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Treatment of Glucantime-resistant/tolerant cutaneous leishmaniasis with *Lucilia sericata* larvae and its larval secretions: The first study in the world

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Abstract. *Lucilia sericata* larval secretions have bacteriostatic and bactericidal effects. We investigated the effect of *L. sericata* larval secretions during passage from the third to the second stage on *Leishmania tropica* under *in vitro* and *in vivo* conditions. The *L. sericata* larvae and their secretions were also used in the treatment of cutaneous leishmaniasis lesions. A total of 29 patients were included in this study. Seven patients were infected with Glucantime-resistant *Leishmania major*. In 22 patients, there was no information about the Glucantime resistance status and infection with *L. tropica or L. major*. All patients were efficiently treated with *L. sericata* larvae and their secretions without leaving scar tissue. Additionally, after 1-2 months of treatment, *Leishmania* spp. was not detected in the samples using PCR.

INTRODUCTION

Cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are the most common clinical forms of leishmaniasis worldwide, and the cutaneous form of this disease is commonly seen in Turkey. *Leishmania tropica* (*L. tropica*) and *Leishmania major* (*L. major*) are etiological agents of CL and infection causes skin sores. The sores may start out as papules or nodules and may end up as ulcers, and skin ulcers are covered by a scab or crust (Desjeux 1996; Unat *et al.*, 1981; Unat *et al.*, 1995; Ozcel *et al.*, 2007).

The first line compounds to treat leishmaniasis are the two pentavalent antimonials, sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), 1940s. However, resistance to antimonials has been reported in different studies especially from Asian countries such as India (Kazemi-Rad *et al.*, 2013; Pourmohammadi *et al.*, 2011; Singh *et al.*, 2007).

In resistant leishmaniasis patients, pentamidine and liposomal amphotericin B are used with good results. Because of its high toxicity, the liposomal form of amphotericin B is used instead of amphotericin B, but liposomal amphotericin B has limited use because of its cost, especially in poor countries. Pentavalent antimonials are used intravenously and/or intramuscularly for visceral leishmaniasis, and depending on the lesion, they are mainly used intralesionally for 12–15 days or in some cases 5 intra-muscular doses 1–2 times a week are used (Ozcel, 2007).

Over 20 years, the 1st and 2nd stages of *L. sericata* larvae have been successfully used to treat different lesions (Sherman 1997; Vistnes *et al.*, 1981; Baer 2011;

Mumcuoğlu 2001; Sherman, 2009; Topalan *et al.*, 2010). *L. sericata* larva dissolves the necrotic tissue over the lesion using their enzymes, disinfects the lesion and stimulates granulation tissue formation. Maggot debridement therapy (MDT) is widely used to treat diabetic lesions. *L. sericata* larvae secrete proteolytic enzymes, antibacterial secretions and some substances that stimulate granulation in necrotic tissues when they pass from the 2nd stage to the 3rd stage. These secretions lyse, kill or inhibit the bacteria, thus disinfecting the lesions (Kerridge *et al.*, 2005; Huberman *et al.*, 2007; Prete 1997).

In our previous study, *L. sericata* 2nd and 3rd stage larval secretions were found to be effective in *L. tropica in vitro* and *in vivo* (Polat *et al.*, 2012). In the present study, the 1st stage of *L. sericata* larvae and the secretions of *L. sericata* 2nd and 3rd stage larvae are used in the treatment of CL.

MATERIALS AND METHODS

A total of 29 patients suspected of having leishmaniasis were sent to our laboratory from the dermatology clinic at Istanbul University. Samples were taken from lesions on their hands, arms, face and forehead, and they were examined both microscopically and using the culture method in N.N.N. medium (Table 1). Patients are diagnosed as having leishmaniasis when the amastigote form is observed in a microscopic examination using Giemsa staining.

Patients and patient groups

The patients were divided into two treatment groups, as follows:

> The first group of 7 patients had lesions on their faces and foreheads, and this group received treatment with Glucantime.
>
> The second group consisted of 22 patients with lesions on their arms and legs, and they received treatment using the 1st stage of *L. sericata* larvae.

All patients were sent home after treatment.

First group

Glucantime was applied intradermally twice per week for a total of 5 doses until the lesion turned white. Patients were followed-up after 1 month of therapy, to check for healing of their lesions. The second therapy program was then initiated, but successful treatment was not obtained. The parasite species and resistant genes from lesion smear material was tested using PCR, and the amastigote form of leishmania was observed under light microscope using Giemsa staining.

