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Therapeutic use of *Lucilia sericata* maggot in controlling bacterial bio-burden in Rat wound model

Borkataki, S.[1*], Katoch, R.[2], Goswami, P.[3], Bhat, A.[4], Bhardwaj, H.R.[5], Chakraborty, D.[6] and Chandrawathani, P.[7]

[1]Division of Veterinary Parasitology, Faculty of Veterinary Sciences, SKUAST-Jammu, R S Pura, Jammu, India
[2]Division of Veterinary Parasitology, Faculty of Veterinary Sciences, SKUAST-Jammu, R S Pura, Jammu, India
[3]Division of Veterinary Pathology, Faculty of Veterinary Sciences, SKUAST-Kashmir, Shuhama, Srinagar, India
[4]Division r of Veterinary Microbiology, Faculty of Veterinary Sciences, SKUAST-Jammu, R S Pura, Jammu, India
[5]Division of Teaching Clinical Complex, Faculty of Veterinary Sciences, SKUAST-Jammu, R S Pura, Jammu, India
[6]Division of Animal Genetic & Breeding, Faculty of Veterinary Sciences, SKUAST-Jammu, R S Pura, Jammu, India
[7]Research & Innovation Division, Department of Veterinary Services, Putrajaya, Malaysia
[*]Corresponding author e-mail: borkataki_sanku@rediffmail.com
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Abstract. Delayed wound healing due to extraneous bacterial contamination, antibacterial resistance and other associated factors are of great concern in dealing patients having chronically infected wound. Medicinal properties of certain maggots of Calliphoridae family are known for its effective wound debridement therapy. The objective of the study was to evaluate the wound healing potential of maggots of *Lucilia sericata* in an experimentally infected cutaneous wound model in Wistar rat. The study was carried out by using male Wistar rats (n=48) by creating excisional wounds and later contaminated with mixed population of gram positive and gram-negative bacteria. Animals were divided randomly in to four groups with 12 individuals each, being denominated as control, antibiotic treated, maggot treated, and antibiotic plus maggot combination treated group. Ten pre-sterilized maggots were applied per centimetre square wound bed for 24 hours. Different wound kinetics in *L. sericata* maggot treated wounds revealed significant reduction in wound area with maximum contraction, early elimination of bacterial bioburden as compared to group of infected control and group of rats receiving only antibiotic treatment. The histopathological examination of wounded tissue of maggot treated groups showed early and better epithelialization, collagenation and neovascularization with complete healing of wound in two weeks. The maggot effects on healing when used singly or in combination with antibiotic were recorded to be similar. The results of the present study clearly demonstrate that the maggots of *L. sericata* possesses a definite antibacterial action along with removal of dead tissues and effectively reduced the bacterial bio-burden in infected wound and induced wound healing quickly.

INTRODUCTION

Wound healing is a complex process of regeneration of tissues. Delayed wound healing is incriminated by various agents, particularly due to extraneous infection by pyogenic bacteria as well as presence of necrotic tissues over the wound surfaces (Guo & DiPietro, 2010). Bacterial infection at wound makes it chronic in nature and conventional application of antibacterial ointment in some cases reported unsuccessful due to the development of resistance bacteria to antibiotic (Filius & Gyssens, 2002). Maggot debridement therapy (MDT) is essentially a controlled therapeutic

myiasis, facultative myiasis or also known as wound myiasis is one of the ancient remedies for modern medicine and considered as an emerging field of alternative medicine that has made a major come back in the 21st century (Sherman *et al.*, 2007; Geary *et al.*, 2009). In the last few decades doctors have used maggot therapy in the world to treat human wound (Sherman 2000, Sherman 2002). The use of maggot was as old as US civil war as Napoleon's Surgeon-in-Chief (Baron Dominic Larrey) reported that maggots developing in infected wound prevented further infection and accelerated wound healing (Goldstein, 1931). The exploring of application of living maggots in ceasing bacterial population in wound and induction of healing may be needed for management of chronic wound or in the unsuccessful cases of healing of wound by conventional antibiotic therapy.

Although several researches have been conducted on effectiveness of maggot in wound healing (Sherman 2002; Sherman *et al.*, 2007), very little interest has been shown in India about use of MDT in Human as well as in veterinary medicine. The present study encompassed fly *L. sericata* as candidate species of maggot therapy in treating contaminated wound by bacteria in Wistar rat. Keeping the prevalence of *L. sericata* fly in the existing area (Kour *et al.*, 2015) as well as therapeutic challenge in management of chronic wound and reducing the risk factors that inhibit wound healing, the present study was designed to evaluate the effectiveness of the fly larvae in an experimental infected excisional wound model in Wistar rat.

