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Effects of local infiltration of peripheral blood mononuclear cells with phonophoresis on wound healing in dogs

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ABSTRACT

A total of six dogs with extensive wounds of 5×5 cm^2 size and above were taken with the objective of evaluating the efficacy of autologous peripheral blood mononuclear cells (PBMC) with phonophoresis on wound healing in dogs. Autologous PBMCs were isolated freshly on 0, 7, 14, 21 and 28th day from blood by using density gradient centrifugation. Obtained PBMC was infiltrated around the wounds. Phonophoresis was done using therapeutic ultrasound massage at 0.5 watts/cm^2 for 10 min for all the six animals. Clinical evaluation and wound planimetry was performed during post treatment period. Most affected breed was mongrel and main aetiology was automobile accident. The wounds were mostly located on limbs, neck and abdominal region. PBMC was noticed to be compatible to the tissues. No signs of infection were noticed in the wounds after administration of PBMC, rather healthy granulation tissue-formation, increasing values of wound contraction, epithelization and healing were noticed. PBMC-phonophoresis in wound healing of canines in present study was found to be effective in extensive wounds.

Keywords: Dogs, Peripheral blood mononuclear cells (PBMC), Phonophoresis, Wound

Healing of extensive wounds in companion animals can be challenging as they need complex management with accurate debridement of damaged and necrotic tissues, local and systemic infection control, protection of tissues beneath the wound and induction of cutaneous tissue regeneration (Zubin *et al*. 2015). Vascular endothelial growth factor (VEGF) is a strong positive regulator of angiogenesis and stimulates endothelial cell functions needed for new blood vessel formation, such as proliferation, migration, differentiation, and survival. It increases the vascular permeability and acts as mitogenic agent for endothelial cells (Leung *et al*. 1989, Hosgood 2003).

Cytokines like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and interleukin IL-10 are released by PBMC. They could influence fibroblast activities in the remodelling phase of the healing process (collagen, collagenase and stromelysin synthesis) (Nami *et al*. 2016). PBMCs enhanced neovascularization and epidermal healing in a model of chronic full-thickness skin wound in diabetic mice (Loukianova *et al.* 2003). Topical application of PBMC secretome improved the epidermal differentiation and angiogenesis of the wounds respectively in a porcine burn model (Hacker *et al*. 2016).

Phonophoresis is a non-invasive, painless method that has less adverse effects and is well tolerated. It is a technique by which therapeutic ultrasound is used to introduce pharmacologic agents and able to kill bacteria by activating the sonosensitizers to produce reactive oxygen species, which are toxic to microbes in wound healing (Fares *et al*. 2017).

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MATERIALS AND METHODS

A total of six dogs irrespective of breed, age and sex presented with skin wounds sized 5×5 cm^2 and above were taken for the study. After wound bed preparation, the PBMC was infiltrated and phonophoresis was done. The animal particulars, viz. breed, age and sex were documented. The complete history was obtained from the owner regarding the probable etiological factor, date and time of occurrence of the wound and medication if any undertaken. Subjective evaluation of the wound was carried out on 0, 7, 14, 21 and 28th day of presentation. Location (Abramo *et al.* 2004), colour, nature of exudate, volume of exudate, odour of exudate, wound edges, type of tissue in the wound, tunnelling, peri wound area, foreign body present in the wound were documented.

Isolation of PBMC: The whole blood (4-7 ml) from cephalic vein was collected in the EDTA coated vacutainer on 0, 7, 14, 21 and 28th day of presentation (Bhattacharjee *et al*. 2017, Vanaki *et al.* 2018). Whole blood (4 ml) was diluted with equal volume of phosphate-buffered saline (PBS) solution and mixed gently. Aseptically transferred 4 ml of HiSep (Himedia- a sucrose density gradient solution LSM 1077) into a 15 ml clean centrifuge tube.