Table 1. Patient demographics

AGE GROUP	Gender (Number)	LESION LOCATION					LESION NUMBER		COUNTRY		TOTAL
		Face	Arm	Feet	Face-Arm	Face-Arm-Feet	One	>One	TURKEY	SYRIA	
3–8	Male (11)	10	5	2	4	2	7 Patient	4 Patient	4	7	11
	Female (6)	4	2	–	–	–	6 Patient	–	4	2	6
8–14	Male (1)	1	–	–	–	–	1 Patient	–	1	–	1
	Female (–)	–	–	–	–	–	–	–	–	–	–
14–20	Male (–)	–	–	–	–	–	–	–	–	–	–
	Female (1)	–	1	–	–	–	1 Patient	–	1	–	1
>20	Male (4)	2	2	–	–	–	4 Patient	–	4	–	4
	Female (6)	2	4	–	2	–	2 Patient	4 Patient	6	–	6
TOTAL	Male (16)	13	7	2	4	2	21 Patient	8 Patient	20	9	29
	Female (13)	6	7	–	2	–					

In the 7 patients treated by Glucantime therapy, the amastigote form of *Leishmania* was observed and the infection was defined as Glucantime-resistant *L. major* using the PCR method. The *Leishmania* isolates identified in patient samples were used in PCR analysis. The secretion was applied intradermally until the lesion turned white after 4-5 doses at one day intervals.

Second group

In the 1st stage, hatched larvae were used to treat the patients. Twenty-two patients who had cutaneous leishmaniasis lesions on their hands or arms were treated by placing 6-7 sterile *L. sericata* larvae at the 1st stage directly on to 1 cm^2 lesions (Fig. 1). Based on the necrotic tissue appearance, the larval treatment was removed after daily, weekly or once-to-twice a week application onto the wounds or ulcers for 48-72 hours. The procedure was approved by the Ethics Committee of Cerrahpasa Medical Faculty, Istanbul University (number 15.06.05/14620). Patients were informed about the treatment and alternative therapies, and volunteers signed an informed consent form.

Preparation of *L. sericata* larvae

In fly cages containing the adult *L. sericata* colonies, a chicken liver was placed into sterile open petri dishes and left for 3–4 h for flies to lay their eggs. The liver were then removed from the cages, and the eggs of *L. sericata* were collected. Eggs were analyzed, and after sterilization, they were transferred to a sterile liver agar culture. The agars, including the eggs were incubated for 36–40 h in incubators at 25-30°C. Sterile larvae passing from the 2nd to the 3rd phase were collected.

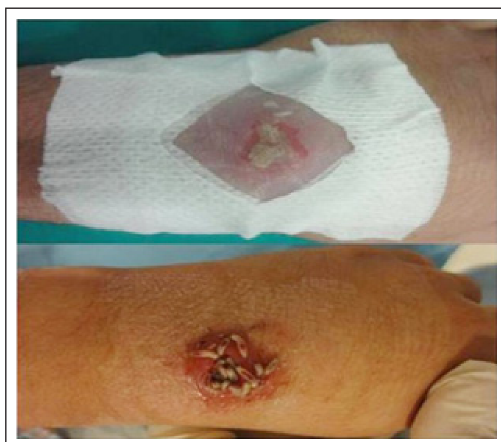

Figure 1. Cutaneous leishmaniasis lesions on patients' hands or arms were treated by placing 6-7 sterile *L. sericata* larvae at the 1st stage directly on 1 cm^2 lesions.

Preparation of *L. sericata* larvae secretions

There were 2,500 2nd and 3rd phase sterile larvae placed into a 1L sterile beaker and 5 ml distilled water was added. Distilled water (n=5) was added at 2 h intervals for 6 h, and the larvae were left to deposit their secretions into the water. After the procedure, the larvae were removed and the secretions were collected (Polat *et al.*, 2012).

PCR targeting resistant *Leishmania* genes

A set of oligonucleotide primers, Leish ReF2, (5'→3' TCGACCGCGAGTGTCTCAGC) and Leish ReR2 (5'→3' TGGCATAGTGCGCAA AAGTG) were designed and used for PCR amplification. This primer amplified a 356-bp piece of the leishmaniasis MDR (Accession NO L08091). The 30 µl reaction mixture contained (final concentration) 2 µl DNA extract as template, 3 µl 1× PCR buffer, 1.5 µl $MgCl_2$, 0.5 µl deoxynucleotide triphosphate (dNTP), 0.25 µl Taq DNA polymerase (5 U/µl; Gibco, BRL), 20.75 µl dH_2O and 2 µl of each of the forward and reverse primers.