MATERIALS AND METHODS

Preparation of experimental animals

The study was conducted in forty-eight healthy male Wistar rat (*Rattus norvegicusalvinus*, Rodentia, Mammalia) weighing 200-250 grams which were purchased from Indian Institute of Integrative Medicine (IIM, Regional Research Laboratory) Jammu and were placed in the divisional laboratory animal house. Animals were placed six in each large polypropylene cage with controlled environment of twelve hours light and dark cycle and provided with standard palletized feed and RO water as per the standard provided by Laboratory Animal house, IIIM (RRL) Jammu. The study protocol was subjected to Institutional Animal Ethics Committee (IAEC) for experimental clearance (AU/FVSJ/14-15/VPA/464), dated 12-01-2015. Animals were divided randomly in to four groups of twelve animals each, categorized as Group I (C) for control without any treatment, Group II (A) for antibiotic treated, Group III (M) for maggot treated and Group IV (A+M) for antibiotic plus maggot combination applied for healing of infected cutaneous excisional wound created in these animals. The wounding procedure was carried out under anaesthesia using Ketamine hydrochloride (100mg/kg) and Xylazine (10mg/kg) in combination cocktail @0.10mL/gm intraperitonially. The dorsal surface of the rats near above the shoulder region was shaved with sterile razor and of $2X2cm^2$ (400 mm^2) excisional wound was prepared and immediately exposed to bacterial infection by inoculating a mixture of 1-2 pure colonies of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* with sterile swab and rubbed over wound surface. These animals were caged individually and left undisturbed. The wound became infected and chronic as characterized by oozing out of pus and formation of scar along with necrotic tissue in wound bed on 4th day of wounding and 4th day of preparation of wound was considered as 0-day, i.e. the day of initiation of treatment.

Disinfection of eggs

Adults fly of *L. sericata* were caught through net from natural environment/field including garden, different meat/fish shop of the locality, live-stock farm etc and were reared in laboratory condition. The colonies of the candidate species were maintained on meat diet in laboratory and used for this study. The eggs were collected and were subjected to disinfection for use in maggot therapy following the method laid previously

(Sherman *et al.*, 2007). Eggs hatched in diet were collected separately with the help of fine brush and deposited in moistened filter paper on a sterile petridish and then disinfected with dilute phenol (3% Lysol). Disinfected eggs were then transferred to sterile nutrient agar plate moistened with cotton and kept it in room temperature for further development of L_1 larvae. These active live maggots were immediately used for application over wound in the present study.

Method of application of treatment

As per the design of the experiment, application of maggots was done in the infected wound bed on the 0-day of initiation of treatment in Group III (M) and Group IV (A+M) animals at a dose-rate of 10 maggots/ cm^2 of wound bed immediately after hatching. Then maggots were left within their dressing for 24 hours (Sherman 2009) by making a cage like dressing of two ply light gauze and glued covering the surrounding skin of the wound. Maggots were recovered alive after 24 hours and disposed of as other infectious dressing waste. Sensitive antibiotic (Ciprofloxacin cream 0.1%) was applied once on the wound of Group II (A) and Group IV (A+M) of the experiment whereas Group I (C) animals were kept as control. The rats were anaesthetized at different days of observation starting from 0 day of initiation of treatment at weekly interval in all the four treatment groups for measurement of wound contraction, assessment of wound bio burden (total bacterial count), collection of granulation tissue from wound area for histological evaluation.

Measurement of wound area and contraction

The area of wound contraction was measured on 0, 7^{th}, 14^{th} day (weekly interval) till experience of early complete healing in any of the group irrespective of treatment. Surface area of wound was measured by tracing paper method (Ahanger *et al.*, 2010) by tracing its contour using a transparent paper on different day's interval starting from 0 day of initiation of treatment. The wound area was calculated by weight method and then converted in percent of wound contraction and calculated by using the following formula (Zhang *et al.*, 2010).

$$\textbf{Wound contraction (\%)} = \frac{Woundareaonday(0) - woundareaonday(n)}{Woundareaonday(0)} X\,100$$

