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Original article

THERAPEUTIC EVALUATION OF ACTIVATABLE MELITTIN FOR SELECTIVE TREATMENT OF LEISHMANIASIS *IN VITRO* AND *IN VIVO*

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Summary

Akhzari, S., S. Nabian, M. Taheri & Y. Salimabadi, 2024. Therapeutic evaluation of activatable melittin for selective treatment of leishmaniasis *in vitro* and *in vivo. Bulg. J. Vet. Med.,* **27**, No 1, 57–67.

Leishmaniasis is a major public health problem worldwide. Because of its high prevalence, the development of an effective treatment is especially important. Drug delivery systems are promising pharmaceutical formulations for improving the therapeutic index of drugs. In this study, activatable melittin (AM) peptide was designed and encapsulated with albumin and liposome for selective targeting of *Leishmania* infected cells. The effect of AM was determined by measuring its cytotoxicity and pathologic effects using Giemsa and haematoxylin/eosin staining, respectively. The results of this study showed that the toxicity of designed AM is reduced compared to wild-type melittin, in addition to the use of AM-loaded albumin nanoparticle and liposome containing AM and their therapeutic role against cutaneous leishmaniasis caused by *L. major*. The encapsulation of AM with albumin and liposome as a non-toxic carrier at a concentration of 25 µg/mL can improve and optimise the antileishmanial effects of this drug, so, infected cells treated with AM in albumin nanoparticle and liposome had less *Leishmania major* promastigotes. These forms of the drug could be a good alternative to the current drugs so performance of further *in vivo* studies is suggested.

Key words: activatable melittin, BALB/c mice, cutaneous leishmaniasis

INTRODUCTION

Leishmaniasis is a parasitic disease transmitted by sandflies infected with the *Leishmania* protozoa, which is still a health problem worldwide, especially in tropical and subtropical areas (Akhzari *et al.*, 2018). Leishmaniasis is endemic in more than 70 countries worldwide with an incidence of 0.5 million cases of visceral leishmaniasis (VL) and 1.5 million cases of cutaneous leishmaniasis (CL). Glo-

bally, there are 70,000 deaths each year and 350 million people are at risk of infection (Sabur *et al.*, 2015).

Despite of the efforts of clinical researchers over the years, little progress has been made in the treatment of cutaneous leishmaniasis with satisfactory clinical improvement; one of the important reasons for this problem is the lack of proper clinical trial studies in this field. One of the problems in the treatment of leishmaniasis is the drug resistance of parasites to some compounds (Ouellette *et al.*, 2004; Mina & Denny, 2018). Due to the high prevalence of leishmaniasis in different parts of the world and the emergence of resistance to current drugs, the discovery of new drugs with lower toxicity and broader range of therapeutic effects seems necessary (Zhang *et al.*, 2009). Melittin is a small peptide, a major component of *Apis mellifera* venom. The N-terminal part of this molecule is predominantly hydrophobic and the its C-terminal part is hydrophilic and strongly basic. It forms a tetramer in water but also can spontaneously integrate itself into cell membranes (Paray *et al.*, 2021). Melittin as an antimicrobial peptide can induce apoptosis in *Leishmania*, and then modulate cell death (Kulkarni *et al.*, 2009). Some studies showed that acylated synthetic cecropin A-melittin hybrid could be safe and effective for treating canine leishmaniasis (Alberola *et al.*, 2004).

Akhzari *et al.* (2021) showed that melittin had inhibitory effect on CHO cell line. Previous studies have shown that melittin induces inhibitory effects on proliferation of various cancer cells via induction of apoptosis, necrosis or lysis. Moreover, increased concentration and incubation time of this peptide can increase cytotoxicity of the melittin. At concentrations of 1 and 1.8 μg/mL melittin induced apoptosis in HeLa cells after 24 h of incubation while melittin at concentrations of 4 μg/mL induces late apoptosis and necrosis of the cells as demonstrated by flow cytometric analyses (Zarrinnahad *et al.*, 2018; Akhzari *et al.*, 2021).

In a previous study of ours, a molecular peptide based on melittin and its inhibitor sequences and matrix metalloproteinase (MMP) degradable linker which could be used for the selective targeting of *Leishmania* infected cells was designed (Akhzari *et al.,* 2021). Generally, leishmaniasis is characterised by strong inflammatory responses with a high-level production of tumour necrosis factor (TNF). The TNF induces matrix metalloproteinase enzymes, so regulation of melittin production due to expression level of MMPs in *Leishmania*-infected macrophages would happen (Akhzari *et al.,* 2021).

