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A new therapeutic approach for the treatment of cutaneous leishmaniasis: Photothermal application of macrophage-specific antibody binding graphene oxide nanoparticles

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ABSTRACT

Background & objectives: Cutaneous leishmaniasis (CL) is a zoonotic and anthropogenic protozoal disease. We aimedto develop a new therapeutic approach for the treatment of CL.

Methods: BALB/c mice have infected *L. major* amastigotes from their footpads. Twenty-one days later after injection, the animals were divided into three control and three experimental groups. The intralesional injection of graphene oxide and photothermal application (GO+PA) were applied to the first experimental group (Group 1); graphene oxide modified with a macrophage-specific antibody and photothermal application (MSA+GO+PA) were applied to the second experimental group (Group 2), and the photothermal application (PA) was applied to the third experimental group (Group 3).Miltefosine was administered orally to the first control group (Group 4); the second control group that is not treated was assigned as the positive control (Group 5) and the third control group was assigned as the negative control (Group 6). Lesions were examined (erythema and edema) after the 5th day and 10th of the treatment, clinically. On the 10th day of the treatment, the level of TNF-α, IL-1, IL-6, and IFN-ɤ were detected histopathologically and immunohistochemically.

Results: In the 5th day of the treatment it was observed that 50% of the animals were completely treated with Group 2, and in the 10th day, the ration raised to 75%.

Interpretation & conclusion: We showed a novel application to treat CL by using MSA modified GO and PA within 10 days. According to our study outcomes, this application could be a new treatment approach for CL cure.

Key words Cutaneous Leishmaniasis; BALB/c; Nanoparticle; Graphene oxide; Phototherapy

INTRODUCTION

Leishmaniasis is a protozoon disease characterized by zoonosis and anthroponosis with a cosmopolitan distribution[1]. *Leishmania* protozoans were first identified and named by William B. Leishman in 1901 which are the cause of disease[2]. Within thirty *Leishmania* species, twenty-one of them can be pathological to human health[3]. *Leishmania* parasites exhibit a clinical presentation that varies from non-lethal and spontaneously healing skin lesions to infections that may spread to internal organs and cause the death of thousands of people in epidemics[1, 4].

Cutaneous leishmaniasis (CL) begins as a small stiff and itchy papule where female sandfly sucked blood and gradually develops a skin rash. Depending on the species of parasite, it becomes turned into a hard and bulgy around, crusted, slightly leaky wound within 1 to 4 months. Symptoms such as fever and shivering, are seen in people when secondary infection gets added to slow-growing wound with 2–3 cm in diameter. The wound heals by leaving scar tissue in time[1, 5]. As a result of the ascending rate of traveling to endemic areas, CL has also begun to be seen in non-endemic areas. In this context, leishmaniasis has spread to non-endemic areas and become a major health problem[6–8]. Because current anti-leishmanial drugs are toxic, ineffective and expensive for some *Leishmania* species, treatments could fail[9–10].

Nanotechnology provides a rapid and effective way for diagnostics and treatments of different diseases. The optical, fluorescent and magnetic properties of nanoparticles are utilized in the prognosis and diagnosis of various pathogenic and genetic diseases and the early detection of tumor cells. Because of its mechanical, physical and optical properties graphene has been subject to many researches and studies[11–14].

The reason why we used graphene oxide (GO) in this study is entirely associated with the structural and functional characteristics of GO. The GO particles have functional oxygen groups in its structure so because of their hydrophilic nature they can disperse in water. Different

nanoparticle sand molecules can bind to GO because of different functional groups present on their surface, GO nanoparticles are small enough to pass the cellular membranes, and because of their hydrophilic nature they can not be held in the cellular membranes. They present high thermal resistance, and they maintain their structure between -75 to +200°C, and they have a strong antibacterial effect due to its high thermal conductivity (5000W / Mk)[14]. In addition, conjugation of macrophage-specific antibody (MSA) to the GO molecules enables GO to being localized to the target cells. Its intracellular entry and thus its cytotoxicity is reduced to a minimum level. Today, the adverse effects of drugs used to treat CL have been prompting researchers to develop new treatment approaches. This is the first study to investigate the co-effect of MSA binding GO nanoparticles and photothermal application (PA) on CL treatment.

MATERIAL & METHODS

Experimental animals and experimental groups

The experiment was designed to use three experimental and three control groups, which consist of 44 female BALB / c (4-8 weeks old, 20 ± 5 grams) mice (Table 1). The mice were fed a standard laboratory diet at Cumhuriyet University, Faculty of Medicine, Animal Laboratory during the study. The growing conditions were set to12h:12h dark/light cycle and 50-70% humidity.

Ethical statement

The research was approved by decision dated 25.03.2016 and numbered 52 decision of Local Ethics Committee for Animal Experiments of Cumhuriyet University, Turkey

Development of experimental CL model

The strain used in this study is *Leishmania major* (*L. major*). MHOM / TR / 2013 / MANISAPB145 were obtained from Parasite Bank of Celal Bayar University. The parasites were kept virulent by continuous passage in BALB/c mice. Amastigotes were isolated from lesions of infected BALB/c mice. Briefly, Infected BALB/c mice were obtained from Parasite Bank of Celal Bayar University and were subjected to general anesthesia. Anesthesia was applied by intraperitoneal injection of ketamine (120 µg/g) combined with xylazine (12 µg/g). The lesion site was carefully cut with sterile scissors and was taken into a sterile falcon tube. 5 mL of RPMI-1640 (Sigma, R8758) medium containing 10% horse serum (Sigma, H0146). was added and homogenized with a sterile glass baguette then filtered through sterile gauze. It was centrifuged at 80 g for 5 min at 4°C and centrifugation pelleted large cellular debris. The supernatant was then centrifuged at 1300g for 10 min at 4°C to pellet amastigotes. The pelleted amastigotes were resuspended in ammonium chloride (168 mM)[15], in order lyse red blood cells and washed 3 times with PBS and adjusted to 1.10^7 organisms per ml in RPMI-1640 containing 10% horse serum. Obtained amastigotes were injected to subcutaneously into the footpads of BALB / c mice. After three weeks of the injection, CL was developed on the footpads of mice.

