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ORIGINAL ARTICLE

Combination of infra-red light with nanogold targeting macrophages in the treatment of Leishmania major infected BALB/C mice

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ABSTRACT

Purpose: In the treatment of cutaneous leishmaniasis (CL), developing drug resistance, existing toxic effects of drugs and failure respond to treatment cause the need to try different treatment methods. We investigated the effect of gold-conjugated macrophage-specific antibody on amastigotes under infra-red light for the treatment of CL.

Methods: Female BALB/c (4–8 weeks old, 20 ± 5 g weight) mice were used in the study. The *L. major* strain was inoculated on the soles of mice in amastigote form and subpassed. Nanogold (Au), Au + macrophage-specific antibody (MSA) modification and near infra-red (NIR) (5 seconds) were applied to mice groups that developed cutaneous leishmaniasis on their soles. On the 5th and 10th days of the treatment, the lesions were examined clinically and pathologically.

Results: When the erythema values were examined, the highest decrease was calculated in the Au + MSA + NIR group in the measurements made on the 10th day ($p < 0.014$). The best improvement in 10th day measurements is in the NIR and Au + MSA + NIR groups when area values were examined ($p = 0.011$, $p = 0.001$). There was a statistically significant difference between the groups in terms of parasite load (PL) ($p < 0.005$) in pathological evaluation. According to PL grouping, the best result is NIR ($p = 0.002$). When both main titles (clinical and pathological) are examined, the Au + MSA + NIR group is thought to have an optimal therapeutical feature.

Conclusions: Au + MSA + NIR combination could be a new treatment approach for CL treatment.

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cutaneous leishmaniasis; nanogold; BALB/c; phototherapy; macrophage-specific antibody

Introduction

Leishmaniasis is a chronic disease caused by intracellular flagellate protozoa and which the treatment failure rate has increased recently. Leishmaniasis has become a health problem in non-endemic regions due to reasons such as climate change, migration, urbanisation, and suppression of immunity[1,2]. Skin lesions in cutaneous leishmaniasis, a clinical form of leishmaniasis, can usually heal spontaneously within months or years without treatment. However, since leishmaniasis is located in open anatomical areas such as the face and arms, bad wound healing causes cosmetic problems[3].

Developing drug resistance, existing toxic effects of drugs and failure to respond to treatment have led researchers to seek different treatment methods.

Thanks to nano medicine, which is considered one of the most promising technologies of recent years, different treatment alternatives are being tried for the treatment of Leishmania. In studies conducted for this purpose, it has been reported that nanoparticles such as silver, gold, chitosan and iron oxide (Fe3O4), titanium, zinc have a growth or inhibitory effect on Leishmania[4]. Gold has strong optical absorption, high stability, and some nanoparticles, such as gold, have a photothermal effect after exposure to near infra-red (NIR) light. Gold absorbs IR energy and converts it into heat, the temperature rises and the cell is damaged as a result[5,6]. Targeted treatments, which open a new era in cancer treatment, act by targeting specific molecules that cause cancer to grow, progress and spread[7]. In the treatment of leishmania, targeted treatments such as macrophages, which are necessary for the life cycle of leishmania, will result in reduced toxicity of drugs and shorter treatment time[8].

Based on all this information, in this study, we investigated the effect of gold-conjugated macrophage-specific antibody on amastigotes under infra-red light. When the literature was examined, no gold-macrophage specific antibody (MSA) treatment was found under near infra-red light, and as far as we know, our study on this subject is the first.

Material and method

Experimental animals and experimental groups

In this study, the approval of Cumhuriyet University Animal Experiments Local Ethics Committee, dated 01.09.2016 and

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Table 1. Experimental groups, mice numbers and treatment plan used in the study.