Campos *et al.* (2014) have reported that cells in cutaneous leishmaniasis (CL) lesions secrete high levels of matrix metalloproteinase-9 (MMP-9), compared to cells in healthy people.

The aim of the present study was to evaluate the effect of activatabe mellitin as a leishmanicidal agent and to determine its cytotoxicity and pathologic effects.

MATERIALS AND METHODS

In this study, melittin peptide was designed as described by Akhzari *et al.* (2021). The attachment of melittin (Bio company) to a neutralising polyanionic peptide (E7) via a matrix metalloproteinase cleavable linker may be a useful approach for therapeutic uses in leishmaniasis, so melittin was inhibited unless the linker is proteolysed in infected cells.

Preparation of AM-loaded liposome

In this study to achieve effective concentrations of peptide-containing liposome for *in vivo* experiments, different concentrations of 100, 50, and 25 µg of peptide per 100 µL of liposome *in vitro* were used. AM was encapsulated in liposome based on film-hydration procedure (Ghanbarzadeh *et al.*, 2013). Lipids were dissolved in chloroform and AM was dissolved in PBS (10 µg/µL) and then they were mixed in glass tubes and dried as a thin film by rotary evaporation (Heidolph, Germany) under reduced pressure. The lipid film was freeze-dried by Freeze drier (VD-800F; Taitech, Japan) overnight to remove the solvents completely. It was then hydrated in HEPES buffer (10 mM, pH 7.2) containing 5% dextrose by intermittent vortexing and bath sonication under argon for a short time (approximately 30 s at 25 ºC) to disperse completely the lipids into the buffer. The resulting multilamellar vesicles (MLVs) were extruded 5 times through 200 nm and 11 times through 100 nm syringe filter at 25 ºC to form 100 nm small unilamellar vesicles (SUVs) with a uniform size. Liposomes were stored at 4 ºC.

Preparation of AM-loaded albumin nanoparticle

The precipitation method was used for this purpose by applying different concentrations of $ZnCl_2$. Different concentrations of activatable melitin (100, 50, and 25 µg) were dissolved in distilled water and albumin solution (0.2 g/mL) was added to the activatable melittin solution at 55 ^oC and pH=7. Drug precipitation was performed using 2 µg/mL of $ZnCl_2$. To enhance the drug loading and to achieve a higher amount of activatable melittin in the albumin nanoparticles, the $ZnCl_2$ was gradually added (Akhzari *et al.*, 2022).

Parasite culture

The promastigote form of the standard strain of *Leishmania major* (MRHO/IR/75/ER) was prepared from the Faculty of Public health, Tehran University of Medical Sciences. For mass cultivation, NNN Medium was used and complete RPMI-1640 medium with L-glutamine, HEPES and penicillin- streptomycin (100 IU/mL) supplemented with 10% heat-inactivated foetal bovine serum was used as previously described (Tempon *et al.,* 2005).

Macrophage culture

RAW cell line (strains of mouse macrophages) was prepared from Pasteur Institute (Tehran, Iran), and cultured in complete RPMI-1640 medium at 37 ^oC and CO_2 (5%) conditions.

Evaluation of the cytotoxicity of melittin on macrophages in vitro

A suspension (1×10^5 cells/mL) was prepared from cultured macrophages and 100 µL was poured into each well of 6-well plates. After 24 h of incubation at 37 ^oC with CO_2 (5%), the supernatant was removed and 100 µL of melittin at different concentrations (0.5, 1.8, 4 µg/mL) were added to the wells and incubated again for 48 h at 37 ^oC. Washing was done separately after 4, 24, 48 hours. The slides were fixed with methanol and stained with Giemsa 10% and macrophages were observed under a light microscope with ×1,000 magnification. Finally, they were compared with the control group.

Evaluation of albumin nanoparticle and liposome cytotoxicity on macrophages

A suspension (1×10^5 cells/mL) was prepared from cultured macrophages and 100 µL was poured into each well of 6-well plates. After 24 h of incubation at 37 ^oC

with CO_2 (5%), the supernatant was removed and 100 µL of drugs in different concentrations (25, 50,100 µg/mL) were added to the wells and incubated again for 48 h at 37 ^{o}C. After 4, 24, 48 hours they were washed separately. The slides were fixed with methanol and stained with 10% Giemsa. Macrophages were observed under a light microscope with ×1,000 magnification and were compared with the control group.