Preparation of MSA binding GO nanoparticles and its binding to macrophage

GO (Sigma, 794341) nanoparticles and MSA (Abnova, MAB5180) were purchased commercially for GO+MSA conjugation that was formed by EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride) / NHS (N-Hydroxysuccinimide)amidation-method[11]. Briefly, the GO was mixed with 400 mM EDC and 100 mM NHS in 1 mL of pH 5.2 MES (2-(N-Morpholino) ethanesulfonic acid hydrate, Sigma, 130672) buffer

Table 1. Experimental groups designed and the number of the animals used in the study.

Groups	Experimental groups	Control Groups	Number of animals
Group 1	The GO were applied at the first day of the study with 20 µL was inoculated near the lesion site subcutaneously one dosage and PA was applied every day at single dose of 15 sec.		8
Group 2	The GO modified with a MSA were applied at the first day of the study with 20 µL was inoculated near the lesion site subcutaneously one dosage and PA was applied every day at single dose of 15 sec.		8
Group 3	Only PA was applied every day at single dose of 15 sec.		8
Group 4		Miltefosine at a dose of 25 mg/kg/ day was orally administred.	8
Group 5		Untreated	8
Group 6		CL not created	4
	Total		44

GO: Graphen oxide, PA: Photothermal application, MSA: Macrophage-specific antibody, CL: Cutaneous leishmaniasis.

and activated for 30 min. The mixture was centrifuged at 13,000 rpm for 5 min, and the supernatant was discarded. The MES buffer wash was repeated to remove excess EDC and NHS. Later, the resulting functionalized mixture was dispersed in 1.0 mL of pH 7.4 PBS and sonicated for 5 min to obtain a homogeneous dispersion. Then, 100 µL MSA (5µg/mL) was added to the dispersion, and the mixture was stirred overnight at 4 °C. The reaction mixture was washed with PBS and centrifuged at 13 000 rpm for 5 min three times, and the supernatant was discarded. The resulting mixture was redispersed in 1.0 mL of pH 7.4 PBS containing 1% BSA and stored at 4°C. The GO+MSA conjugation was validated by using fouirer-transform infrared spectrophotometer (FT-IR) and elemental analysis (EA) in Anadolu University, Plant, Pharmaceutical and Scientific Research Center.

FT-IR and EA

The GO nanoparticles were separately centrifuged at 13,000 rpm. The supernatant was discarded. It was homogenized with 2mL of deionized purified water. The wash was repeated five times. Then the obtained materials were lyophilized on lyophilization apparatus (Christ Freeze Dryer Alpha 1-2 LD, Germany) for 24 hours. KBr tablets prepared nanoparticle were analyzed in FT-IR spectrometer (Perkin Elmer, Spectrum 100, USA). The elemental analysis of the nanoparticles was performed using a micro elemental analyzer (LecoTruSpec, USA). GO+MSA modification FT-IR and elemental analysis were also analyzed by the same method.

Tissues were analyzed by indirect immunofluorescence staining to confirm that synthesized GO+MSA nanoparticles were bound to macrophages. For this purpose, 20 µL of GO+MSA was inoculated near the lesion site subcutaneously to two mice with CL. Mice were sacrificed under general anesthesia (ketamine (120 µg/g) combined with xylazine (12 µg/g)) after 24 hours. Freshly harvested samples were analyzed by immunofluorescence staining. Briefly, 20 µL of GO+MSA was injected subcutaneously into the footpad of two mice with CL. Mice were sacrificed under general anesthesia after 24 hours. Samples taken from fresh tissue were frozen at -22°C in the cryotome (Leica, Germany) device. Samples section from the frozen tissue were kept in the same device for one night. Monoclonal antibody (anti-MSA, ab177226) prepared (ratio of 1/15) on the tissues was added dropwise and incubated for 1.5 hours. Then samples kept in PBS for 5 min. The samples were then covered with the closing solution and evaluated in the fluorescence microscope.

Administration of treatment

The experiment was carried out ten days, and after five days, mice were examined clinically. After the experiment finished, mice were examined clinically and analyzed immunohistochemically and histopathologically.The GO and GO+MSA were applied on the first day of the study with 20 µL were inoculated near the lesion site subcutaneously one dosage. PA was applied to Group1-3 every day at a single dose of 15 sec in the form of near infra-red radiation using the PL-E Pro 808 nm, 0.05 J/cm^2, 50 mV/cm^2 Infrared Laser (Jet, China) device (Fig. 1). Miltefosine at a dose of 25 mg/kg/day was orally administered to mice with CL (Group 4). Mice with CL (Group 5) and mice without CL (Group 6) were not treated. The effect of treatment was evaluated on the 5th day and the 10th day. All mice were sacrificed under general anesthesia after ten days of the treatment (Table 1).

Evaluation of treatment effect

The efficacy of the treatment was evaluated in three ways.

1. Physical appearance: Mice were weighed at the beginning and at the end of the study to see if there was a general weight gain or loss.

