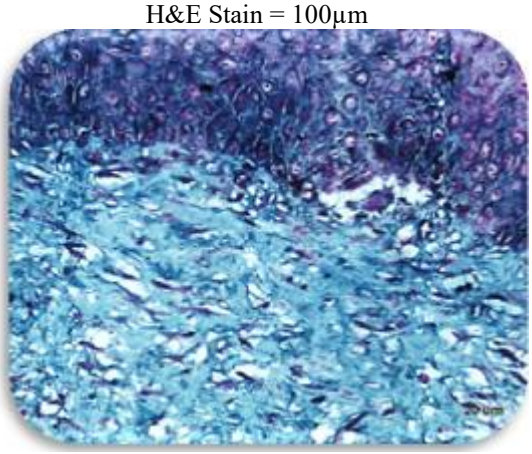

Plate 6: Group I -14th Day
Wound Bed - Mature collagen Fibres with Moderate Neovascularisation
Masson's Trichrome Stain = 20µm

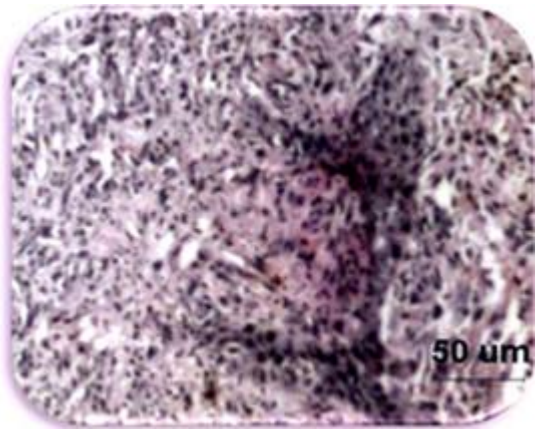

Plate 6: Group II – 3rd Day
Wound Bed -Moderate Angiogenesis with Neutrophilic and Macrophage infiltration
H&E Stain = 50µm

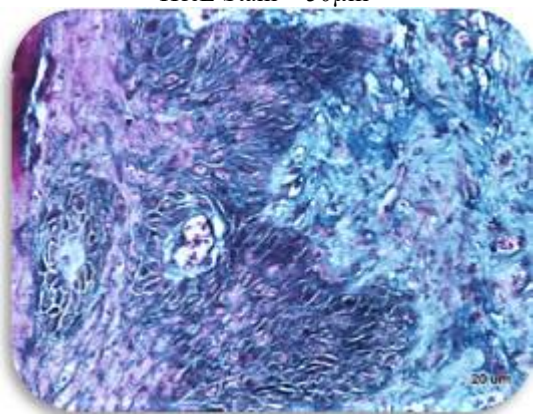

Plate 7: Group II- 3rd Day
Wound Bed - Epidermal peg with Moderate Neovascularisation
Masson's Trichrome Stain = 20 µm

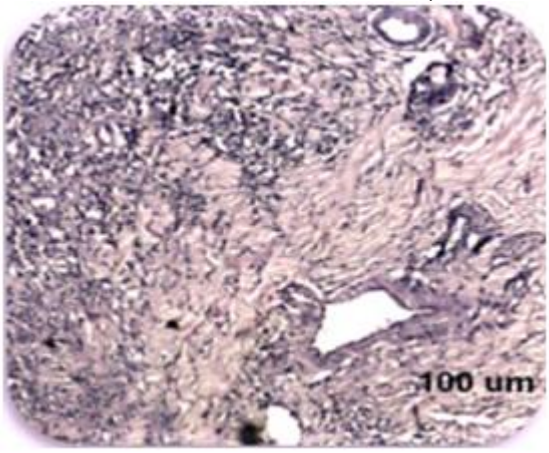

Plate 8: Group II – 7th Day
Wound Bed - Moderate Epithelisation and Neutrophilic Infiltration
H&E Stain = 50µm

