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THE IMMUNE DEFENSE AGAINST MOUSE PARA COCCIDIOIDOMYCOSIS OFFERED BY PEPTIDE P10 IS SIGNIFICANTLY ENHANCED BY POLY (LACTIC ACID-GLYCOLIC ACID) NANOPARTICLES

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ABSTRACT

Background and goal: This study describes the development and testing of a sustained delivery method for the immunomodulatory peptide P10 with the goal of lowering the peptide's in vivo degradation and the quantity needed to trigger an immunological response that protects against Para coccidioidomycosis. ***Methods of the experiment***: To simulate the chronic form of Para coccidioidomycosis, BALB/c mice were infected with the yeast Paracoccidioides Brasiliense's. Every day, the animals received treatment with either sulfamethoxazole/ trimethoprim alone or in combination with peptide P10, which was either encapsulated in poly (lactic acid-glycolic acid) (PLGA) nanoparticles at varying doses (1 µg, 5 µg, 10 µg, 20 µg, or 40 µg·50 µL−1) or emulsified in Freund's adjuvant. The quantification of cytokines in the immune response and the fungal burden in tissues were used to evaluate the therapeutic efficacy. ***Important Findings:*** Animals provided when compared to the animals that were not treated, the fungal load in the lungs was significantly reduced by the combination of chemotherapy and P10 nanotherapy. Following a 30-day course of therapy, P10 encased in PLGA (1 µg·50 µL−1) demonstrated superior efficacy as an adjuvant to chemotherapy when compared to "free" P10 emulsified in Freund's adjuvant (20 µg·50 µL−1). The highest efficacious concentrations of P10 entrapped inside PLGA (5 or 10 µg·50 µL−1) were seen after a 90-day treatment period. High levels of interferon-gamma were observed in the lung following treatment with P10 emulsified in Freund's adjuvant (20 µg·50 µL−1) or P10 entrapped within PLGA (1 µg·50 µL−1). ***Inferences and conclusions:*** The use of PLGA-encapsulated P10 peptide in conjunction with sulfamethoxazole/ trimethoprim showed improved treatment efficacy against Para coccidioidomycosis. The addition of P10 to PLGA nanoparticles significantly decreased the quantity of peptide required to produce a protective effect.

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INTRODUCTION

The development of effective adjuvants to vaccines for immunological protection against a variety of diseases has been the subject of extensive research. The development of antifungal vaccines has received attention more lately due to the sharp rise in the occurrence of systemic mycoses (Cutler *et al*., 2007). Among other immunogens, peptide antigens show particular promise in eliciting efficient immune defense reactions against these illnesses (Taborda *et al*., 1998; Travassos *et al*., 2008a). Peptides can be protected from degradation and released at preset rates by incorporating them into controlled release systems (Johansen *et al*., 2000). Due to their ability to progressively regulate the release of an antigen, biodegradable polymers are appealing as vaccine delivery systems (Commandeur *et al*., 2006). Following in vivo treatment biocompatible polymers, they decompose into molecules that participate in regular metabolic processes before being removed. When polymer biodegradation takes place, under specific circumstances like pH and temperature changes, and depending on the composition of the polymer, any polymer-entrapped molecule—a medicine or a peptide—is released (Commandeur *et al*., 2006; Vicent and Duncan, 2006). Adjuvants made of polymeric systems, like nano- and microparticles, are suitable for use in the preparation of single-shot vaccines. According to Vincent and Duncan (2006), antigens enclosed in polymers can be released over an extended length of time at a regulated rate of polymer degradation. Many antigenic proteins, such as ovalbumin, cholera and tetanus toxoid, as well as malarial and pneumotropic