Evaluation of the inhibitory function of albumin nanoparticle and liposome on Leishmania amastigotes in macrophages

The activity against *Leishmania major* (*L. major*) intracellular amastigotes was determined in infected macrophages. The latter were dispensed into 6-well plates containing glass cover slips at a density of 4×10^4 cells/well and were incubated for 24 h prior to infection. *L. major* promastigotes were added at a ratio of 1:10 (macrophage/promastigote) and were incubated for 24 h. Non-internalised parasites were removed by washing once with medium followed by incubation and treatment with mentioned components for 5 days at 37 ^{o}C in 5% CO_2. Cells were fixed in methanol, stained with Giemsa stain and observed under a light microscope to determine the number of intracellular parasites. The parasite burden was determined as the number of infected macrophages in a total of 400 cells from drug-treated and control cultures and compared with the number of counted amastigotes in the untreated cultures (Tempon *et al.,* 2005).

Animals

Twelve female BALB/c mice, aged 7 weeks (18–25 g) were purchased from Pasteur Institute of Iran, Tehran and housed in the animal facility of the Faculty of Veterinary Medicine, University of Tehran. A 7-day acclimatisation period before the challenge was respected.

In vivo experiments

All of BALB/c mice were infected subcutaneously with 2×10^6 *L. major* promastigotes cultured for 6 days in 500 µL of RPMI-1640 medium, as previously described (Tempon *et al.,* 2005). Fourteen days after infection (day 14), animals were treated daily subcutaneously for a period of 8 days. Mice were divided into 3 groups (4 mice per group) as followed: group I (control group): with PBS injection; group II: treated with 100 µL liposome injection containing 25 µg/mL peptide (1.2 mg/kg body weight); group III: injected with 100 µL albumin nanoparticle containing 25 µg/mL peptide (1.2 mg/kg body weight) and were treated every other day. The volume of each injection was 400 µL per mouse. Animals were euthanized 7 days after the end of treatment (day 28). The animals were euthanised and tissue specimens (skin and spleen) were fixed in the 10% neutral buffered formalin (pH. 7.26) for 48 h, then embedded in paraffin, and sectioned to 5 mm thickness. Finally, the sections were stained with haematoxylin and eosin (H&E). The histological slides were evaluated using light microscopy (Olympus BX51; Olympus, Tokyo, Japan). Inflammatory cell infiltration and presence of parasite amastigote were comparatively assessed in the different groups.

RESULTS

Treatments of macrophage cells with melittin for 48 h indicated that at different concentration (0.5, 1.8, and 4 µg/mL) melittin caused damages in different levels to cell membrane including cell shrinkage,

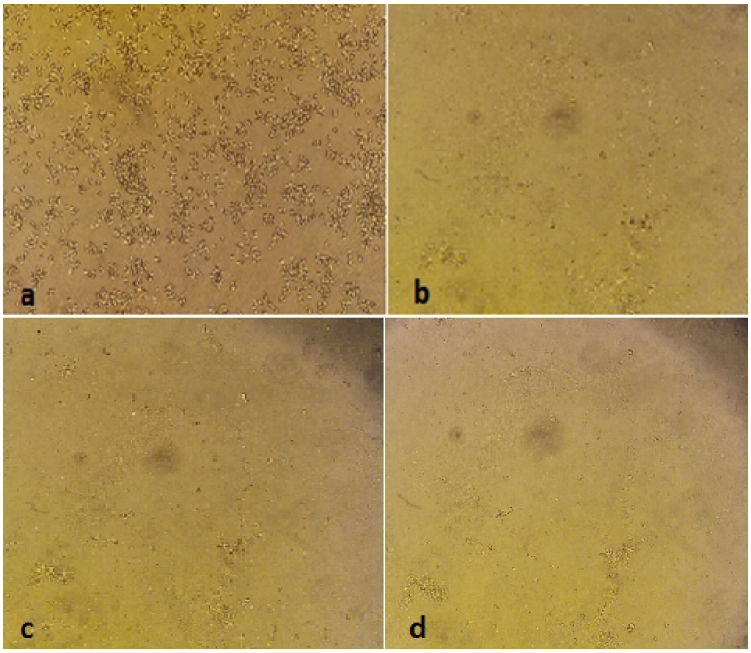

Fig. 1. Treatments of macrophage cells with melittin for 24 h. A) control; B) cells treated with melittin at concentration of 0.5 µg/mL; C) cells treated with melittin at concentration of 1.8 µg/mL; D) cells treated with melittin at concentration of 4 µg/mL.