Fig. 1: Photothermal application using PL-E Pro 808 nm infrared laser.

2. Clinical conditions: The presence of erythema and edema, and healing situation were classified on the 5th and 10th days of the study [Grade 0: no erythema and edema, complete healing (Fig. 2A), Grade 1: partial erythema and edema, partial healing (Fig. 2B), Grade 2: severe erythema and edema, no healing (Fig. 2C)].

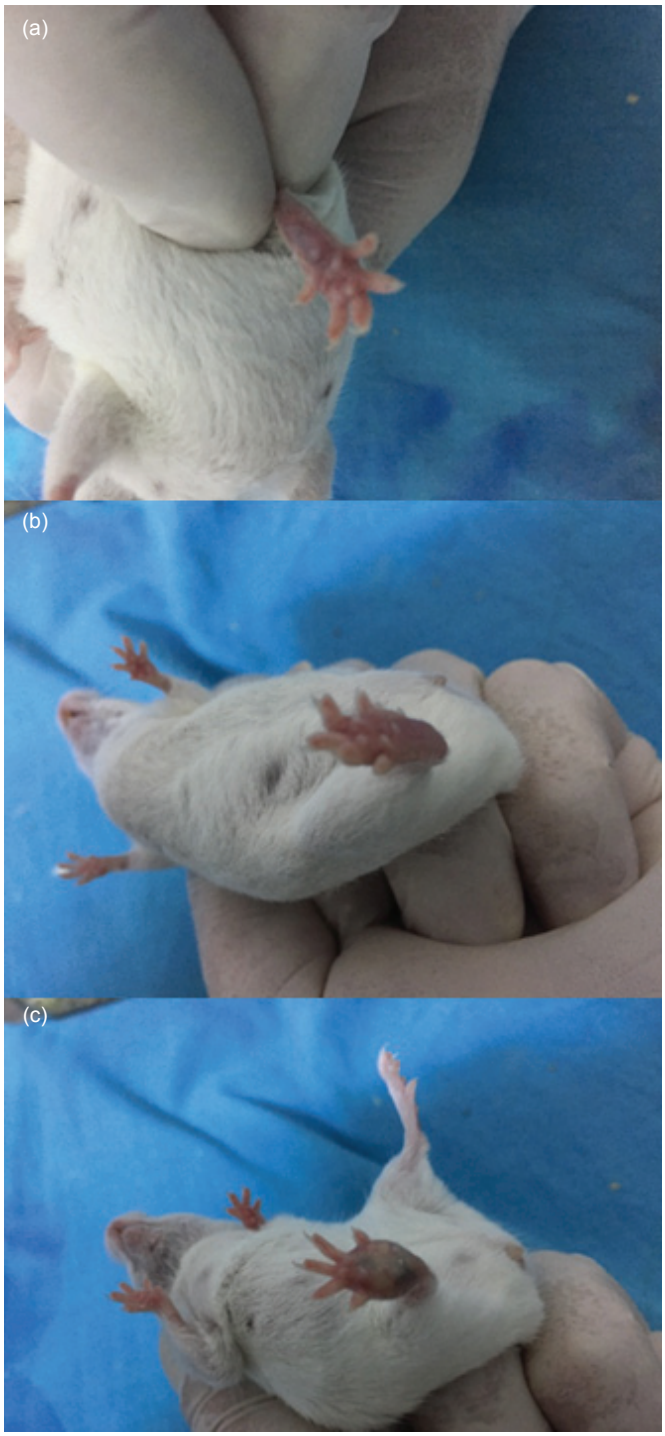

Fig. 2: The view of lesions and clinical grading. A. Grade 0: Complete healing (erythema: 0, edema: 0); B. Grade 1: Partial healing (erythema: 1, edema: 1); C. Grade 2: No healing (erythema: 2, edema: 2).