Experimental groups	Mice numbers
PC	7
Au	7
Au + NIR (5 sec)	7
Miltefosine	7
NIR (5 sec)	7
Au + MSA + NIR (5 sec)	7
	42

Au: Gold; NIR: Near infra-red light; MSA: Macrophage specific antibody; PC: Positive Control.

numbered 65202830-050.04.04–86 was obtained for the use of experimental animals.

Female BALB/c (4–8 weeks old, 20 ± 5 g weight) mice were used in the study. During the study, mice were housed in a well-ventilated environment with a 12-h light/12-h dark cycle, 40–60% relative humidity, and a temperature of 20–24 °C, in separate cages for each group. In the study, 4 experimental groups and two control groups were formed, and 7 mice were included in each group (Table 1). Experimental groups were formed to measure the effect of Au, the effect of Au + MSA modification and the effect of NIR. Miltefosine, which is preferred in the treatment of leishmania, was used in the control group, while the other groups were not given treatment. NIR was applied to the groups for 5 s.

Parasite inoculation

From Celal Bayar University Parasite Bank, *L. major* MHOM/TR/2013/MANISAPB145 strain was obtained to be used in our study. Amastigotes were isolated from lesions of infected BALB/c mice. The parasite strain was inoculated on the soles of the feet of 10 BALB/c mice in amastigote form under sterile conditions and passaged. The density of amastigote was evaluated by histological examination of the lesions, which occurred after an average of 3 weeks after the passage. Afterwards, the passages were made from animal to animal to ensure the continuity of the strain. For the study, amastigotes were inoculated into the soles of 60 mice and CL developed in the feet of these mice at the end of 21th day. Foot diameters were measured with calliper (measurement unit inch). Mice close to each other in measurements were included in the study (Figure 1(a)). Experimental animal with ulcers and severe edoema were excluded from the study (Figure 1(b)) and 42 mice were randomly divided into study groups with 7 mice in each group.

Preparation of macrophage specific antibody-bound gold nanoparticles

5 nm size Au nanoparticles (Sigma, 765449) and Macrophage Specific Antibody (Abnova, MAB5180) were purchased. AuNPs coated with MSA (Macrophage SpecificAntikor) were prepared according to Zhao et al.[9] Briefly, 8 µL of MSA was added to 1 ml of AuNPs and incubated on a shaker for 30 min at room temperature. 100 µL of 10% bovine serum albumin was added to the mixture and incubated again for 10 min at room temperature. The suspension was then

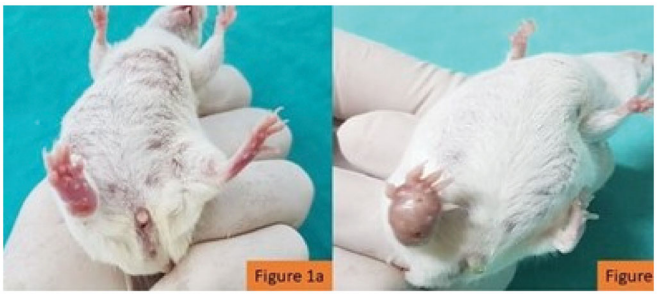

Figure 1. The soles of mice after three weeks of the parasite strain injection. (a) experimental mice (b) excluded mice

washed with wash solution [PBS, pH7, containing 1% (v/v) BSA] by centrifugation at 20 000 g for 1 h at 4 °C. The pellet containing antibody-coated AuN-Ps was re-suspensiond in a 1 ml washing buffer and the washing process was repeated 3 times. The prepared suspension was stored for use in the study at +4 °C.

Confirmation of Au + MSA synthesis and elemental analysis

Elemental analyzes of the substances were performed using a micro elemental analyser (Leco TruSpec, USA). Whether the Au + MSA modification was successful or not was verified by the SEM method (Hitachi TM3030 desktop SEM-EDX) at Anadolu University Plant, Pharmaceutical and Scientific Applications and Research Centre (Figure 2(a,b)). The difference in the chemical position between the two samples as a result of the elemental analysis, and the detection of Ca, Mg, Fe, S substances that were not present in the gold nanoparticle analysis in the analysis results after antibody binding confirms that MSA binds to Au nanoparticles.