bacterial antigens, have been demonstrated to respond well to polymers as adjuvants (Dhiman and Khuller, 1998; Jaganathan *et al* According to Jiang *et al*. (2005), poly (lactic acid-glycolic acid) (PLGA) is a polymer whose rate of degradation and antigen release are predictable. The quantity and frequency of antigen exposures needed for protection can be greatly decreased by releasing an immunomodulatory peptide at a predefined rate. The main diagnostic antigen secreted by Paracoccidioides brasiliensis (Puccia *et al*., 1986) is the glycoprotein 43 kDa glycoprotein (Gp43), and Taborda *et al*. (1998) discovered a 15-amino acid peptide that bears the T-cell epitope of Gp43. P10 is a peptide that stimulates an immunological response to experimental Para coccidioidomycosis (PCM; Taborda *et al*., 1998; Travassos *et al*., 2004) that is dependent on interferon-gamma (IFN-γ). When paired with other antifungals such as sulfamethoxazole/trimethoprim, vaccination with P10 increased the treatment efficacy against PCM, indicating a significant role for P10 in enhancing result. Shortening the duration of PCM treatment (Marques *et al*., 2006). In Latin America, around 10 million people may be infected with the dimorphic human pathogenic fungus P. Brasiliense's, and 2% of those persons may develop acute or chronic forms of Para coccidioidomycosis (PCM) (Brummer *et al*., 1993). In chronic PCM, the lung is primarily impacted by a granulomatous inflammatory response, which serves as an excellent defense against fungal spread (Brummer *et al*., 1993; de Camargo and de Franco, 2000).

The acute form of PCM involves the lymphoreticular system and may be fatal. PCM is typically treated with polyenes, azoles, and sulphonamides. In instances that are severely disseminated, amphotericin B is recommended. This treatment must be continued with azoles and sulfamethoxazole/trimethoprim for an extended period of time. References: Brummer *et al*. (1993); Litholatry *et al*. (1999). This work describes the development, fabrication, and in vivo evaluation of a sustained delivery system that loaded the immunoprotected peptide P10 onto PLGA nanoparticles, a biodegradable polymer. The aforementioned compound was given in conjunction with trimethoprim/sulfamethoxazole to treat a mouse model of severe PCM.

Techniques

PLGA particles loaded with the P10 peptide are prepared: PLGA polymeric blends containing 50:50 poly-lactic acid (PLA):poly-glycolic acid (PGA) were used to create the particles. Prior to being added to an aqueous solution containing 1% polyvinyl alcohol and the P10 peptide, the polymers were first dissolved in dichloromethane. The ultimate preparation was intended to contain 1, 5, 10, 20, or 40 μg·50 μL−1. To achieve the water-in-oil emulsification, the mixture was subjected to strong agitation in a blender (10000–15000 rpm). By swirling the mixture at room temperature and letting it evaporate under low pressure, the organic solvent was extracted from the mixture.