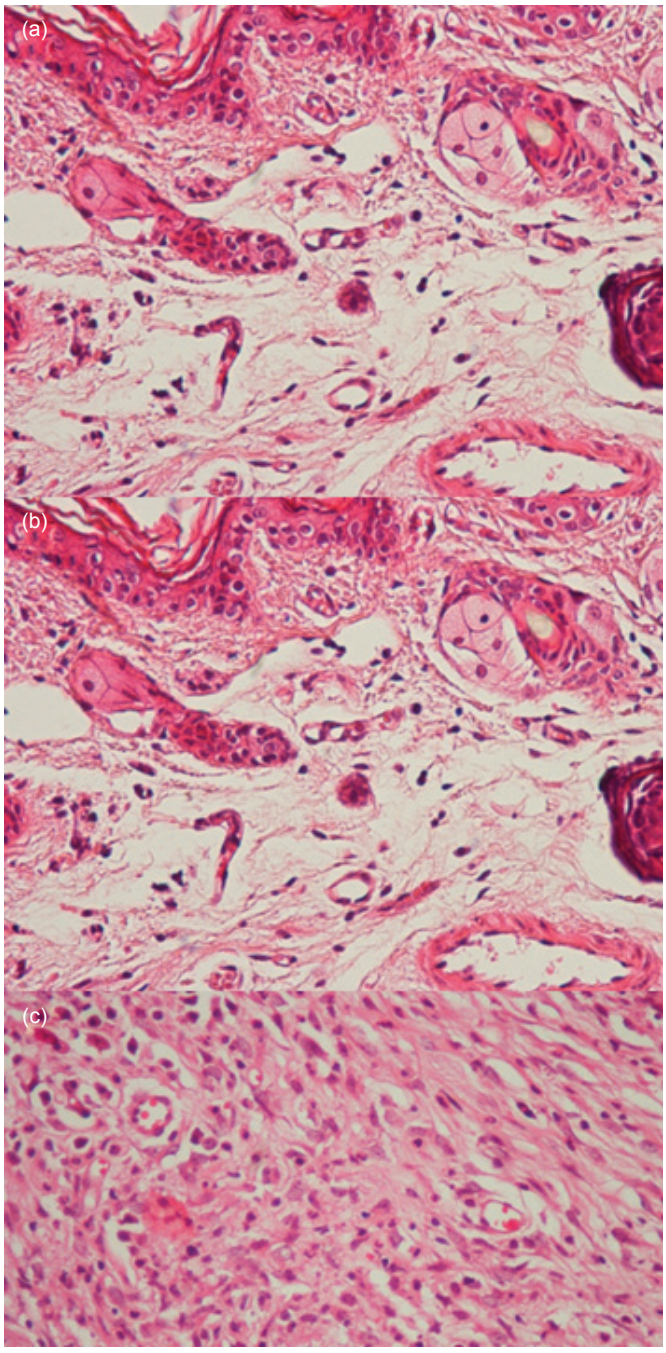

Fig. 3: Grading the inflammatory reactions in tissues as a result of hematoxylin & eosin stating. A. Grade 0: no inflammatory reaction, B. Grade 1: moderate inflammatory reaction, C. Grade 2: severe inflammatory reaction.

3. Tissue samples from the experimental and control groups were evaluated histopathologically (hematoxylin & eosin staining) and immunohistochemically (TNF-α, IL-1, IL-6, and IFN-ɤ) on the 10th day of treatment. While the inflammatory reaction was classified as Grade 0: not detected (Fig. 3A), Grade 1: moderate (Fig. 3B) and Grade 2: severe (Fig. 3C)

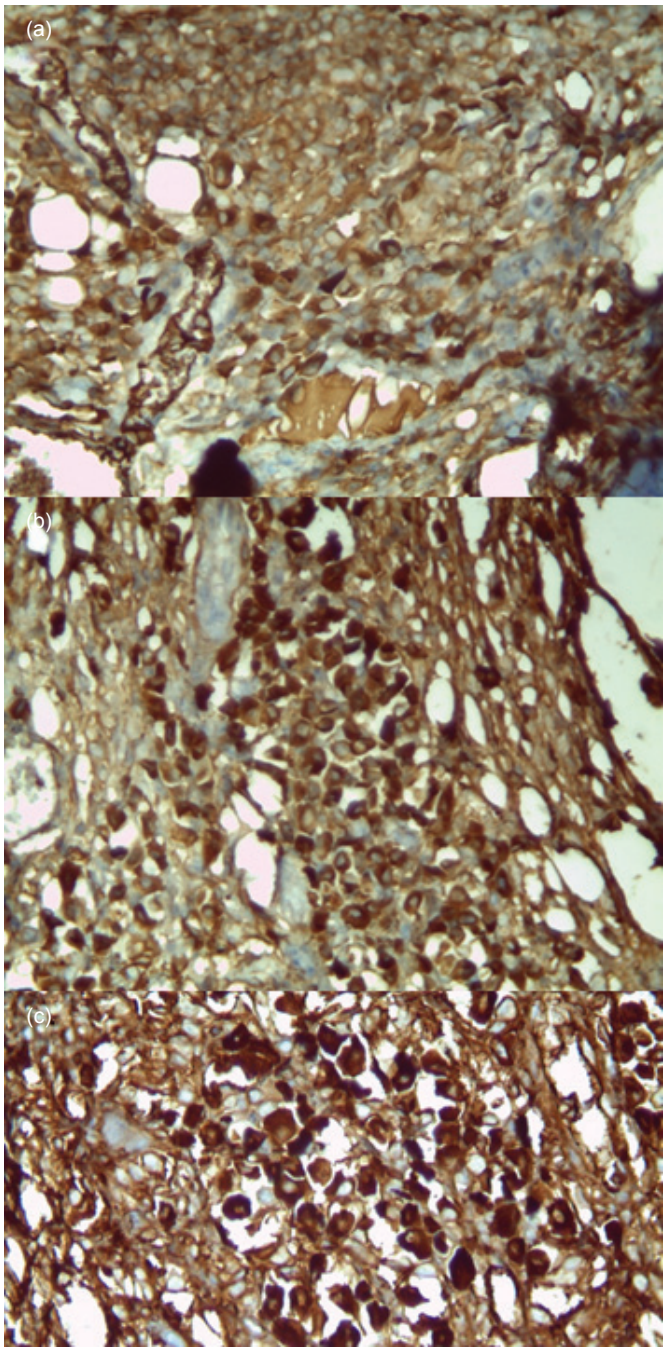

Fig. 4: Grading the intensity of TNF-α, IL-1, IL-6 and IFN-ɤ reactivity as a result of the immunohistochemical staining. A. Grade 1: mild cytokine, B. Grade 2: moderate cytokine, C. Grade 3: intense cytokine.

in hematoxylin and eosin staining of tissues, intensity of TNF-α, IL-1, IL-6, and IFN-ɤ was classified as Grade 1: mild cytokine (Fig. 4A), Grade 2: moderate cytokine (Fig. 4B) and Grade 3: severe cytokine (Fig. 4C) in the immunohistochemical staining of tissues. To detect the number of amastigotes in the groups, 10 different areas were analyzed under 40X magnification to detect the average number of amastigotes in samples. The immunohistochemical, histopathological, and indirect immunofluorescent staining analysis was done by Sivas Cumhuriyet University, Faculty of Medicine, Department of Pathology, Turkey.