Treatment plan

After CL formation, 20 µL of Au and Au + MSA nanoparticles were injected subcutaneously into the feet of the mice in the experimental group, and the treatment procedure was applied 24 h later. Positive control groups did not receive treatment, and miltefosine groups were administered orally miltefosine at a dose of 50 mg/kg/day for the first five days and 25 mg/kg/day for the last five days. Due to mouse deaths, doses were reduced in the treatment of miltefosin. NIR was applied with the PL-E Pro 808 nm Infra-red Laser (Jet, China) device in the form of near infra-red radiation at a dose of 5 s/day. Experimental groups were arranged as only Au, Au + NIR, Au + MSA + NIR, NIR as indicated in Table 1. On the 5th and 10th days of the treatment, the mice were evaluated clinically, and on the 10th day histopathologically and immunohistochemically. At the end of the 10-day treatment period, the animals were sacrificed after general anaesthesia.

Determining the effect of treatment

Clinical appearance

On the 5th and 10th days, CL lesions were evaluated in terms of erythema and edoema, and foot diameters were measured

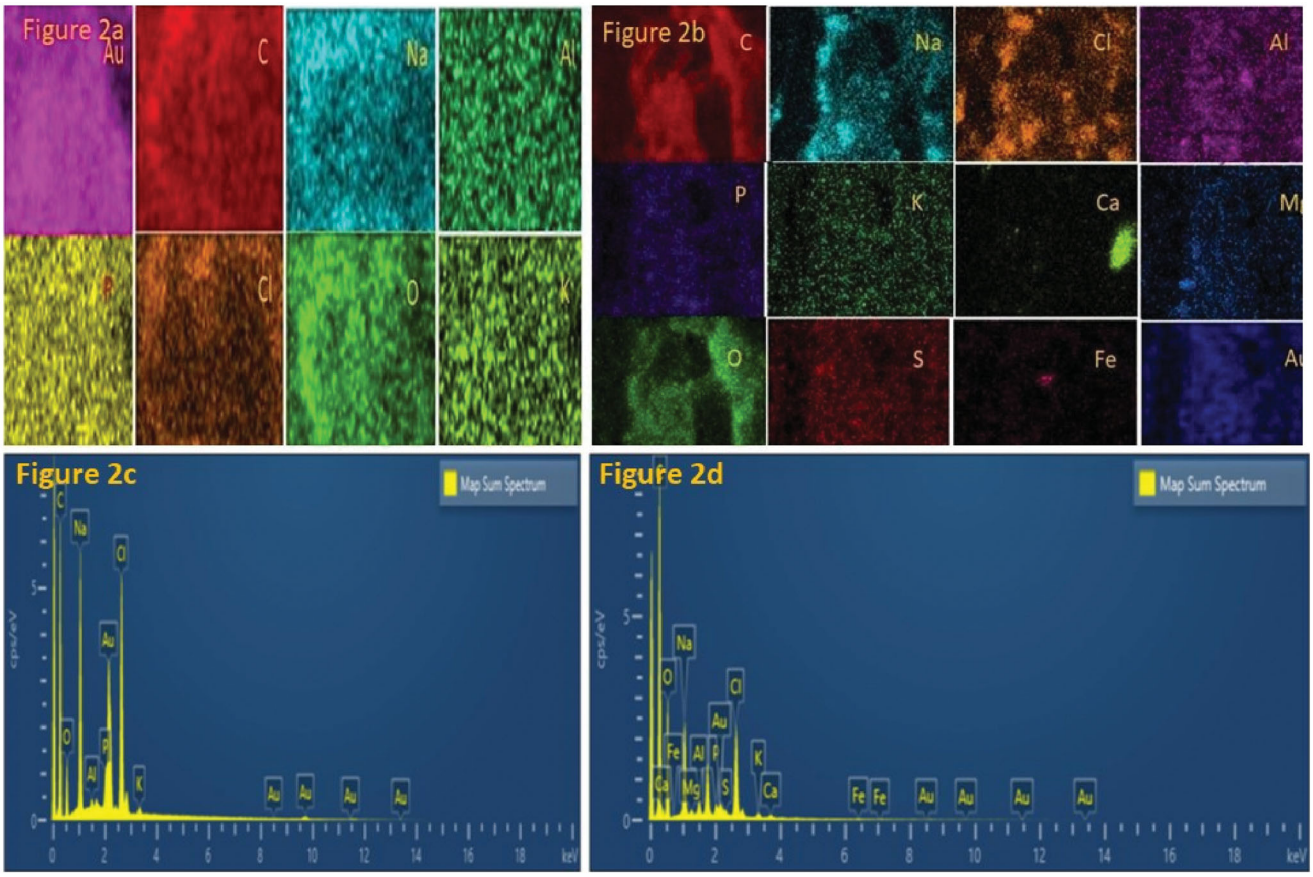

Figure 2. SEM image and elemental analysis of MSA-binding Au nanoparticles (When the (c) and (d) are examined (the (c) is without antibodies, (d) is with antibodies) the fact that the Ca, Mg, Fe, S substances between the two figures are not in the (c) but in the (d) shows that the antibody is bound to the gold nanoparticle). (a,c) unbound of nanogold particles to MSA. (b,d) binding of nanogold particles to MSA.

with calliper (measurement unit inch). (no erythema:0, mild erythema:1, moderate erythema:2, severe erythema:3; no edoema:0, mild edoema:1, moderate edoema:2, severe edoema:3). In the clinical evaluation, the values formed by the multiplication of the foot diameter and edoema values were evaluated statistically.