irregularity in cellular shape, cellular detachment but there were no changes in the control group (Fig. 1). Treatments of macrophage cells with AM-loaded albumin nanoparticle and liposome for 48 h demonstrated cell membrane damages at peptide concentration 100 µg/mL including cell shrinkage, irregularity in cellular shape and detachment. 50% of macrophage cells showed irregularities at peptide concentration 50 µg/mL but there were no changes at peptide concentration of 25 µg/mL.

The inhibitory effects of processed nanoparticle and liposome containing AM on amastigote forms of parasites in macrophages after 48 h appeared at concentrations 50 and 100 µg/mL. However, at concentration of 25 µg/mL of each treatment, the number of *Leishmania* amastigotes in macrophages decreased significantly compared to the control group (Fig. 2, 3).

Regarding pathological test results, in the spleen of BALB/c mice from the infected group without treatment, lymphoid follicles (lymphocytes) were almost replaced by *Leishmania*-infected macrophages (histiocytes) (Leishman body) (Fig. 4). Furthermore, in the red pulp, several parasitised histiocytes were seen along with presence of giant cells (Fig. 4), indicating a severe disease. Infected animals treated with peptide loaded-albumin nanoparticles and peptide loaded-liposome showed preservation of the lymphoid follicles in white pulp, suggesting their proper effects (Fig. 4). For these groups, presence of infected macrophages was

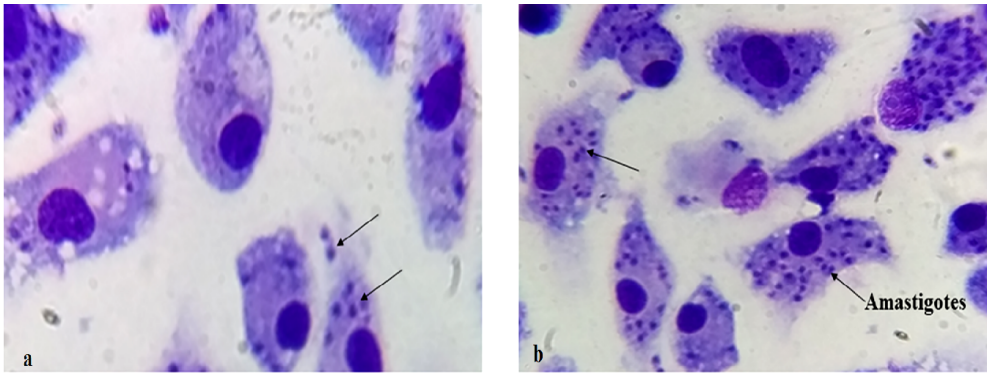

Fig. 2. Comparison of infected macrophages with *Leishmania major* amastigotes with a group of albumin nanoparticle treated with peptide dose of 25 µg/mL for 48 h, Giemsa stain: A) treated macrophages and B) control macrophages.

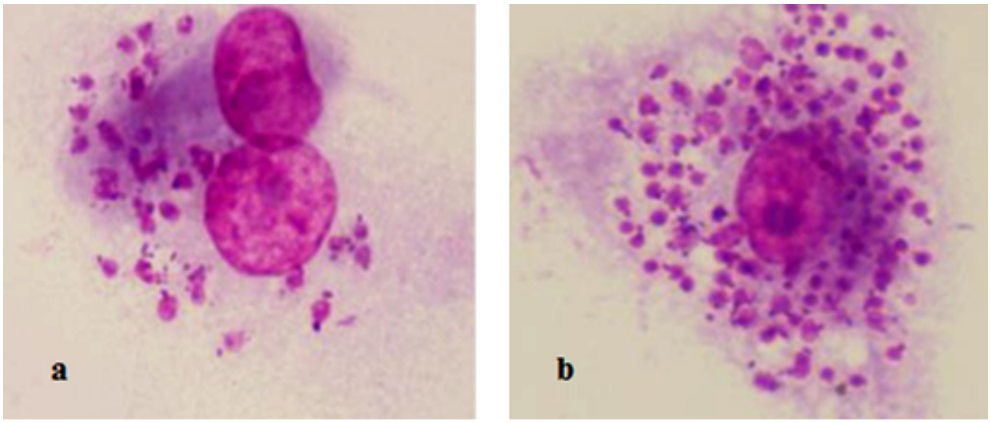

Fig. 3. Comparison of infected macrophages with *Leishmania major* amastigotes with a group of liposomes treated with a peptide dose of 25 µg/mL for 48 h, Giemsa stain: A) treated macrophages and B) control macrophages.

also evident in the red pulp, but they were fewer in comparison to the control group.