Statistics

Histopathological and immunohistochemical data obtained from our study were analyzed using SPSS (Ver: 22, IBM, USA). Man-Whitney U test was used to evaluate these data, and the level of significance was considered as 0.05.

RESULTS

Experimental CL model

On the 5th day of the amastigote inoculated to the footpads of BALB / c female mice, less pronounced erythema was detected in the footpads (Fig. 2A). While the lesion began to show itself as the grade 1 erythema and edema on the 10th day (Fig. 2B), the grade 2 erythema and edema developed on the 21st day (Fig. 2C). The CL lesions developed in the mice were measured with calipers, and close sized lesions were included in the study.

Preparation of MSA binding GO nanoparticles and its binding to macrophage

According to FT-IR analysis results, GO contains two basic chemical structures. These are carboxylic acid (-COOH) groups and two aromatic benzene rings in the middle of the molecule (Fig. 5A). At GO+MSA spectrum, -COOH groups in the outermost region of the molecule must first be converted to the carboxylate anion (-COO-) and then to the amide (-COONH) structure after bonding. In these two bindings, the amide bond was observed at both 1515 cm^{-1} and 1625 cm^{-1} (Fig. 5B). In conclusion, replacing –COOH bods with –COONH bonds prove that the successful binding has occurred between GO and antibodies.

According to elemental analysis results, in the GO+MSA particles 7,1109% of nitrogen, 25,196% of carbon, 3,6028% of hydrogen and 3,8936% of sulfur were detected. The presence of nitrogen and sulfur in GO+MSA was not present in GO, was confirmed the successful binding of MSA to GO.

Binding of GO+MSA molecule to macrophage

After 24 hours of the subcutaneous injection of GO+MSA into mice with CL, the binding of the antibody to target macrophage in the lesion site was confirmed by using indirect immunofluorescence staining. The intensi-

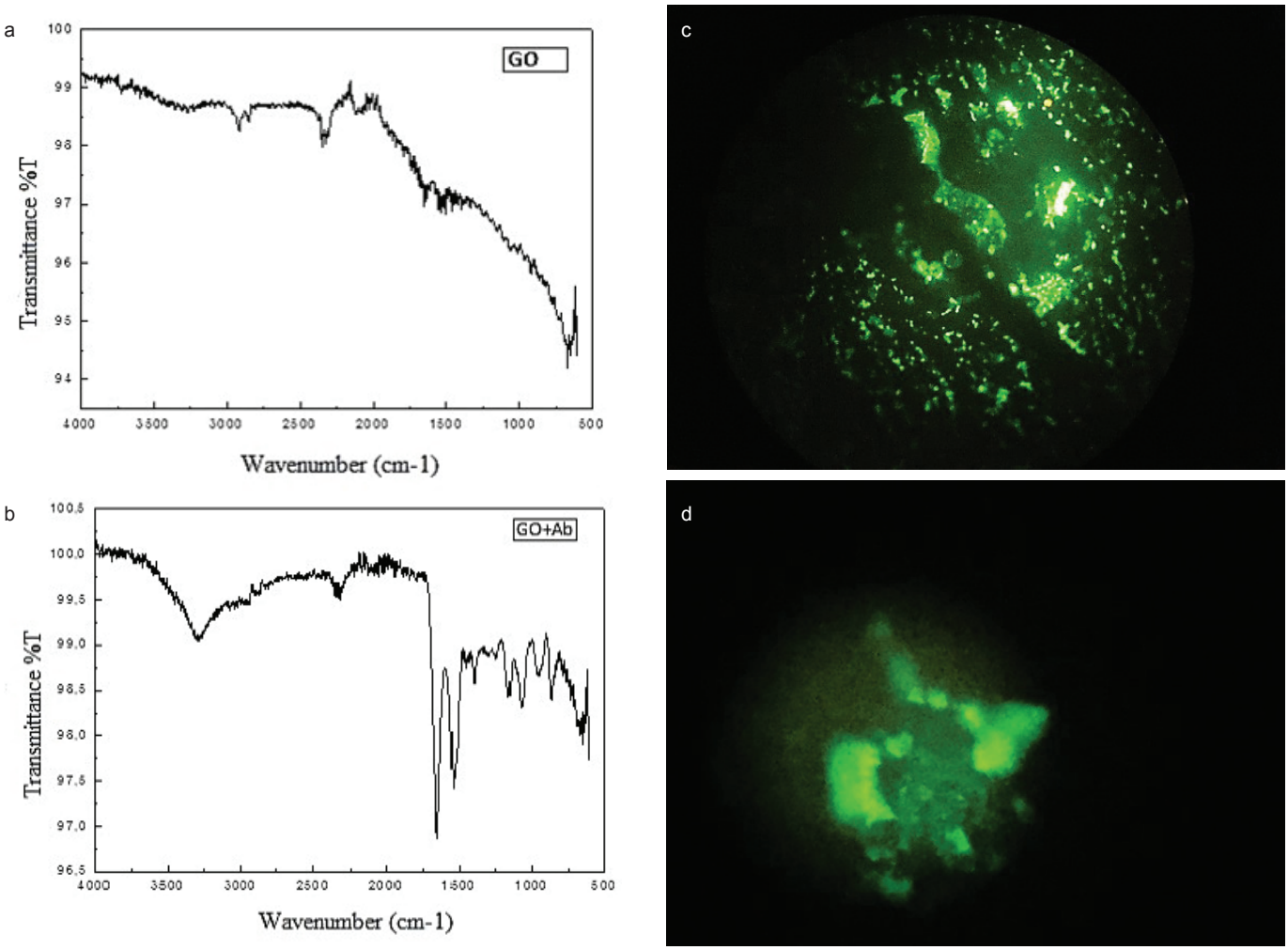

Fig. 5: FT-IR diagram and immunofluorescence staining. A. GO FT-IR diagram; B. GO + MSA FT-IR diagram; C, D. 10X (C) and 100X (D) magnification views of the immunofluorescence staining showing that GO + MSA binds to the target macrophages in the tissue. Arrow indicates the macrophage.

ty of green fluorescent indicates the amount of GO+MSA in the tissue (Fig. 5C, D).