Histopathological and immunohistochemical evaluation:

At the end of the study, tissue samples taken from the experimental and control groups were evaluated histopathologically (haematoxylin & eosin staining) and immunohistochemically (TNF-α, IL-6 and IFN-γ). In haematoxylin & eosin staining, lymphocyte and histiocyte density in tissues (1: mild, 2: moderate and 3: severe) and TNF-α, IL-6 and IFN-γ density in immunohistochemical staining were evaluated (1: mild, 2: moderate, 3: severe). Amastigote density was grouped as 0– 1/4: mild, 1/4 – 2/4: moderate, 2/4– 3/4: severe, 3/4 – 4/4: very severe at 40X magnification (Nikon Eclipse E600 Microscope)

Statistics

The data were evaluated using the SPSS version 22.0 package program. Data were analysed by Chi-square analysis, Kruskal-Wallis H test and Mann Whitney U test. P values < 0.05 were considered statistically significant. Normality test was not performed due to the condition $n < 30$. Analysis comments were made at the 95% significance level.

Results

Clinical evaluation

Erythema:

When the erythema values were examined, a decrease was calculated in all treatment modalities except photothermal treatment. The highest decrease was calculated in the Au + MSA + NIR group in the measurements made on the 10th day (Table 2), (Figure 3(a)).

Area

A statistically significant difference was calculated in all groups compared to the previous period ($p < 0.005$). There was a statistically significant difference between the groups during the first measurement (post-procedure) ($p < 0.005$). It is seen that the best improvement in 10-day measurements is in the NIR and Au + MSA + NIR groups. Among these two methods, Au + MSA + NIR group with the smallest IQR value can be preferred (Table 3), (Figure 3(a,c)).

Table 2. Evaluation in terms of erythema.