Fig. 5 illustrates the morphology of the wound lesions in different experimental groups. The number of infected inflammatory cells in the tissue was considerably higher in control lesions. Plasma cells and macrophages were the predominant cell type of infiltrated inflammatory cells. Moreover, the numbers of parasitised macrophages in the group treated by albumin were higher than in group of treated liposomes. The lesions were covered by a thick crusty scab in all experimental groups.

DISCUSSION

It seems that evaluation of the effectiveness of any therapeutic factor in the diseases with spontaneous recovery such as cutaneous leishmaniasis is very difficult.

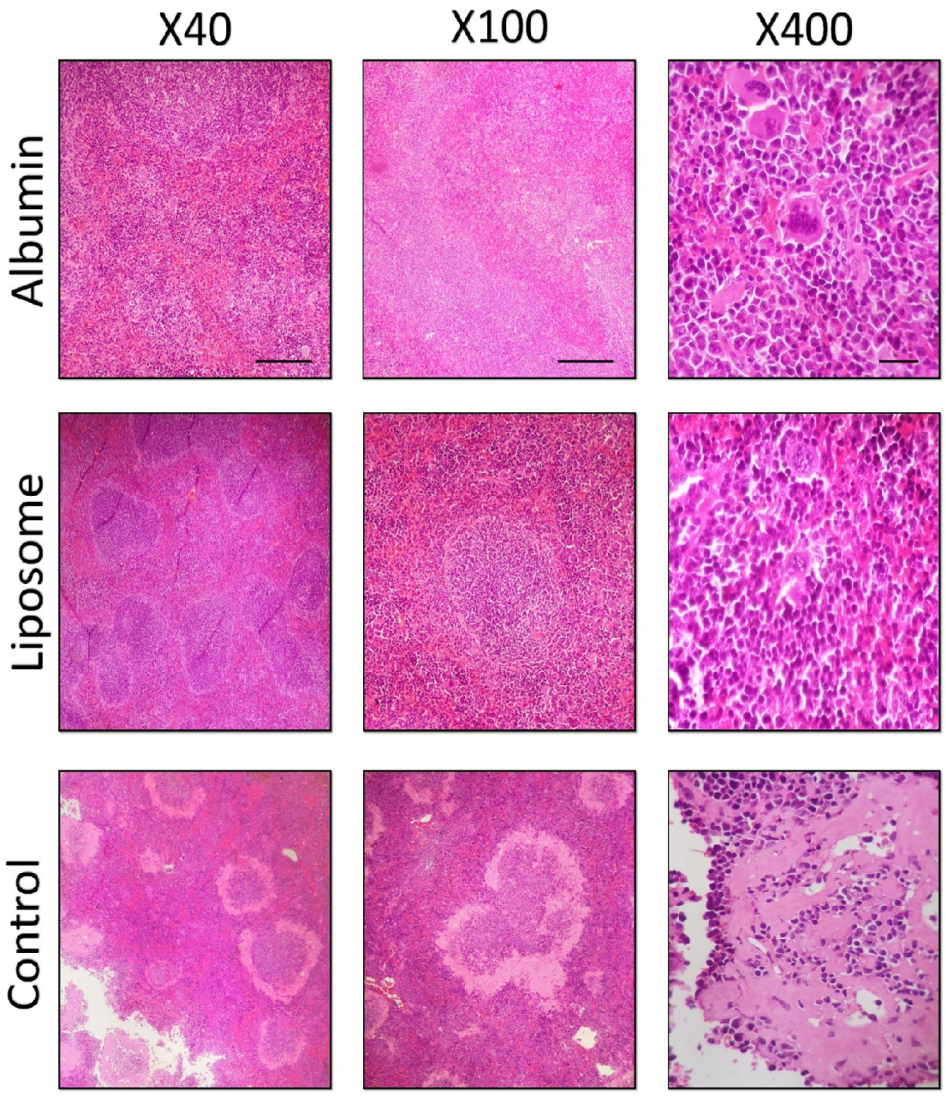

Fig. 4. Microscopic sections of spleen. Lymphatic follicles atrophy and considerable reduction of white blood cells in white pulps are evident in the control group**.** Control group: injected with PBS; liposome group: injected with 100 µL liposome containing 25 µg/mL peptide; albumin group: injected with 100 µL albumin nanoparticle containing 25 µg/mL peptide.
H&E; bar=200 µm (×40); 100 µm (×100) and 50 µm (×400).