Total bacterial count for assessment of wound bio-burden

The total viable count of bacteria (CFU=colony forming units) from wound bed in different days were determined by following the standard plate counting method (Mendes *et al.*, 2012) by maintaining aseptic condition. For each treatment group four numbers of animals were selected randomly for total viable bacterial count assessment of wound. The wound was debrided by mechanical removal of scab and bacterial collection was performed by using one-point method (Sullivan *et al.*, 2004). The centre of each wound was scrubbed carefully by rotating sterile swab 3 times clockwise using manual pressure to produce small amount of exudates. The inoculated swab was inserted into a pre-sterilized tube containing 4mL sterilized phosphate buffer solution (pH-7.4) and vortexed for 30s. A 200µL aliquot of the suspension was transferred to 1.8 ml of phosphate buffer solution (pH-7.4) and the same was used for serial 10-fold dilution like 1:10, 1:100, 1:1000, 1:10 000 etc for quantification of bacteria. A 200 µL of each dilution was placed on the nutrient agar plate and spread uniformly with the help of 'L' shaped spreader. Then the plates were incubated under aerobic conditions at 37°C for 24 hours, and subjected to counting of CFU. The entire procedure was repeated in all the treatment groups (Group I, II, III, and IV) at weekly interval till observed complete healing in any of the treatment group. The results were expressed in the number of colonies forming units per ml of initial suspension. Quantitative results were presented in log as the mean ± standard error of total viable bacteria and expressed as logarithm-transformed values (log[cfu/ml]) from initial suspension of samples.

Histopathological examination of wound tissues for wound closure kinetics

Around 50 mg of granulation tissue sample from wound area of four randomly selected animals of each treatment group were collected in 10% neutral buffered formalin (NBF) at different days of observation under anaesthesia (as described above) from all the treatment groups for histopathological study of wound closure kinetics. Tissue section were also stained with Masson Trichrome (for connective tissues), in addition with routine H&E (Luna 1968). Stained sections were observed under light microscope at different objectives of 10X, 20X, 40X, 100X and digital photography was done accordingly.

The inference data was subjected to two ways ANOVA, calculating the mean and standard error of each parameter (Snedecor 1994) for statistical significance (p=0.05) between different groups.

RESULTS

Effect of maggot on wound contraction in Wistar rat

Table 1 shows the effect of maggot in reduction of area and contraction in excisional infected wound in Wistar rat. The wound area decreased in the entire treated groups significantly ($p \leq 0.05$) over the days of observation within the group (Table 1). The results showed amongst all the 4 groups, maggot treated wound area reduced in size more quickly and completely than antibiotic treated wound and control wound (Fig. 1). Above 95% contraction of wound was achieved within 14th day of wounding in maggot treated group with complete healing, while non maggot treated were still not completely healed after 14th day of observation (Table 1). In the maggot application groups i.e. in Group III (M), the mean wound area was decreased from (551.06±10.77 mm^2) to (25.67±1.22 mm^2) and similarly in Group IV (A+M) i.e. (530.27±12.50 mm^2) to (23.87±2.25 mm^2) on day 14th of wounding with complete healing. In terms of wound contraction, higher contraction (>95%) tuning to wound closure was observed in Group III (M) and Group IV (A+M) at 14th day of wounding. In both 7th and 14th day of wounding, wound contraction between the Group III (M) and Group IV (A+M) was not found significant.

Wound bioburden in different groups of Wistar rat

The average total bacterial colony counts or quantitative results expressed in mean logarithm-transformed values in log (CFU) per millilitre of initial suspension presented in Table 2. The result showed, contamination of wound decreased significantly ($p \leq 0.05$) at 7th and 14th day for maggot application Group III (M) and Group IV (A+M), but not significant ($p > 0.05$) in the case of group without maggot i.e. control infected Group I (C) and Group II (A) receiving only antibiotic at 7th and 14th day.

The average total bacterial counts log (CFU) per millilitre of initial suspension was found similar amongst the all groups at 0-day of initiation of treatment and do not

Table 1. Wound area and per cent contraction in different days of observation in different groups of Wistar rat

Group	0 day	7th day		14th day	
	Area (mm^2)	Area (mm^2)	% Contraction	Area (mm^2)	% Contraction
Gr I (C)	553.37^{Ac}±11.37	359.08^{Cb}±9.68	34.97^{Aa}±1.66	185.57^{Ca}±7.05	66.36^{Ab}±1.33
Gr II (A)	532.33^{Ac}±12.73	249.48^{Bb}±9.85	52.72^{Ba}±2.31	129.62^{Ba}±7.17	75.41^{Bb}±1.57
Gr III (M)	551.06^{Ac}±10.77	137.06^{Ab}±6.34	74.90^{Ca}±1.45	25.67^{Aa}±1.22	95.34^{Cb}±0.20
Gr IV(A+M)	530.27^{Ac}±12.50	136.55^{Ab}±10.02	74.05^{Ca}±2.12	23.87^{Aa}±2.25	95.52^{Cb}±0.38

Similar superscript does not differ significantly (P<0.05). Smaller superscript denotes within the groups between the days and capital superscript denotes between the groups within different days.