Group	Erythema 0th day Med.±IQR (%25-%75)	Erythema 5th day Med.±IQR (%25-%75)	Erythema 10th day Med.±IQR (%25-%75)	*p Value
PC	3 ± 1 (2–3)	2 ± 0 (2–2)	2 ± 1 (1–2)	0.024[a]
Au	3 ± 1.5 (2.5–4)	2 ± 1.5 (1.5–3)	2 ± 2 (1–3)	0.023[b]
Au + NIR	3 ± 1 (2–3)	2 ± 1 (2–3)	2 ± 0 (2–2)	0.023[c]
Miltefosine	3 ± 1 (3–4)	3 ± 0 (3–3)	3 ± 0 (3–3)	0.050
NIR	2.5 ± 2 (2–4)	2.5 ± 2 (1.5–3.5)	2.5 ± 1.5 (2–3.5)	0.223
Au + MSA + NIR	3 ± 1 (2–3)	2 ± 0 (2–2)	2 ± 1 (1–2)	0.014[d]
p[&]	0.439	0.346	0.002	

a: 0th day-5th day $p = 0.046$.
b: 0th day-5th day $p = 0.046$.
c: 0th day-5th day $p = 0.046$.
d: 0th day-10th day $p = 0.038$; 5th day-10th day $p = 0.046$.
*: Kruskal-Wallis H Test, Post Hoc Mann-Whitney U Test.
&: Friedman F Test.

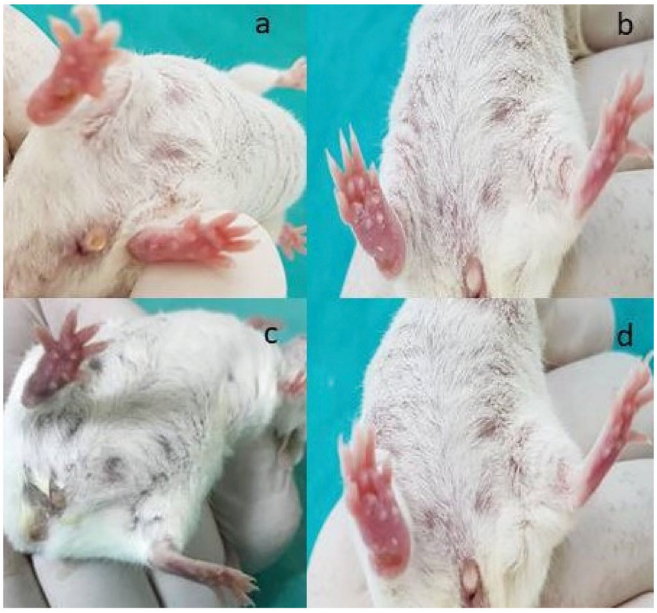

Figure 3. Clinical appearance after 10 days of treatment. (a) Au + MSA + NIR (healing), (b) miltefosine (non healing), (c) NIR (healing), (d) positive control.

Pathological evaluation

Histiocytes

When evaluated in terms of histiocytes, a statistically significant difference was found in all pathology results ($p < 0.005$) (Figure 4).

There was a statistically significant difference between the groups in terms of parasite load (PL) ($p < 0.005$). According to PL grouping, the best result is NIR ($p = 0.002$) (Figure 4(c)).

When IL-6 was evaluated, the best result was seen in the NIR group again ($p = 0.019$). When evaluated in terms of TNFα, the best result among the treatment groups was Au + MSA + NIR ($p = 0.004$). When the IFN-ɤ treatment groups were evaluated, there were mice in the Au group that were evaluated both severely and mildly. For this reason, the randomness of the measurements in the Au group is remarkable. Since there is no difference between other treatment methods in terms of IFN-ɤ, any treatment method can be preferred (Table 4).

Lymphocytes

When evaluated in terms of cytokine measurements, no statistically significant difference was calculated between the groups ($p > 0.005$). In terms of PL, the best recovery was calculated for NIR ($p = 0.002$).

Clinical and pathological evaluation

When the clinical examination was evaluated, it was seen that the Au + MSA + NIR group stood out compared to the other groups and in the pathological examinations, the NIR group gave better results, followed by the Au + MSA + NIR group. Accordingly, when both main titles are examined, the Au + MSA + NIR group is thought to have an optimal therapeutical feature (Figures 3 and 4).