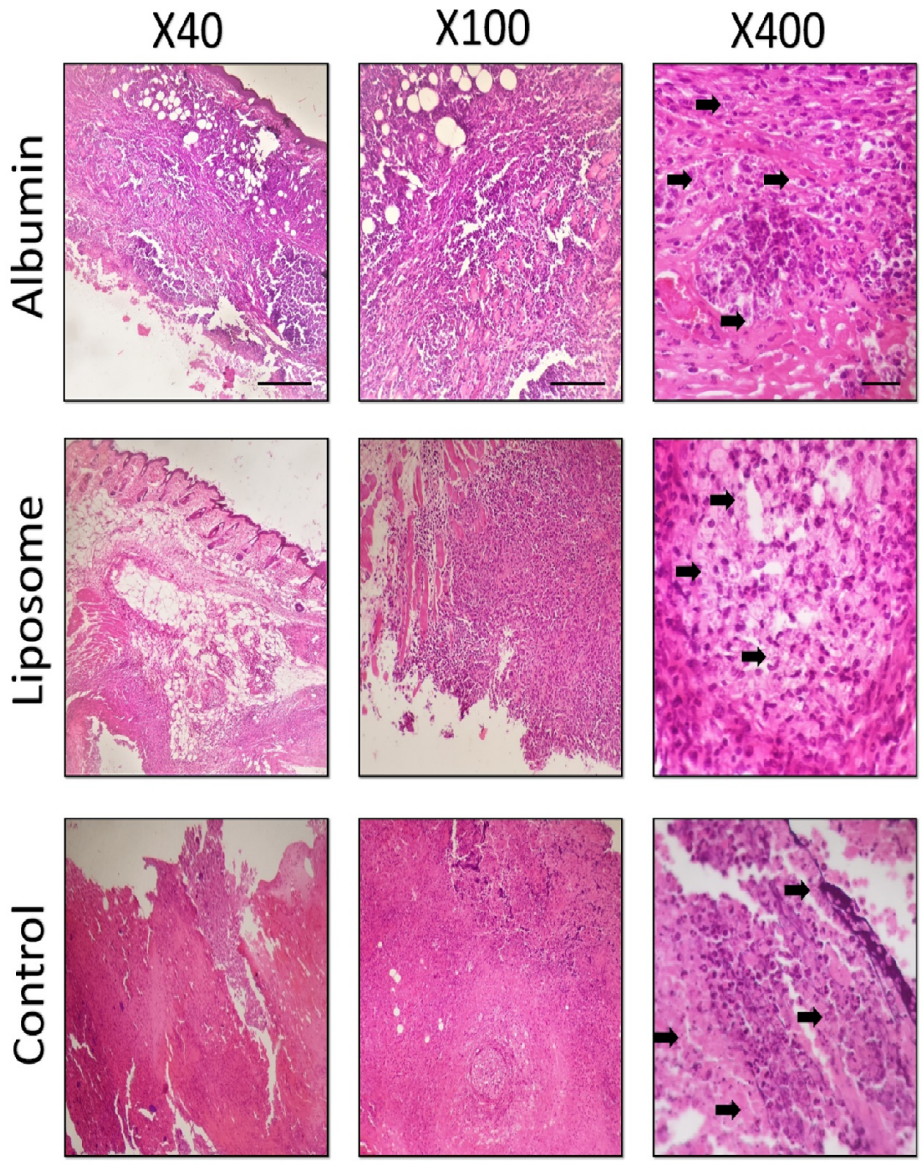

Fig. 5. Cutaneous leishmaniosis, diffuse infiltration of inflammatory cells including histiocytes, plasma cells and lymphocytes. Numerous gray-blue bodies within the vacuolated cytoplasm suggestive of *Leishmania* amastigote (arrows). Control group: injected with PBS; liposome group: injected with 100 µL liposome containing 25 µg/mL peptide; albumin group: injected with 100 µL albumin nanoparticle containing 25 µg/mL peptide. H&E;
bar=200 µm (×40); 100 µm (×100) and 50 µm (×400).