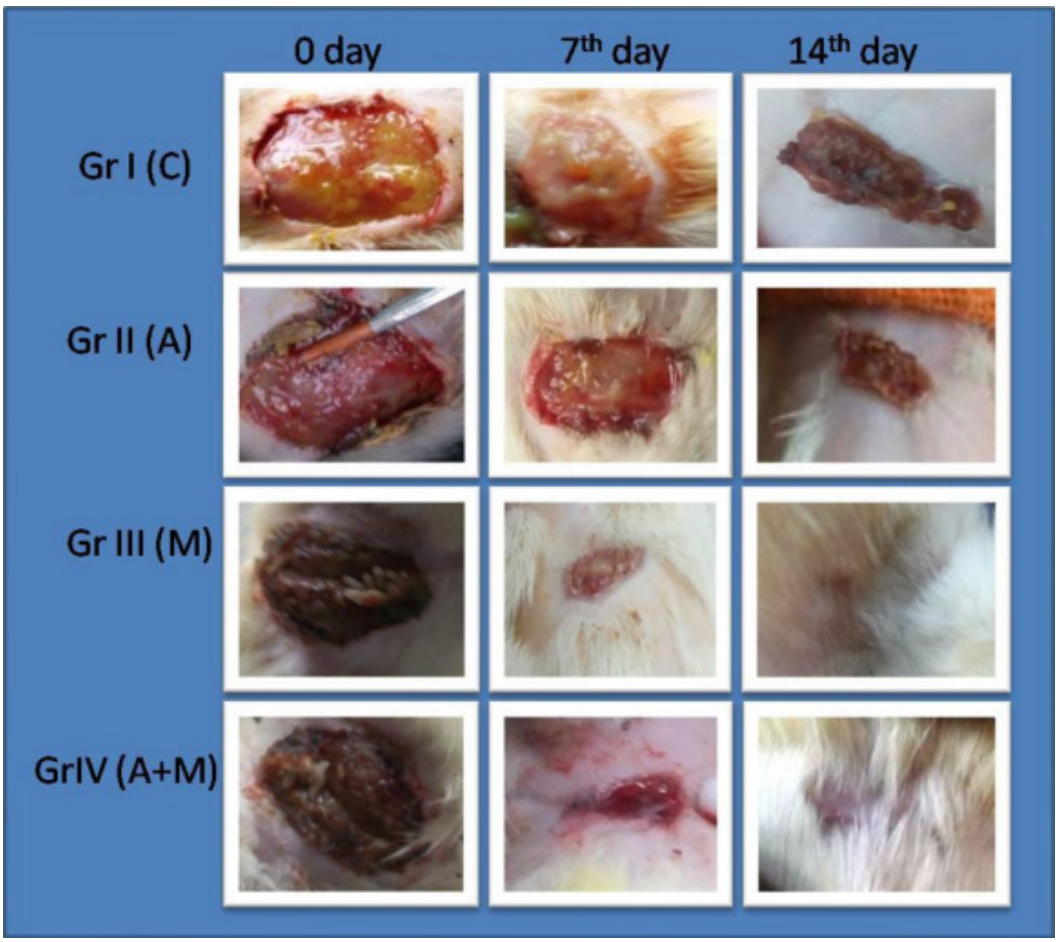

Figure 1. Wound contraction in different days of healing in different treatment groups in Wistar rat.

Table 2. Total bacterial colony counts in log (CFU)/ml of initial suspension in different days of observation in Wistar rat

Group	log (CFU) /ml of initial suspension		
	0 day	7th day	14th day
Gr I (C)	$7.73^{Ab}\pm0.01$	$6.99^{Ca}\pm0.01$	$6.14^{Ca}\pm0.07$
Gr II (A)	$7.71^{Ab}\pm0.01$	$5.58^{Ba}\pm0.03$	$4.90^{Ba}\pm0.08$
Gr III (M)	$7.69^{Ac}\pm0.02$	$3.73^{Ab}\pm0.05$	$0.59^{Aa}\pm0.59$
Gr IV (A+M)	$7.73^{Ac}\pm0.02$	$3.62^{Ab}\pm0.11$	$0.57^{Aa}\pm0.57$

Similar superscript does not differ significantly ($P<0.05$). Smaller superscript denotes within the groups between the days and capital superscript denotes between the groups within different days.