Discussion

In this study, we aimed to investigate the effect of gold-conjugated macrophage-specific antibody on amastigotes under infra-red light. As a result of the study, while clinically significant improvement was observed in the Au + MSA + NIR group for 5 s in BALB/c mice with leishmania compared to the other groups; in pathological examinations, we found positive results primarily in the NIR group and secondly in the Au + MSA + NIR group. When the mice were evaluated both clinically and pathologically, we found that the NIR group showed optimum therapeutical properties.

In the European region of the World Health Organisation, Israel, Turkey, Uzbekistan and Turkmenistan have been reported as endemic areas for cutaneous leishmaniasis. It is estimated that Leishmaniasis affects 0.7–1 million people worldwide. Leishmaniasis has become a major health problem also in non-endemic regions due to reasons such as global warming, migration, urbanisation, and suppression of immunity[1,10]. In some cases of CL, spontaneous recovery can be observed in less than 2 years, but these wounds will heal with scarring and the presence of this scar in a visible area such as the face may cause cosmetic problems[3]. While 5-valent antimony and sodium stibogluconate are used in the first-line treatment of CL, pentamidine, amphoteracin B and miltefosine are also among the drugs used. However, the toxic effects of these drugs, their intolerance and the recent

Table 3. Evaluation in terms of area.

Group	Area 0th day Med.±IQR (%25-%75)	Area 5th day Med.±IQR (%25-%75)	Area 10th day Med.±IQR (%25-%75)	*p Value
PC	0.05 ± 0.01 (0.05–0.06)	0.04 ± 0.02 (0.02–0.04)	0.01 ± 0 (0.01–0.01)	<0.001[a]
Au	0.04 ± 0.01 (0.04–0.05)	0.03 ± 0.01 (0.02–0.03)	0.01 ± 0.01 (0–0.01)	<0.001[b]
Au + NIR	0.06 ± 0.02 (0.05–0.07)	0.03 ± 0 (0.03–0.03)	0.01 ± 0 (0.01–0.01)	<0.001[c]
Miltefosine	0.05 ± 0.01 (0.05–0.06)	0.03 ± 0.01 (0.03–0.04)	0.02 ± 0.01 (0.01–0.02)	<0.001[d]
NIR	0.06 ± 0.03 (0.05–0.08)	0.05 ± 0.04 (0.03–0.07)	0.01 ± 0.02 (0.01–0.03)	<0.001[e]
Au + MSA + NIR	0.05 ± 0.01 (0.04–0.05)	0.03 ± 0.02 (0.02–0.04)	0.01 ± 0.01 (0–0.01)	<0.001[f]
p[&]	0.011	0.161	0.027	

a: 0th day-5th day $p = 0.005$; 0th day-10th day $p = 0.005$; 5th day-10th day $p = 0.005$.
b: 0th day-5th day $p = 0.011$; 0th day-10th day $p = 0.011$; 5th day-10th day $p = 0.011$.
c: 0th day-5th day $p = 0.001$; 0th day-10th day $p = 0.001$; 5th day-10th day $p = 0.001$.
d: 0th day-5th day $p = 0.005$; 0th day-10th day $p = 0.005$; 5th day-10th day $p = 0.005$.
e: 0th day-5th day $p = 0.011$; 0th day-10th day $p = 0.011$; 5th day-10th day $p = 0.011$.
f: 0th day-5th day $p = 0.001$; 0th day-10.th day $p = 0.001$; 5th day-10th day $p = 0.001$.
*: Kruskal-Wallis H Test, Post Hoc Mann-Whitney U Test.
&: Friedman F Test.