PCR reaction was carried out using 35 cycles of denaturation at 94°C for 30 sec (3 min in cycle 1), annealing at 56°C for 30 sec and elongation at 72°C for 30 sec (5 min in cycle 30). The PCR products were subjected to electrophoresis using a 1.5% agarose gel, stained by ethidium bromide and visualized under a Transilluminator.

RESULTS

All samples that were taken from the patients' lesions were stained using Giemsa and were cultured in N.N.N. medium. All samples that were microscopically

confirmed to have the amastigote form of *Leishmania* were successfully grown in the N.N.N. medium.

First group

In 7 patients treated with Glucantime therapy, the amastigote form of *Leishmania* was observed and was determined to be Glucantime-resistant *L. major* using the PCR method (Fig. 2). These patients were treated with subcutaneous injections of *L. sericata* larval secretions during passage from the 2nd to the 3rd stage (4–5 doses at one-day intervals).

Second group

Twenty-two patients who had cutaneous leishmaniasis lesions on their hands and arms were treated using the 1st stage of *L. sericata* larvae within 8-10 days (shown in Fig. 3). After a 1-2-month treatment period, *Leishmania* spp. could not detected in the samples using PCR methods. Moreover, there was no scar tissue observed in the lesion areas at the 5-6 month-follow-up after treatment (Fig. 4).

DISCUSSION

L. sericata larvae and the secretions have been successfully used in our laboratory since 2007. Our *in vitro* and *in vivo* studies have shown that *L. sericata* larval secretions at the time of passing from the 2nd to the 3rd stage were effective on *L. tropica* (Polat *et al.*, 2012). Our *in vivo* studies have demonstrated that the secretions have an accelerated effect on lesion healing by changing mRNA and miRNA expression levels and stimulating the production of extracellular matrix and epithelial cells (Aksoz 2013, Arkan 2013).

Twenty-two patients who had cutaneous leishmaniasis lesions on their hands or arms were treated by direct application of stage 1 *L. sericata* larvae. In this way, larvae could attach onto the necrotic tissue and easily enter deeply into necrotic tissues. The 7 patients treated with Glucantime therapy received 4-5 subcutaneous injections of secretions from *L. sericata* larvae that were passing from the 2nd to the 3rd stage at one-day intervals.

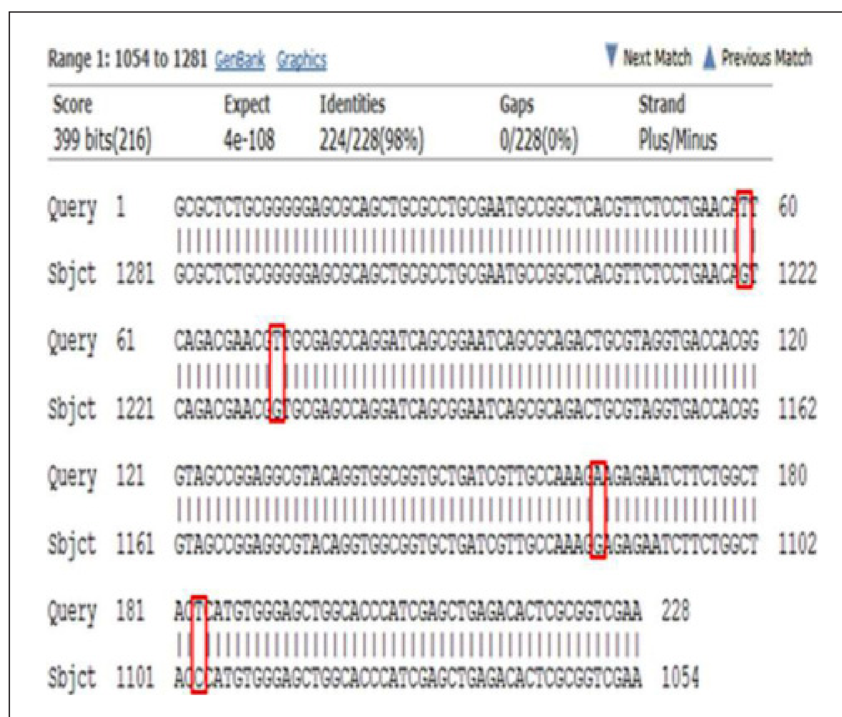

Figure 2. Glucantime resistance gene.