differ significantly. In Group II (A), where antibiotics was used, bacterial count decreased significantly when compared to Group I (C) at 7th and 14th day of wounding, but appreciable bacterial count i.e. (4.90±0.08) was existed even on 14th day of wounding in Group II (A). A parallel reduction of bacterial count at 7th day and negligible count at 14th day of wounding in maggot treated groups Group III (M) i.e. (0.59±0.59)

and Group IV (A+M) i.e. (0.57±0.57) was observed indicating maggots' effects on reducing the bacterial load at wound surface. These low bacterial counts were significantly differing with control Group I (C) i.e. 6.14±0.07 and with Group II (A) i.e. 4.90±0.08.

Histopathological examination of wound healing

The histopathological examination of wounded tissue at different days of observation showed varied changes in formation of granulation tissues. There was an abscess formation along with necrotic tissues involving deep dermis in wound tissues/bed at 0-day of initiation of treatment in all the groups of rats. The superficial necrotic mass was also admixed with bacterial colonies with evidence of haemorrhage in deep dermis (Fig. 2). Abundant polymorphonuclear (PMN) cellular infiltration was noted with characteristics of sub-acute inflammation in all the group of rat at 0-day of initiation of treatment (Fig. 2).

In maggot treated groups (maggot singly and in combination with antibiotic), the histopathological observation of granulation tissue at 7th day showed lightly infiltrated PMN cells along with macrophages/ monocytes indicative of ending of inflammatory phase at wound. Purulent inflammation was replacing by granulation tissue along with neovascularisation was prominent at 7th day of maggot treatment (Fig. 3). The wound granulation tissue was rich in fibroblasts, which was infiltrated perpendicular became parallel and abundant newly formed blood vessels (Fig. 4). The appearance of connective tissue, collagen apparent on histological strain with Masson's trichrome indicated in Fig. 5 at 7th day of maggot application group. The similar changes were not observed for Group I (C) and Group II (A) at 7th day instead there was evidence of chronic active inflammation. The wound tissue collected at 14th day of wounding from maggot application groups (Group III & IV) showed complete closure of wound revealing organized fibrous tissue formation at dermis and new hair follicle formation and re-epithelization (Fig. 6). In the other, i.e. in Group II (A) and Group I (C), initiation of healing was noticed with formation of collagen and neovascularisation, but complete closure of wound was not observed in any of the animals of these groups at 14th day of observation.

DISCUSSIONS

Basic information of healing is known by measurement of wound area and volume (Romanelli 2002). Wound area and wound contraction measured in the present study in different group of rats in different days of observation showed maggot (alone and with antibiotic) treated wound area reduced in size more quickly and completely than were with antibiotic treated and control wounds.

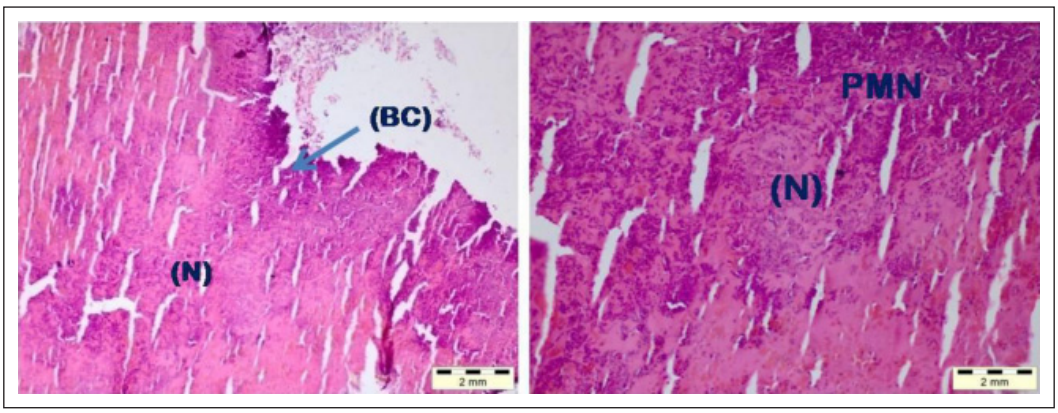

Figure 2. Sub-acute inflammation of dermis at 0-day (without treatment) evidence of necrotic mass (N)with PMNs cells admixed with bacterial colonies (BC). H&E, 200X.