The HiSep solution was gently overlaid with 8 ml diluted blood without breaking the surface plane and centrifuged at 2400 rpm (slow centrifuge) at room temperature (25°C) without brake for 30 min. Following centrifugation, the supernatant containing the plasma and platelet above the interface band was discarded by aspiration using the micropipette leaving the granulocytes and erythrocytes settled as red pellet. Using a micropipette, the mononuclear cells were carefully aspirated and transferred to a clean centrifuge tube and 10 ml of phosphate buffered saline was added to the mononuclear cells in the centrifuge tube and mixed by gentle aspiration. The cells were washed by centrifuging at 1,400 rpm at room temperature (15-25°C) for 10 min to remove the HiSep solution and to reduce the number of platelets. Washing the cells was done twice with isotonic phosphate buffered saline and then finally the PBMC pellet was resuspended in the same PBS buffer for the further application. Microscopic examination of PBMC smear was done using Leishman stain (Adewoyin 2014). Enumeration of the PBMCs was done to determine cells and their numbers using trypanblue dye and neubauer chamber (Bhattacharjee *et al*. 2017, Vanaki *et al.* 2018).

*Infiltration of peripheral blood mononuclear cells and phonophoresis:*The skin around the wound was prepared aseptically and 1 ml of freshly prepared autologous PBMC was infiltrated locally at 5 different point (Fig. 1). Ultrasound massage was done in the peri wound area on 0, 7, 14, 21 and 28th day of presentation. Therapeutic ultrasound massage was given after infiltration of PBMC with HMS medical systems, Indsonic machine (1-3 MH) at 0.5 watts/cm^2 for 10 min after smearing adequate amount of ultrasound gel, the probe was placed gently against the skin surface, moved slowly and deliberately (Fig. 2).

Wound planimetry (Fig. 3) was done using a black, extra-fine and felt-tipped marking pen to trace the border of intact epidermis onto a sheet of clear acetate. The measurements were taken non sequentially for each wound. After completion of each tracing, number of squares within the traced area of each acetate grid were counted using graph sheet (Wunderlich *et al*. 2000).

Linear wound measurement was performed by measuring the distance between the wound edges in a straight line in centimetres. Wound length was measured first and taken as the longest distance between the two edges of the wound and the width was the longest distance measured perpendicular to the wound length.

Wound depth (Fig. 4) was measured as the distance from the visible surface to the deepest area by placing a cotton tip applicator into the deepest portion of the wound (Murthy *et al*. 2013). Area was calculated. These values were utilized for the calculation of percentages which aimed to measure the healing process using the percent wound healing, percent epithelialization, and percent wound contraction (Stanley *et al.* 2013).

Wound dressing: Wounds were protected with povidone iodine impregnated gauze and bandage**.**

Percent of wound epithelialization day (n) =

$$\frac{\text{Area of epithelium day (n)}}{\text{Total wound area day(n)}} \times 100$$

Percent of wound contraction

Step 1: Total wound on day (n) as % of original =

$$\frac{\text{Total wound area day (n)}}{\text{Original wound area day (0)}} \times 100$$

Step 2: Percent of wound contraction day (n) = 100 – Total wound on day (n) as % of original

Percent of total wound healing

Step 1: Open wound day (n) as % original =

$$\frac{\text{Open wound area day (n)}}{\text{Original wound area day (0)}} \times 100$$

Step 2: Percent of total wound healing day(n) =100 – open wound day(n) as % of original

RESULTS AND DISCUSSION

A total of six dogs irrespective of breed, age, sex and etiology presented with skin wounds sized 5×5 cm^2 and above were taken for the study (Table 1). Out of 6 dogs, three were Mongrel, one each was Spitz cross, Labrador retriever and German shepherd. Age group was between 2-8 years. Five male dogs and one female. Etiology was automobile accident in four dogs (Aithal *et al. 1*999, Akinrimade 2014) and self-mutilation wound and dog bitten wound in one each. Dogs were presented between 1-10 days past injury.

Clinical evaluation of the wound: Location of wounds was abdomen in two cases, limbs in two, neck in one and head in one. On the day of presentation, colour was red which turned into pink and pale pink on day 21 and 28

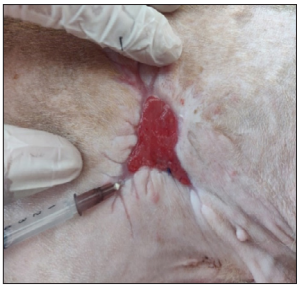

Fig. 1. Infiltration of PBMC around the wound.