Evaluation of treatment effect

Physical Evaluation

After five days of the treatment there was an average of 1 g weight gain detected both in control end the experimental groups, and no other physical changes were detected. After ten days of the treatment, no change was observed in the weight of group 4, yet there was an average of 1.25 g weight gain observed in other groups.No other physical changes were observed in the groups at the end of the 10th day.

Clinical evaluation

Half of the mice in group 2 (4 out of 8) showed complete healing after five days of the treatment and developed no complications during the treatment (Table 2). In all mice in group 1 (8 out of 8) superficial burn wounds were detected in the lesion region of the footpaths. 75% of the mice in group 2 (6 out of 8) showed complete healing after ten days of the treatment. The rest of the mice in group 2 (2 out of 8) also showed partial healing (Table 2). There was no complication detected during the 10-day treatment period. Statistical analysis of the data showed that there was a significant difference between group 2and group 5 after both five days' treatment and ten days' treatment ($p < 0,05$).

Histopathological and immunohistochemical evaluation

Group 2 revealed a dense polymorphonuclear leukocyte infiltration with first-degree inflammation in the wounded area. Myofibroblastic cells seen in some locations in the wound area and angiogenesis indicate that

Table 2. Clinical evaluation data of the groups after 5-day and 10-day treatment.

	Groups	Complete healing (erythema:0, edema:0)	Partial healing (erythema:1, edema:1)	No healing (erythema:2, edema:2)
5th day	Group 1	2 (%25)	2 (%25)	4 (%50)
	Group 2	4 (%50)	2 (%25)	2 (%25)
	Group 3	2 (%25)	2 (%25)	4 (%50)
	Group 4	2 (%25)	2 (%25)	4 (%50)
	Group 5	0	0	8 (%100)
10th day	Group 1	4 (%50)	3 (%37,5)	1 (%12,5)
	Group 2	6 (%75)	2 (%25)	0
	Group 3	4 (%50)	3 (%37,5)	1 (%12,5)
	Group 4	3 (%50)	1 (%16,6)	2 (%33,4)
	Group 5	0	0	8 (%100)

Table 3. Infection/inflammation grade and mean amastigote counts in groups as a result of the histopathological evaluation.

Groups	Infection/inflammation grade	Mean amastigote counts
Group 1	2	2
Group 2	1	0
Group 3	2	5
Group 4	2	>20
Group 5	2	>20

Table 4. The situation of cytokines by immunohistochemical staining results.

Groups	TNF-α	IL-1	IL-6	IFN-γ
Group 1	3	2	3	2
Group 2	1	1	1	1
Group 3	3	2	2	3
Group 4	3	2	2	3
Group 5	3	3	3	3

wound healing has initiated and continued. Closer examination showed that both inflammatory reaction and wound healing process have continued in the wound area. On examination under 40x magnification, no amastigotes could be observed (Table 3).

Statistical analysis of the severity of infection and the number of amastigotes suggests that there was a significant difference between group 2 and group 5 in both conditions ($p<0,05$). Immunohistochemical results revealed that TNF-α and IFN-γ exhibited intense reactivity, and IL-1 and IL-6 exhibited moderate reactivity in both the 4 and 5 groups. In the group 1, TNF-α and IL-1 exhibited intense reactivity, and IL-6 and IFN-γ exhibited moderate reactivity. In the group 2, TNF-α, IL-1, and IL-6 exhibited mild reactivity, and IFN-γ exhibited moderate reactivity. Intense TNF-α, moderate IL-1 and IL-6, and mild IFN-γ reactivity were detected in the group 3 (Table 4). Analysis of the immunohistochemical data indicated that there was a statistically significant difference between 2 and 5 groups ($p <0.05$), but there was no statistically significant difference between group 5 and other experimental groups ($p >0.05$). Comparing group 2 with other experimental groups also showed a significant difference after ten days of treatment ($p <0.05$).

DISCUSSION

According to World Health Organization (WHO) reports, leishmaniasis is endemic in 98 countries[16]. The usage of chemotherapeutics is still the first choice for the treatment of this disease because vaccine against leishmaniasis has not been developed so far. Although there are new chemotherapeutics used for leishmaniasis, many of them are derived from previous chemotherapeutics. In addition, intralesional and systemic antimony compounds still are the gold standard for treatment[5]. Many researches and cases have been performed in parallel with the WHO recommendations. Various therapeutic agents have been applied locally or systemically, in combination and alone. Positive results of wound healing have been obtained. However, detailed examination of these studies revealed that complete healing was not achieved except for a few cases. During the treatment, systemic toxicity was developed and at the end of the treatment, a scar tissue remained at the wound site. When comparing treatment duration, it differs from 20 days to 6 months[17–21]. It is known that chemicals used in CL treatment have more side effects and toxicity[22–23]. Oral administration of miltefosine and parenteral administration of meglumine antimoniate have been shown to lead to liver and kidney toxicity[19]. In addition to the side effects of chemicals used in CL treatment, high costs and difficulties with their accessibility have prompted researchers to seek different treatment methods. Electrotherapy is one of these treatment methods. In a study the effects of low electrical potentials on *L. major* were investigated both in culture and in experimentally infected BALB/c and NMRI mice. According to study findings, exposure to direct-current potentials of 3, 6, 9 and 12 V killed all promastigotes. When electrodes were used in skin lesions of infected mice, all but the lowest voltage (3 V) caused unwanted ulceration. At 3 V, however, 3 weeks of electrotherapy, for 10 min twice weekly, initially appeared to cure all the lesions and the therapy[24].