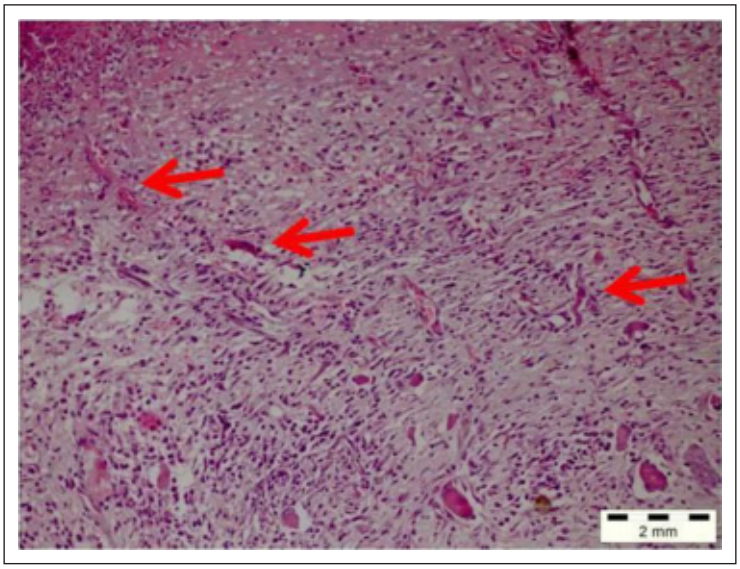

Figure 3. Granulation tissue formation with fibroplasia and neovascularization (red arrow) in dermis at 7th day on maggot treatment. H&E, 200X.

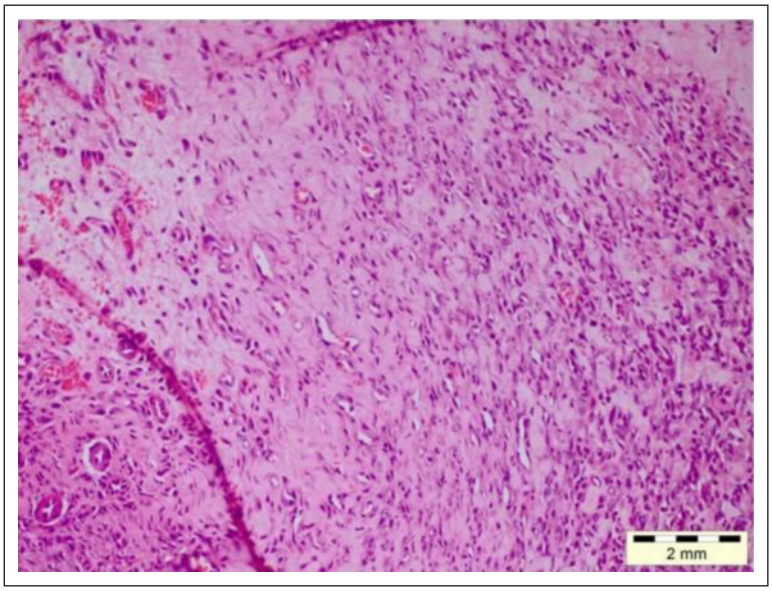

Figure 4. Infiltrating fibroblast perpendicular becomes parallel and formation of blood vessels noted at 7th day of Maggot treated group. H&E, 200X.

Similarly, contraction was found maximum (>95%) led closure of wound in maggot treated achieved in two weeks with complete healing when compared to antibiotic treated (75.0%) and control (66.0%) wounds respectively. It implies that maggot therapy was more effective and efficient in controlling septic contaminated wound than routine antibiotic treatment. The present study used pre-sterilized *L. sericata* maggot for treating

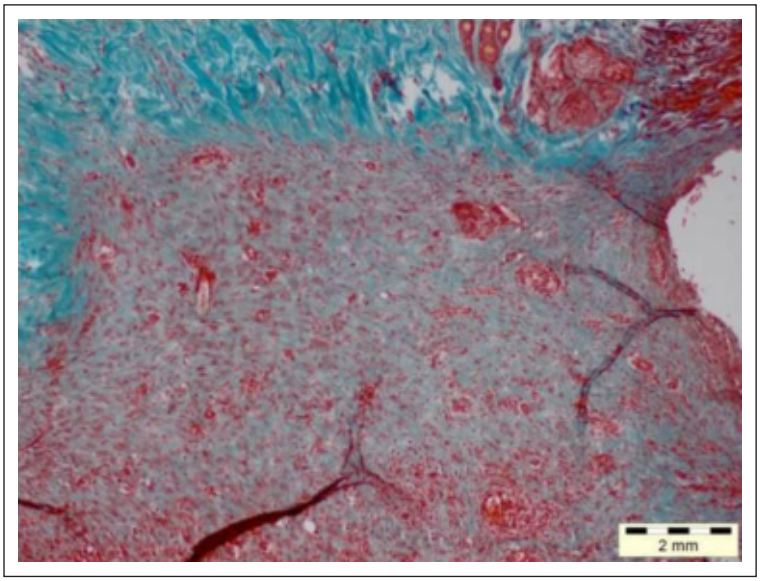

Figure 5. Granulation tissue stained with Masson's trichrome (MST) indicated collagen tissue formation at wound of 7th day in maggot treated group. Collagen tissue at unaffected healthy skin differentiated. MST, 200X.