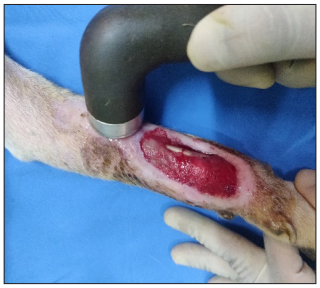

Fig. 2. Phonophoresis around the wound.

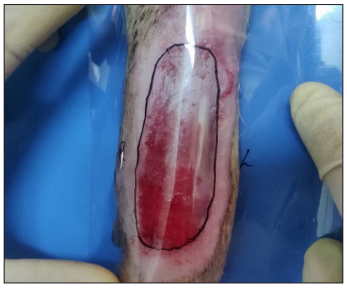

Fig. 3. Wound planimetry.

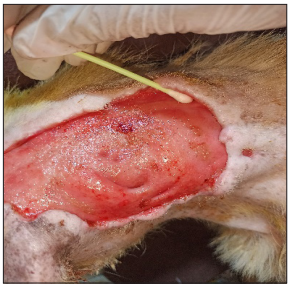

Fig. 4. Measurement of wound depth.

Table 1. Signalment and anamnesis of the cases.

Animal No.	Breed	Age (in years)	sex	Etiology	Day of occurrence of the wound	Previously treated	Location of the wound
1	German shepherd	3	M	Dog bite	10	Yes	Left lateral aspect of neck
2	Spitz cross	4	M	Automobile accident	7	No	Dorsal aspect of left radius and ulna (Forearm)
3	Mongrel	3	M	Automobile accident	10	No	Dorsal aspect of head
4	Labrador	8	F	Self-mutilation after surgery	5	No	Right ventral abdomen
5	Mongrel	4	M	Automobile accident	7	Yes	Lateral aspect of right Knee
6	Mongrel	2	M	Automobile accident	1	No	Right ventral abdomen

which indicate re-epithelialisation and wound healing (James and Bayat 2003, Jothil *et al.* 2006). Nature and volume of exudate was serosanguinous and moderate on day 0, which reduced and became nil on day 28th in five cases which indicated that the PBMC with phonophoresis helps in wound healing, due to anti-inflammatory cytokines (Chen *et al*. 2017). Odour of exudate was foul and musty on day of presentation**,** after treating with PBMC and phonophoresis on day 28, no odour was noticed in all the dogs.

Wound edges were well defined, firm and attached. Peri wound area was also dry and healing was noticed on 28th post operative day. Complete healing ranged from 14 to 60 days based on the size and depth of wounds rather than on the etiology.

PBMC concentration: Mean±SE of PBMC concentration was 2.90±0.18.

Type of tissue present in the wound: The animals showed healthy granulation (bubbly and smooth) and epithelialization by 14th day of observation. On day 28, smooth granulation and epithelial tissue was seen in five and epithelial tissue was noticed in one.

Tunnelling/Undermining (cm): The Mean±SE of the undermining of the wound (cm) was 0.55±0.35 and 0±0 on day 0 and 28 respectively.

Wound planimetry: Length, width and depth of the wounds decreased gradually throughout the period of study.

*Percent of wound epithelialisation***:** The Mean±SE of the wound epithelialisation (cm) was 28.27±2.27, 54.97±6.83, 68.27±6.88, 76.18±5.12 and 82.84±3.93 on 0, 7, 14, 21 and 28th day of presentation respectively.

Percent of wound contraction: The Mean±SE of the contraction of the wound (cm) was 0±0, 44.36±4.23, 69.67±7.52, 83.77±3.69 and 93.08±2.26 on 0, 7, 14, 21 and 28th day of presentation. Wound contraction depended on adherence of the skin edges to the underlying granulation tissue as the edges are actively moved over the wound (Bohling *et al*. 2004). Epithelialization favoured mobilization of the epidermal cells by centripetal migration across the granulation bed and initiated wound contraction as stated by Dart *et al.* (2002).