Recently, nanotechnological application is being used in an ascending rate in medicine. The optical, fluorescent and magnetic properties of nanoparticles are exploited particularly in the early diagnosis of cancer, pathogens

and genetic diseases[11–14]. Starting from the last decade nanomaterials have started to be used as treatment agents. The number of nanoformulations emerging as a new approach for leishmaniasis treatment is also ascending gradually. In a recent study to investigate *in vitro* effects, *L. major* promastigotes and amastigotes were exposed to different concentrations of 20nm sized ZnO nanoparticles. The result revealed that the number of promastigote and amastigote reduced significantly in a dose-dependent manner compared to the control group[25]. The *in vitro* anti-leishmanial effects of titanium dioxide, zinc oxide, magnesium oxide, silver and gold nano particles were examined on *L. major* amastigote and promastigote forms.

Research results indicated that nanoparticles had strong toxicity to promastigotes and were toxic to macrophages. Based on the different toxicity mechanisms of nanoparticles, it has been suggested that all nanoparticles damage many structures such as macrophage membranes, various enzymes, and nucleicacids as well as promastigotes. This means that the nanoparticles do not have a specific target. It has therefore been emphasized that they should not be used in parenteral treatments due to their high toxicity. Although nanoparticles exhibit high anti-leishmanial activity on *L. major*, they prevent the proliferation of cells and reduce the infection index. To be used parenterally or locally, these nanoparticles have to be conjugated with a carrier molecule such as lectin or antibody, which will direct the nanoparticle to specific targets[26].

In our treatment protocol, GO nanoparticles modified with MSA were used locally, and no parenteral treatment was applied.Current literature shows that no treatment method that allows CL to heal within a short period of 5 days. It is thought that regression of CL lesions within five days may be due to the transport of GO nanoparticles modifiedwith MSA to the target cells and that the administered photothermal therapy,are directly effective on macrophages and amastigotes engulfed by these macrophages. In the treatment, intralesional single injection of synthesized GO+MSA was sufficient, and the treatment was supported by photothermal therapy administered in the form of near-infrared radiation. This method is an easier and cost-effective treatment method than other treatment methods.Our study shows that the toxic effects of nanoparticles were not parasite-specific and could affect other cells, enzymes, and nucleic acids.The nanoparticles have been made possible to concentrate at its target through the binding of GO nanoparticles used in the present study to MSA.

The report of WHO states that thermotherapy is an alternative treatment approach to CL. It is indicated that increasing the temperature of the lesion area locally to 50°C for 30 sec once or twice a day is as effective as intralesionally-administered antimony compounds. In this application, it occurs as a complication and disadvantage that second-degree burns can occur and the application should be applied under anesthesia[16]. There are many *in vivo* studies that investigate the effect of thermotherapy on CL[27–30]. It has been reported that there was no significant difference in wound healing rate between patients receiving thermotherapy and those receiving sodium stibogluconate and that thermotherapy may be preferred because of greater parenteral toxicity in patients receiving sodium stibogluconate[27–30].

In mice footpaths, the unmodified GO nanoparticles (group 1) were not directed to the CL lesions and accumulated subcutaneously because of the absence of MSA, and for that reason superficial skin burns were observed. It is known that burning wounds are caused by extreme heat conduction of GO. Histopathological examinations showed that GO is transported to the target cell via MSA and that GO+MSA is phagocytosed by macrophages. Thus, target cells were directly heated by GO nanoparticles and killed together with amastigotes engulfed by macrophages.

CONCLUSION

This is the first research that MSA modified graphene oxide (GO) nanoparticles are used to treat cutaneous leishmaniasis (CL). Based on our research outputs CL started to heal within five days, and the treatment was succeeding in 10 days. The suggested protocol for CL treatment is effective in the short-term application and is applied easily.