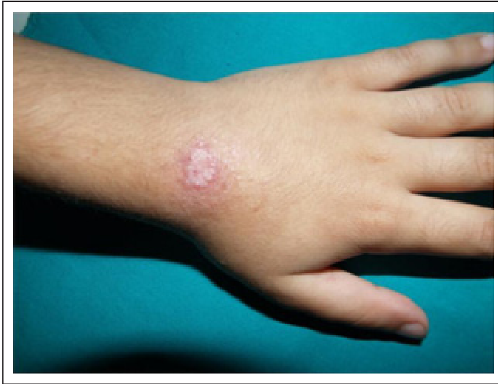

Figure 3. Cutaneous leishmaniasis lesions on patients' hands and arms were treated using the 1st stage of *L. sericata* larvae within 8-10 days.

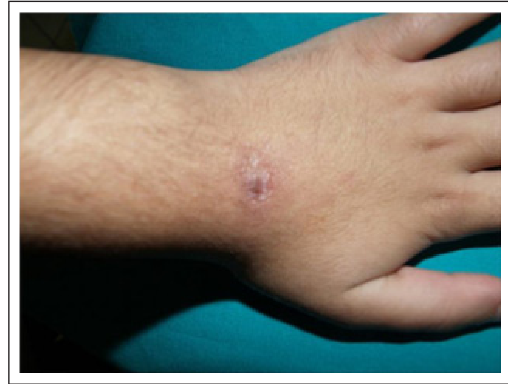

Figure 4. The location of lesions at the 5-6 month-follow-up time point after treatment.

With their enzymes, larvae lyse the necrotic tissues in ulcerated lesions and stimulate granulation in the healthy tissue. The larvae disinfect the lesions by eating the microorganisms or they lyse, kill or prevent their growth by substances they secrete (Prete, 1997).

Worldwide, Glucantime-resistant leishmaniasis patients are treated with the liposomal form of amphotericin B. Because of these drug's high toxicity, the liposomal form of amphotericin B increases the cost of treatments. These drugs are used intravenously or intramuscularly to treat VL, and they are used parenterally for 12-15 days or 5 doses once or twice a week intralesionally. The secretions of *L. sericata* larvae when they are passing from the 2nd to the 3rd stage are effective against the promastigote form of *L. tropica in vitro* and against the amastigote form *in vivo*. CL treatment can, thus, be accomplished by placing the larvae directly on the lesions and/or injecting the larval secretions intralesionally.

Some studies have demonstrated that secretions from the 2nd and 3rd stages of *L. sericata* larvae contain at least two antibacterial substances. One of these is a 3-10 kDa hydrophobic peptide-like structure, and the other is a <1 kDa weight hydrophilic substance. *L. sericata* larvae also secrete preolytic enzymes. These substances eliminate the infection by killing and inhibiting the growing microorganisms (Huberman *et al.*, 2007). Because the substances are natural, there is no toxicity.

Sherman *et al.* showed that MDT is generally effective on lesions that are infected with antibiotic-resistant bacteria. *In vitro* studies have demonstrated that the larvae killed or inhibited growth of pathogenic bacteria, especially *S. aureus* and Group A or B *Streptococcus* spp. (Sherman 1997).

The larvae include the same secretions that have antibacterial effects on Gram-positive [Methicilin-susceptible *S. aureus* (MSSA), Methicilin resistant *S. aureus* (MRSA)] and Gram-negative (*Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli* and *Klebsiella pneumoniae*) bacteria (Balaban 2011).

In conclusion, patients with CL that results from *L. tropica* or *L. major* infection, even with resistance to sodium stibo-gluconate, were treated by directly placing the 1st stage of *L. sericata* larvae and by intralesionally injecting the *L. sericata* 2nd and 3rd stage larval secretions for 10 days.

The results of the studies suggest that fresh and pure larval secretions may cure the developed cutaneous lesions, and also have a high number of secretion applications. This treatment technique is also completely natural, has no side effects, and is a cost-effective alternative compared to other traditional treatment options (Glucantime or liposomal amphotericin B). Our study was the first to show this relationship.

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REFERENCES

Aksoz, I. (2013). Analysis mRNA profiles in wound healing treated *Lucilia sericata* larvae secretion in experimental rat wound model. Istanbul University, Institute of Health Science, Medical Biology Department, master thesis, Istanbul.

Alizadeh, R., Hooshyar, H., Bandehpor, M., Arbabi, M., Kazemi, F., Talari, A. & Kazemi, B. (2011). Detection of drug resistance gene in Cutaneous *Leishmaniasis* by PCR in some endemic areas of Iran. *Iranian Red Crescent Medical Journal* **13**(12): 863-867.