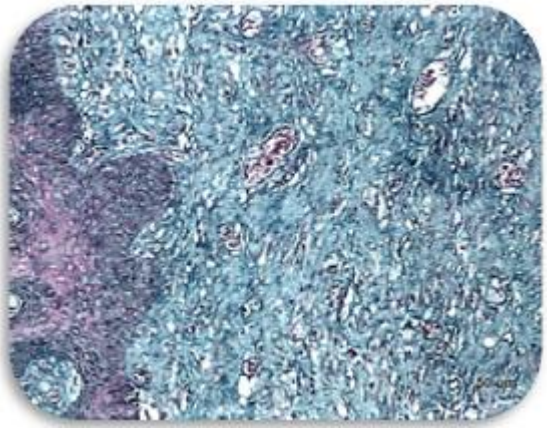

Plate 9: Group II- 7th Day
Wound Bed - Moderate immature collagen fibres with Epidermal Peg
Masson's Trichrome Stain = 50µm

Plate 10: Group II- 14th Day
Wound Bed - Hyperplastic Hyperkeratinised Epidermis and Dermis
H&E Stain = 100µm

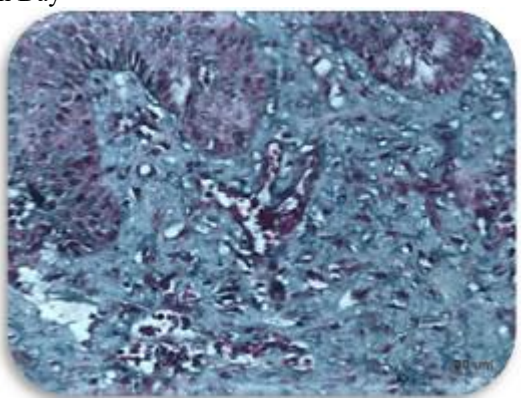

Plate 11: Group II- 14th Day
Wound Bed - Mature collagen fibres with Moderate formed Epidermal peg
Masson's Trichrome Stain = 20 µm

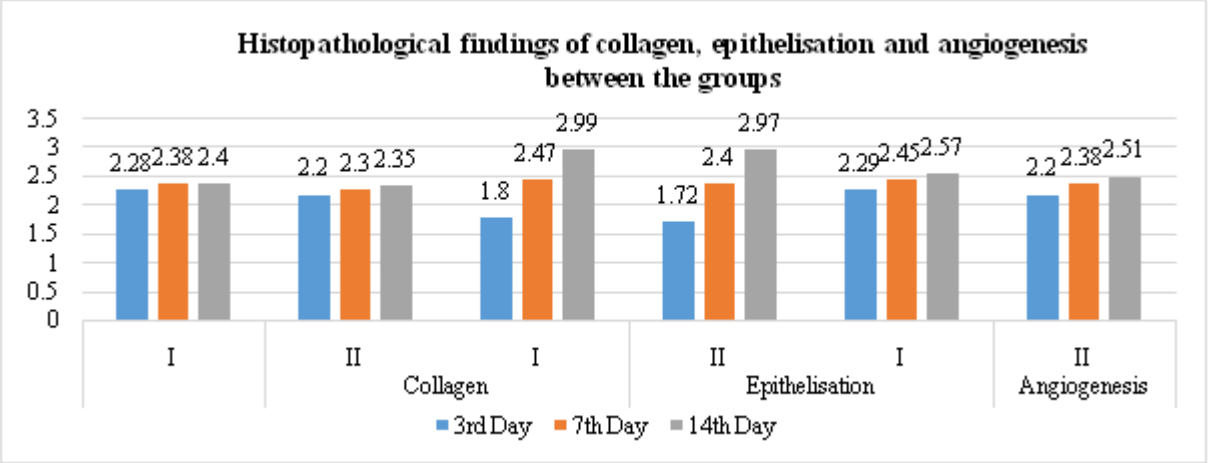

Table: Histopathological findings collagen, epithelisation and angiogenesis of recipient wound bed

Parameters	Groups	Treatment Days (Recipient Wound Bed) Mean ± S.E.		
		3rd Day	7th Day	14th Day
Collagen	I	2.28 ± 0.83	2.38 ± 0.72	2.40 ± 0.62
	II	2.20 ± 0.63	2.30 ± 0.51	2.35 ± 0.71
Epithelisation	I	1.80 ± 0.78	2.47 ± 0.62	2.99 ± 0.00
	II	1.72 ± 0.80	2.40 ± 0.63	2.97 ± 0.03
Angiogenesis	I	2.29 ± 0.11	2.45 ± 0.98	2.57 ± 0.02
	II	2.20 ± 0.01	2.38 ± 0.19	2.51 ± 0.62