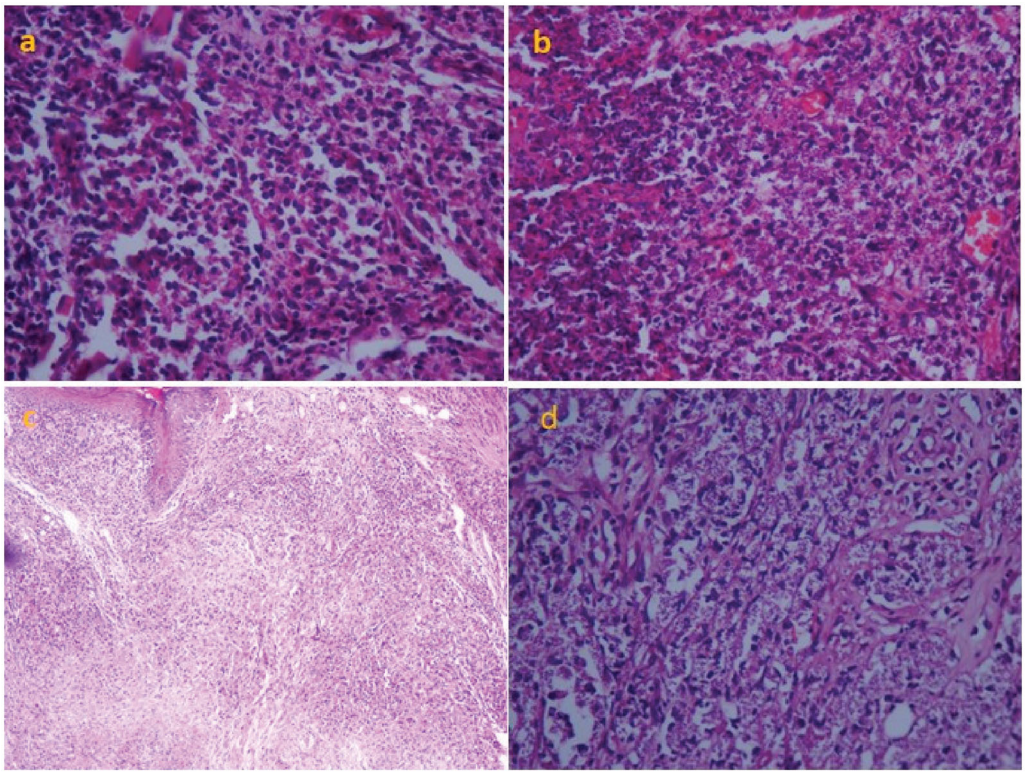

Figure 4. Histopathological examinations. (a) Au + MSA + NIR (moderate lymphoplasmacytic chronic inflammation, histiocytes with few amastigotes. H&E X 50). (b) Miltefosine (mild lymphoplasmacytic chronic inflammation, histiocytes with dense amastigotes. H&E X 50). (c) NIR (severe lymphoplasmacytic chronic inflammation, histiocytes with few amastigotes. H&E X 25). (d) PC (histiocytes with dense amastigotes. H&E X 50).

Table 4. Evaluation in terms of histiocytes.