Arkan, H. (2013). Analysis of micro-RNA profiles in healed wounds by the use of *Lucilia sericata* larvae in experimental rat wound model. Istanbul University, Institute of Health Science, Medical Biology Department, master thesis, Istanbul.

Baer, W.S. (2011). The Classic: the treatment of chronic osteomyelitis with the maggot (larva of the blow fly). *Clinical Orthopaedics and Related Research* **469**: 920-944. doi: 10.1007/s11999-010-1416-3.

Desjeux, P. (1996). Leishmaniasis: public health aspects and control. *Clinics in Dermatology* **14**: 417-423.

Huberman, L., Gollop, N., Mumcuoglu, K.Y., Block, C. & Galun, R. (2007). Antibacterial properties of whole body extracts and haemolymph of *Lucilia sericata* maggots. *Journal of Wound Care* **16**: 123-127. doi: 10.12968/jowc.2007.16.3.27011.

Kazemi-Rad, E., Mohebali, M., Khadem-Erfan, M.B., Saffari, M., Raoofian, R. & Hajjaran, H. *et al.* (2013). Identification of antimony resistance markers in *Leishmania tropica* field isolates through a cDNA-AFLP approach. *Experimental Parasitology* **135**: 344-349. doi: 10.1016/j.exppara.2013.07.018.

Kerridge, A., Lappin-Scott, H. & Stevens, R. (2005). Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata*. *Medical and Veterinary Entomology* **19**: 333-337. doi: 10.1111/j.1365-2915.2005.00577.x.

Mumcuoglu, K.Y. (2001). Clinical applications for maggots in wound care. *American Journal of Clinical Dermatology* **2**: 219-227.

Ozbel, Y. & Toz, S.O. (2007). Leishmaniosis. In *Ozcel's medical parasitic diseases* (ed. Ozcel, M.A.), pp. 197-244. Meta Publishers, Izmir, Turkey.

Polat, E., Cakan, H., Aslan, M., Sirekbasan, S., Kutlubay, Z. & Ipek, T. *et al.* (2012). Detection of anti-leishmanial effect of the *Lucilia sericata* larval secretions *in vitro* and *in vivo* on *Leishmania tropica*: First work. *Experimental Parasitology* **132**: 129-134. doi: 10.1016/j.exppara.2012.06.004.

Pourmohammadi, B., Motazedian, M.H., Handjani, F., Hatam, G.H., Habibi, S. & Sarkari, B. (2011). Glucantime efficacy in the treatment of zoonotic cutaneous leishmaniasis. *The Southeast Asian Journal of Tropical Medicine and Public Health* **42**(3): 502-508.

Prete, P.E. (1997). Growth effects of Phaenicia sericata larval extracts on fibroblasts: mechanism for wound healing by maggot therapy. *Life Sciences* **60**: 505-510. doi: 10.1016/S0024-3205(96)00688-1.

Sherman, R.A. (1997). A new dressing for use with maggot therapy. *Plastic and Reconstuctive Surgery* **100**: 451-456.

Sherman, R.A. (2009). Maggot therapy takes us back to the future of wound care: new and improved maggot therapy for the 21st century. *Journal of Diabetes Science and Technology* **3**: 336-344.

Singh, N., Almeida, R., Kothari, H., Kumar, P., Mandal, G. & Chatterjee, S. *et al.* (2007). Differential gene expression analysis in antimony-unresponsive Indian kala azar (visceral leishmaniasis) clinical isolates by DNA microarray. *Parasitology* **134**(6): 777-787. doi: 10.1017/S0031182007002284.

Polat, E. (2010). Maggot Debridement Therapy (MDT). In *Current Characteristics of the Chronic Wound* (ed. Topalan, M., Aktas S.), pp. 181-193. 1st edition, Aygul Offset, Ankara, Turkey.

Unat, E.K., Yasarol, S., Orhan, V., Dogan, F., Solak, S. & Gezen, C. *et al.* (1981). Leismaniasis. *Turkish Journal of Parasitology* **2**: 59-65.

Unat, E.K., Altas, K. & Samasti, M. (1995). Unat's Medical Parasitology. 5th edition. Cerrahpasa Medical Faculty Foundation Publishers, Istanbul, Turkey.

Vistnes, L.M., Lee, R. & Ksander, G.A. (1981). Proteolytic activity of blowfly larvae secretions in experimental burns. *Surgery* **90**: 835-841.