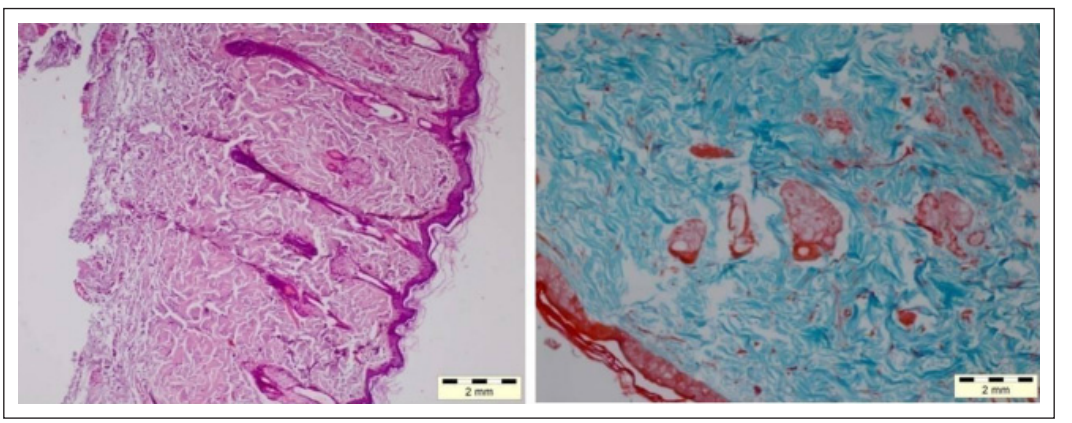

Figure 6. Complete closure of wound revealing organized fibrous tissue formation at dermis and new hair follicle formation and re-epithelization. H&E, 200X (left) and MSTX200 (right).

chronic septic wound and were proven safe for therapeutic application, removing devitalized tissues, purulent exudates of the wound of animals, preserving healthy tissue unharmed. Thus, the maggot of *L. sericata* was shown promising for therapeutic application in animals. The action of *L. sericata* on wound have been studied by other researcher and opined that maggot of this species ingested necrotic tissues only, sparing healthy tissues and also ingest bacteria and kill them in the gut of the maggots (Gottrup and Jorgensen 2011; Sherman 2014) and thereby increased oxygen perfusion, rapid spread of granulation tissues, cellular proliferation, fibroblast migration and matrix remodelling (Sherman 2009). Similar tissue reaction was also evident in the granulation tissue at histopathological examination in the present study. Anti-

microbial effects of maggots' excretion and secretion on inhibiting and breaking down biofilm formation of various bacteria (Cazander *et al.*, 2009) could be helped of healing of septic wound in the present study. The faster healing in the present study might be associated with the use of free ranged maggots directly on wound, supporting the findings of early debridement on use of larval therapy in free ranged maggots when compared with use in biobags (Sherman 2014; Dumville *et al.*, 2009). Maggots having minute spines over their bodies and with the help of wriggling movement of the body causes loosening of debridement and rasping of necrotic tissues and thereby helping the excretory secretary enzymes gained access to the deeper tissues and dissolving the necrotic tissues which was then imbibed by maggots of *L. sericata* along with bacteria and making the wound bed clean and thereby reducing the time of healing in maggot treated wound (Sherman 2009; 2014). Another reason of faster wound healing in maggot treated groups might be due to application of maggot promotes stimulation of angiogenesis which is one of the complex series of events and thereby causing proliferation of endothelial cells in the injured area (Bexfield *et al.*, 2010).