Due to the high prevalence of leishmaniasis in different parts of the world and the emergence of resistance to current drugs, the discovery of new drugs with lower toxicity and more therapeutic effects seems necessary (Zhang *et al.*, 2009). The inhibitory effect of melittin on the cell lines has been investigated by Olson *et al.* 2009 (Akhzari *et al.*, 2021). In this study, it was shown that melittin in different concentrations causes some damages to the macrophage cells line which were dose-dependent. As previously mentioned, melittin induces inhibitory effects on proliferation of various cancer cells via induction of apoptosis, necrosis or lysis. Moreover, increased concentration and incubation time of the peptide can increase cytotoxicity of the melittin. In order to selective targeting, we designed a construct for AM (Fig. 4). Melittin was fused to an MMP-2, 9 substrate peptide linker, and a polyanionic peptide inhibitor. Thus, melittin was inhibited unless the linker was proteolysed (Akhzari *et al.*, 2021). Melittin includes a cationic motif called KRKR. Binding of melittin to a neutralizing polyanionic peptide (E7) via a matrix metalloproteinase cleavable linker may be a useful approach for treatment of leishmaniasis (Akhzari *et al.*, 2022). The MMPs are a family of proteolytic enzymes characterised by their overexpression or over-activity in several pathological processes. Generally, leishmaniasis is characterised by strong inflammatory responses with high-level production of TNF. The TNF induces matrix metalloproteinase enzymes. Campos *et al.* (2014) have reported that cells in cutaneous leishmaniasis (CL) lesions secrete high levels of matrix metalloprotease-9 (MMP-9), in comparison to healthy people. Cytoplasmic MMP-2 can be activated by reactive oxygen species (ROS) (Campos *et al.*, 2014), thus we suggested that designed AM would be activated in infected macrophage cells and infection microenvironment sites. In this study encapsulation of AM with albumin and liposome as a non-toxic carrier at concentrations of 25, 50, 100 µg/mL were used and the concentration of 25 µg/mL showed proper antileshmania effect.

Various strategies have been described for the use of MMPs in targeting therapeutic entities (Sobczak, 2022). In a previous study of ours, different methods to load the AM onto the albumin nanoparticle and liposome as a drug carrier and compare their antileishmania effects with the control group were used.

According to the design of activatable melittin for selective targeting of *Leishmania* infected cells and their transfer by liposome and albumin nanoparticle into infected macrophages, the results showed that the toxicity of the drug would be greatly reduced compared to melittin alone. The number of *Leishmania* promastigotes in infected macrophages was greatly reduced due to higher MMP expression in respective microenvironment. Although some studies have demonstrated that melittin includes no significant haemolytic activities at concentrations lower than 0.25 µg/mL, 90% haemolysis of human RBCs has been reported at concentrations more than 1 µg/mL (Vartak *et al.*, 2007).

The *Leishmania* is an obligate intracellular parasite that resides and reproduces in the macrophages. After macrophage phagocytosis, the parasites enter the phagolysosomal vacuoles which not only restrict drug access to the parasite, but also require a relatively high dose of infusion for inhibiting the parasite. Consequently, side effects of the drug would occur, so it seems that the selection of

macrophages as a target of drug can minimise the side effects (Mahmoodzadeh *et al.*, 2015). So, drug delivery system can be used to send the drug to a specific target where the parasite is present (Veerareddy *et al.*, 2004).

In recent years, the focus was on providing liposomes and nanoparticles as carriers for drug delivery (Malam *et al.*, 2009). It seems that these nanostructures can control drug release and protect the molecules of drugs. Also, being of smaller size than cells, they are able to cross biological barriers to deliver the drug to the target site. Also, they are maintnained in the bloodstream for a long time (Gour *et al.*, 2009).

In the present study on the cytotoxicity of designed product on macrophages, the lowest toxicity was shown by AM-loaded albumin nanoparticles and AM-loaded liposomes in comparison of the control group.

CONCLUSION

It seems that encapsulation of AM with albumin and liposome as non-toxic carrier at a concentration of 25 µg/mL, can improve and optimise the antileishmanial effects of this drug. These forms could be a good alternative to the current drugs so performance of further *in vivo* studies is suggested.

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