histiocyte	pathology	category	PC	Au	Au + NIR	miltefosine	NIR	Au + MSA + NIR	*p* Value
	P.density	0–1/4	0 (%0)	0 (%0)	3 (%42.9)	**0 (%0)**	**3 (%75)**	4 (%57.1)	0.002
		1/4–2/4	0 (%0)	2 (%50)	4 (%57.1)	2 (%50)	1 (%25)	3 (%42.9)	
		2/4–3/4	1 (%20)	0 (%0)	**0 (%0)**	2 (%50)	0 (%0)	0 (%0)	
		3/4–4/4	4 (%80)	2 (%50)	0 (%0)	0 (%0)	0 (%0)	0 (%0)	
	IL6	light	0 (%0)	0 (%0)	0 (%0)	0 (%0)	2 (%50)	0 (%0)	0.019
		medium	5 (%100)	3 (%75)	7 (%100)	4 (%100)	2 (%50)	7 (%100)	
		severe	0 (%0)	1 (%25)	0 (%0)	0 (%0)	0 (%0)	0 (%0)	
	TNF	light	4 (%80)	0 (%0)	0 (%0)	0 (%0)	**0 (%0)**	1 (%14.3)	0.004
		medium	1 (%20)	3 (%75)	7 (%100)	4 (%100)	4 (%100)	6 (%85.7)	
		severe	0 (%0)	1 (%25)	0 (%0)	0 (%0)	0 (%0)	0 (%0)	
	IFN	light	**4 (%80)**	1 (%25)	0 (%0)	0 (%0)	0 (%0)	0 (%0)	0.003
		medium	1 (%20)	2 (%50)	7 (%100)	4 (%100)	4 (%100)	7 (%100)	
		severe	**0 (%0)**	1 (%25)	0 (%0)	0 (%0)	0 (%0)	0 (%0)	

development of resistance to drugs have led researchers to seek new treatment methods[4]. The field of nanomedicine, where therapeutic and diagnostic nanoparticles are used, is of great importance in terms of meeting the clinical needs in many disease[6]. Nanomaterials can be used as drug carriers, as contrast agents in imaging, in gene therapies, for diagnosis and treatment of cancer disease[11,12]. In the field of dermatology, nanomolecules are used for sunscreens, emollients, topical treatments, diagnostic approach and therapeutic purposes[13]. As well as, studies are carried out with nanoparticles for the treatment of leishmaniasis. It has been reported that nanoparticles such as silver, gold, chitosan, iron oxide (Fe3O4), titanium, and zinc have growth or inhibitory effects by damaging mitochondrial enzymes and cell cycle proteins of promastigotes[4,8]. Nanoparticles such as graphene are predicted to be used to treat leishmaniasis through interference with infra-red radiation[14].

The use of heat is another alternative treatment for CL. At high temperature (over 39 °C) showed that some leishmania species could not reproduce, and methods such as hot baths, infra-red light, direct electrical stimulation, laser, photodynamic therapy, ultrasound and radiofrequency were used for therapeutic purposes[15]. The WHO report mentions thermotherapy as a treatment approach for CL. It is stated that locally warming the lesion area to 50 °C for 30 s once or twice a day is as effective as intralesional antimony compounds[16]. Gold does not react with biological tissues and is a molecularly stable metal[17]. Along with infra-red light, gold nanoparticles are also highly effective in converting the absorbed light into heat and causes desired damage to the parasite[18]. Likewise, in an *in vitro* study, it was reported that iron oxide-mediated magnetic hyperthermia was effective against leishmania parasites[19]. Recently, targeted therapies are preferred especially in cancer treatments and successful results are obtained[20]. Macrophages play a key role in disease progression. The macrophage provides a safe environment for the replication of the parasite, which completes part of its life cycle within the macrophage, while on the other hand it is responsible for the destruction of the parasite[21]. In the light of all this information, we thought that macrophage-specific antibody conjugated with nanogold under infra-red light would be effective in the treatment. In our study, we used 6 groups of Balb/c mice with leishmania in their feet. When these experimental groups were evaluated both clinically and pathologically, we found that Au + MSA + NIR treatment method was effective on the 10th day. The regression of CL lesions suggests that MSA-modified gold nanoparticles absorb IR energy, converting it to heat, and exert a damaging effect on macrophages and amastigotes. In our study, the importance of NIR application is also revealed. Among the treatment options, NIR should also be considered.

Conclusion

When the literature was examined, MSA conjugated with nanogold was not found in the treatment of leishmaniasis. In this study, in which targeted therapy and nanotechnology were used together, a therapeutic effect was observed in the BALB/c animal model with leishmaniasis. Combination of infra-red light with nanogold targeting macrophages may create new and positive prospects in the treatment of cutaneous leishmaniasis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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