The addition of mixed colonies of virulent bacteria to the wound bed established infection in the test groups. The understanding and control of microbial infection of wound is very important for better healing and management (Muhammad and Muhammad 2005). In the present study, the antibacterial efficacy of maggot was appreciated in Wistar rat revealing elimination of bacterial burden at 14th day of wounding. The fasten reduction of bacterial counts in the wound of group receiving maggot treatment was possibly due to activities of *L. sericata* itself or maggot's excretion/secretion as the reduction of bacterial count was not observed in the groups which receive only antibiotic and with respective control group. The antibacterial activity of excretion/secretion of *L. sericata* was reported for various pathogenic bacteria (Bexfiled *et al.*, 2004; Jaklic *et al.*, 2008). One of the suggested mechanisms is by simple mechanical irrigation of wound by increased exudates, caused by ingestion of liquefied necrotic tissues by the larvae results in wound lavage and dilution of bacterial concentration over the wound area (Sherman *et al.*, 2000; Beasley and Hirst 2004). Another reason of early reduction of bacterial count from maggot treated wounds might be due to secretion of antibacterial compounds such as phenylacetaldehyde and phenylacetic acid, that are secreted by the midgut commensals of maggot- *Proteus mirabilis* (Beasley & Hirst 2004; Erdmann and Khalil 1986). It was observed that maggot treated wound effectively removed the diverse groups of micro-organisms that infected the chronic wound. Cerovsky and Bem (2014) reported that maggot responds to bacterial challenge or injury by rapid production of antimicrobial peptide (AMPs) that have broad spectrum of activity against gram-positive and gram-negative bacteria. Larval secretion also contains deoxyribonuclease (DNAse), which can also able to degrade microbial DNA and thereby inhibiting the microbial growth and biofilms (Brown *et al.*, 2012). There was no significant difference found in reducing the bacterial load in wound healing in maggot alone and maggot-antibiotic combination treated group. Therefore, it can be possible to treat chronic wound with maggot alone.

The healing process largely depends on the regulated biosynthesis, deposition and maturation of collagen (Nayak *et al.*, 2006). In the present study, the histopathological examination of wounded tissues at initiation of treatment showed pus formation/ suppurative inflammation characterized by abundant infiltration of PMN cells as well as necrotic tissues on wound bed. The pyogenic inflammation possibly resulted from bacterial colonization. The present study used mixed bacterial colonies to contaminate the wound and consequently higher bacterial load on wound bed was also recorded, supporting the histopathological findings of pyogenic/ suppurative inflammation in wound. In this study, the maggot treated groups showed better epithelialization, collagenation and neovascularization as compared to control wounds. Neovascularization and inflam-

matory response in maggot treated groups indicate the entire process of inflammation results in stimulation of fibroblasts in synthesizing collagen. There is evidence to suggest that maggots not only debride wounds effectively but also stimulate the growth of granulation tissue. It is believed that the movement of the maggots across the wound bed acts as a stimulant to promoting the growth of granulation tissue (Rayner 1999). However, Thomas (1998) suggested that the stimulation of granulation tissue was more likely to be as a result of macrophage activity being enhanced by maggots' secretions, which in turn stimulates the production of growth stimulating hormones. Additional substances produced include allantoin, thought to stimulate healthy granulation tissue (Robinson 1935) and other antibacterial substances which help in killing bacteria and thereby providing a better environment for healing (Thomas *et al.*, 1997) of wound. The healing of wound, at 7th day of application of maggot treatment showed stimulation of healing by formation of granulation tissue with neovascularisation along with collagen formation in the dermis. There were also decreases in cellularity of inflammatory cells. These changes were minimal in other group of animals, receiving antibiotic treatment and in control. The maturation and remodelling phase of wound healing is characterized by a decrease in the cell population and an increase in collagen organization in granulation tissue which forms a scar (Kumar *et al.*, 2003). Significant changes in this time period occur during epidermis regeneration as well. The organized formation of collagen with well-developed matrix and re-epithelization reported in the present study at 14th day clearly showed the effect of maggot responsible in promoting healing. The effect of maggot and maggot with antibiotic combination used in the present study didn't show any difference of histological characteristics suggesting stimulation of healing is independent of maggots only. The better angiogenesis or neovascularisation in maggot associated treatment groups observed in the present study might be effect of maggot's action or its excretions secretions on the wound bed. Pro-angiogenic compounds were detected within maggots' E/S including the amino acids L-histidine, 3-guanidinopropionic acid (GPA) and L-valinol (bexfield *et al.*, 2010) and these three identified compounds stimulated the proliferation of human endothelial cells.

In conclusion, the present study suggests that the live application of maggots of *L. sericata* in chronic infected wound plays a significant role in the healing of wounds through the process of reducing the bacterial bioburden and collagen formation. Therefore, larval therapy using *Lucilia* fly could be used as a potential therapy for the treatment of chronic infected wound.

Conflict of interest statement

We declare that we have no conflict of interest.

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