Conflict of interest: None

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REFERENCES

1 Garcia LS. *Diagnostic medical parasitology*: American Society for Microbiology Press, 2006.

2 Vincent H. William Boog *Leishman*: parasitologist and politician. *Parasitol* 2017; *144*: 1582–9.

3 Satoskar RS, Bhandarkar SD, Rege NN. *Pharmacology and pharmacotherapeutics*, 24 edn., Vol. 58. Chapter: *Elsevier* 2015.
4 Zangger H, Ronet C, Desponds C, Kuhlmann FM, Robinson J, Hartley MA, *et al*. Detection of Leishmania RNA virus in *Leishmania* parasites. *PLOS Negl Trop Dis* 2013; *7*(1): 1–11.
5 Kumar A. *Leishmania and leishmaniasis*: *Springer* 2013.
6 Balai M, Gupta LK, Khare AK, Srivastava A, Mittal A, Singh A. Cutaneous *leishmaniasis* in a nonendemic area of South Rajasthan: A prospective study. *Indian J Dermatol* 2016; *61*(5): 521.
7 Balogun A, Weigel M, Cevallos W, Puebla E, Rodriguez R. Epidemiology of cutaneous *leishmaniasis* in an endemic Pacific coastal rainforest area of Ecuador. *Ann Glob Health* 2017; *83*(1): 61–2.
8 Gupta LK, Meena S, Khare AK, Balai M, Mittal A, Mehta S. Lupoid cutaneous *leishmaniasis*: A report of three cases from nonendemic area. *Indian J Dermatol* 2017; *62*(62): 548.
9 Scott P, Novais FO. Cutaneous *leishmaniasis*: immune responses in protection and pathogenesis. *Nat Rev Immunol* 2016; *16*(9): 581–92
10 Bhargava P, Singh R. Developments in diagnosis and *antileishmanial* drugs. *Interdiscip Perspect Infect Dis* 2012; 1–13.
11 Du D, Wang L, Shao Y, Wang J, Engelhard MH, Lin Y. Functionalized graphene oxide as a nanocarrier in a multienzyme labeling amplification strategy for ultrasensitive electrochemical immunoassay of phosphorylated p53 (S392). *Anal Chem* 2011; *83*(3): 746–52.
12 Hong H, Yang K, Zhang Y, Engle JW, Nayak TR, Goel S, *et al*. In vivo targeting and imaging of tumor vasculature with radiolabeled, antibody-conjugated nanographene. *ACS nano* 2012; *6*(3): 2361–70.
13 Jung HS, Kong WH, Sung DK, Lee MY, Beack SE, Keum do H, *et al*. Nanographene oxide-hyaluronic acid conjugate for photothermal ablation therapy of skin cancer. *ACS nano* 2014; *8*(1): 260–8.
14 Lu B, Li T, Zhao H, Li X, Gao C, Zhang S, *et al*. Graphene-based composite materials beneficial to wound healing. *Nanoscale* 2012; *4*(9)*:* 2978–82.
15 Glaser TA, Wells SJ, Spithill TW, Pettitt JM, Humphris DC, Mukkada AJ. *Leishmania* major and L. donovani: a method for rapid purification of amastigotes. *Exp Parasitol* 1990; *71*(3): 343–5.
16 WHO. Control of the *leishmaniasis*. In: *World Health Organization technical report series*), 2010: xii.
17 Ben Salah A, Ben Messaoud N, Guedri E, Zaatour A, Ben Alaya N, Bettaieb J, *et al*. Topical paromomycin with or without gentamicin for cutaneous *leishmaniasis*. *New Eng J Med* 2013; *368*(8): 524–32.
18 Islam S. Rapidly progressing facial *leishmaniasis*: effective treatment with liposomal amphotericin B and a review of the management of Old World cutaneous leishmaniasis. *Paediatr Int Child Health* 2017; 1–4.
19 Velez I. Meglumine-antimonate/miltefosine. *Reactions* 2016; *1594*: 131–26.
20 Aerts O, Duchateau N, Willemse P, Lambert J. Cutaneous *Leishmaniasis*: Treatment with low-dose oral fluconazole with an excellent cosmetic result. *Pediatr Dermatol* 2015; *32*(1): 154–5.
21 Van Bocxlaer K, Yardley V, Murdan S, Croft SL. Topical formulations of miltefosine for cutaneous *leishmaniasis* in a BALB/c mouse model. *J Pharm Pharmacol* 2016; *68*(7): 862–72.
22 Oryan A. Plant-derived compounds in treatment of *leishmaniasis*. *Iran J Vet Med* 2015; *16*(1): 1–19.
23 Croft SL, Sundar S, Fairlamb AH. Drug resistance in *leishmaniasis*. *Clin Microbiol Rev* 2006; *19*: 111–26.
24 Hejazi H, Eslami G, Dalimi A. The parasiticidal effect of electricity on Leishmania major, both in vitro and in vivo. *Annals of tropical medicine and parasitology* 2004; *98*(1): 37–42.
25 Delavari M, Dalimi A, Ghaffarifar F, Sadraei J. In vitro study on cytotoxic effects of ZnO nanoparticles on promastigote and amastigote forms of *Leishmania* major (MRHO/IR/75/ER). *Iran J Parasitol* 2014; *9*(1): 6–13.
26 Jebali A, Kazemi B. Nano-based antileishmanial agents: a toxicological study on nanoparticles for future treatment of cutaneous *leishmaniasis*. *Toxicol In Vitro* 2013; *27*: 1896–904.
27 Reithinger R, Mohsen M, Wahid M, Bismullah M, Quinnell R, Davies C, *et al*. Efficacy of thermotherapy to treat cutaneous *leishmaniasis* caused by Leishmania tropica in Kabul, Afghanistan: a randomized, controlled trial. *Clin Infect Dis* 2005; *40*(8): 1148–55.
28 Aronson NE, Wortmann GW, Byrne WR, Howard RS, Bernstein WB, Marovich MA, *et al*. A randomized controlled trial of local heat therapy versus intravenous sodium stibogluconate for the treatment of cutaneous *Leishmania major* infection. *PLoS Negl Trop Dis* 2010; *4*(3): 628.
29 Lopez L, Robayo M, Vargas M, Velez ID. Thermotherapy. An alternative for the treatment of American cutaneous *leishmaniasis. Trials* 2012; *13*: 58.
30 Safi N, Davis GD, Nadir M, Hamid H, ARNG AJCM. Evaluation of thermotherapy for the treatment of cutaneous *leishmaniasis* in Kabul, Afghanistan: a randomized controlled trial. *Mil Med* 2012; *177*(3): 345–351.

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