Percent of wound healing: The Mean±SE of the percent of wound healing was 0±0, 77.27±5.04, 90.10±2.54, 93.51±1.58, and 97.98±0.96 on 0, 7, 14, 21 and 28th day of presentation respectively.

Infiltration of PBMC with phonophoresis showed uncomplicated wound healing in all the cases. PBMC could have influenced fibroblast activities in the remodelling phase of the healing process (collagen, collagenase and stromelysin synthesis). The growth factors from PBMC stimulated the fibroblastic proliferation and favoured the wound contraction and healing (Nami *et al*. 2015). Consistent increase in the wound healing might be due to paracrine effect of PBMC as suggested by Kado *et al*. (2018) where the authors demonstrated the direct involvement in vasculogenesis and indirect paracrine

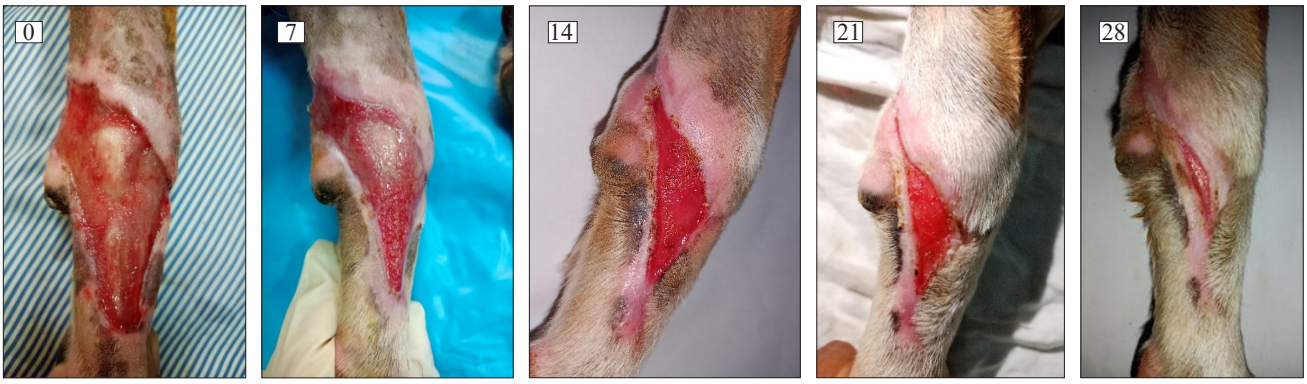

Fig. 5. Animal No. 5 on 0-28 days.

Table 2. Mean±SE values of percent of epithelialization, contraction and wound healing

Particular	Day 0	Day 7	Day 14	Day 21	Day 28
Percent of wound epithelialisation	28.27±2.27	54.97±6.83	68.27±6.88	76.18±5.12	82.84±3.93
Percent of wound contraction	-	44.36±4.23	69.67±7.52	83.77±3.69	93.08±2.26
Percent of total wound healing	-	77.27±5.04	90.10±2.54	93.51±1.58	97.98±0.96

upregulation of several mediators known to participate in wound healing, such as IL-6, IL-4, IL-10, and FGF 32 in porcine.

All the animals showed uncomplicated wound healing. Bensinger *et al*. (1995), Yu *et al*. (1920), Hassan *et al*. (1996) and Urbano-Ispizua *et al*. (1997) also stated that PBMC was superior to other cell sources for cell-based therapy.

Based on the findings of the study, it may be concluded that wound planimetry was used to calculate the wound area and to gauge the healing process in response to treatment were found to be effective. Isolation of peripheral blood was minimally invasive. PBMC was noticed to be compatible to the tissues. No signs of infection were noticed in the wounds after administration of PBMC. Healthy granulation tissue formation was noticed, increasing values of wound contraction, epithelization and wound healing were noticed. On the perusal of the available literature, minimal reports were found on the use of PBMC-phonophoresis in wound healing in canines. It could be inferred that the use of PBMC with phonophoresis on wound healing is effective in promoting angiogenesis and quick wound healing in extensive wounds. Hence it can be recommended for treatment of large wounds.

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