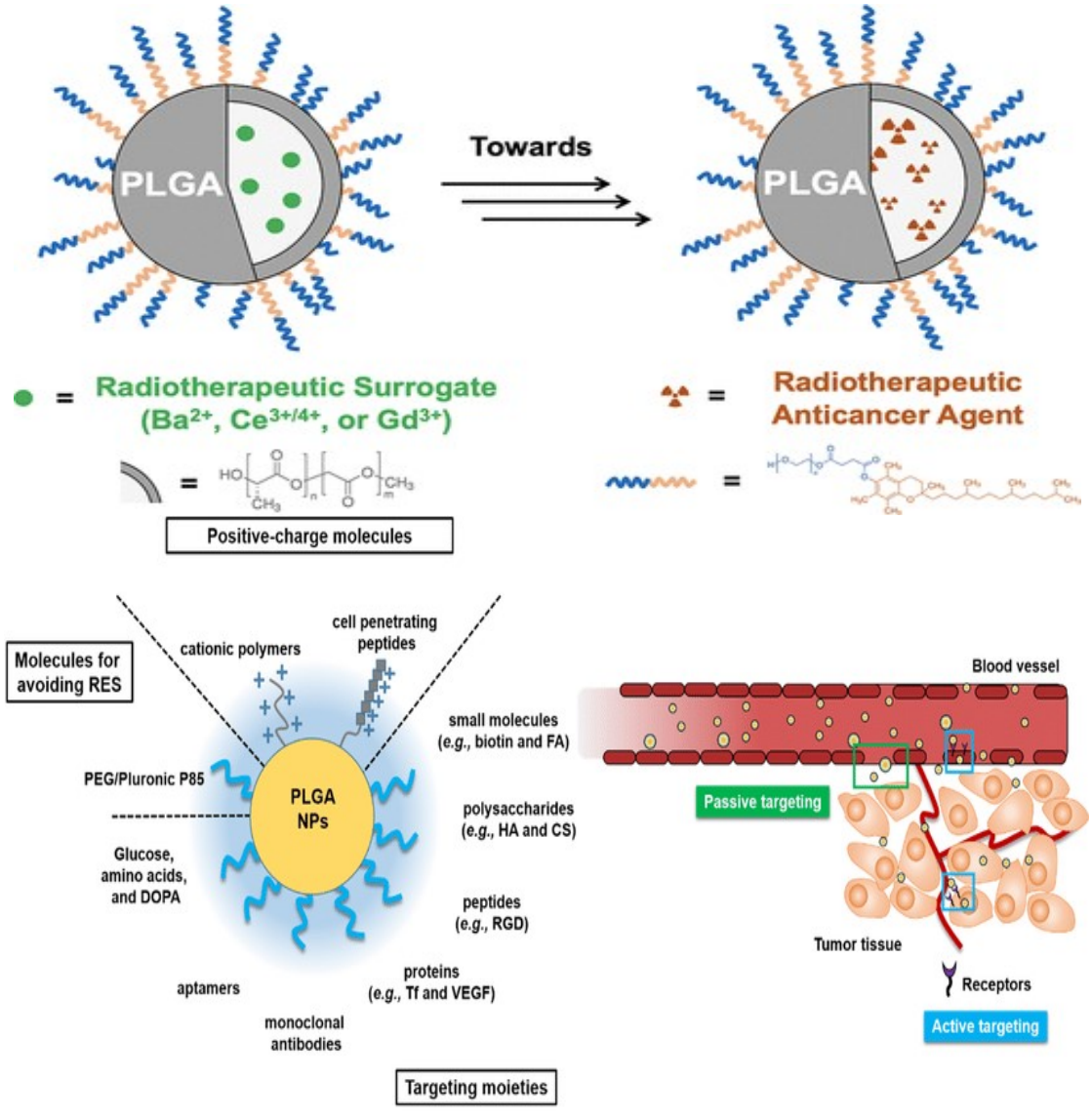

Figure 1. Various surface-engineered poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) for passive or active tumor targeting. Arg-Gly-Asp (RGD); chitosan (CS); dopamine (DOPA); folic acid (FA); hyaluronic acid (HA); poly(ethylene glycol) (PEG); reticuloendothelial system (RES); transferrin (Tf); vascular endothelial growth factor (VEGF).

The particles underwent 10-to 20-minute interval centrifugation (4–10°C; 1100–4600×g). The mixture was suspended in 1.0 and rinsed three times in distilled water mL of phosphate-buffered saline (PBS), which can be used for up to a week and kept at 4°C. Every operation was carried out in a sterile chamber, and every manipulation took place under a sterile hood. Animals were infected with P. brasiliensis, isolate Pb18, a fungal inoculum intended for in vivo investigations. According to Restrepo and Arango (1980), the strain was subculture in McVeigh-Morton culture media, a specified liquid medium, at 35°C and 220 rpm in a rotatory shaker. Following five or seven days of cultivation, the yeast cells were centrifuged to extract the cells, discarding the supernatant and washing the cells three times in sterile PBS at a pH of 7.4. Using a hematocytometer, we counted the cells and changed the inoculum suspension to 3 × 105 viable fungus per 50 µL. Evans blue vital staining (Sigma, St. Louis, MO, USA) was used to test the cellular viability in these investigations, and the results showed a range of 90–95%.

Infection inside the throat: In order to resemble the most typical infection in PCM's chronic state, P. brasiliensis Pb18 was injected intratracheally into BALB/c mice, and following a 30-day inoculation period, the animals were treated with a combination of trimethoprim/sulfamethoxazole and a variety of P10 (1–40 µg) within PLGA. An intraperitoneal injection of 200 µL containing a solution of 80 mg·kg−1 ketamine and 10 mg·kg−1 xylazine (União Química, Brazil) was used to anesthetize the animals. Their necks were hyperextended and incised to barely reveal the trachea after ten minutes. Using a 26.5-gauge needle, 3 × 105 viable fungus were given to each animal in 50 µL PBS. As soon as the fungus were delivered, the incisions were sutured.

Antifungal medication: Nine groups of six diseased mice each were randomly assigned, and the animals were treated with sulfamethoxazole/trimethoprim (15 mg·kg−1 and 3 mg·kg−1 correspondingly) in PBS at pH 7.4, with or without 20 µg P10 solubilized in 50 µL Freund's adjuvant designated as "free" or P10 encased in PLGA at 1 µg, 5 µg, 10 µg, 20 µg, or 40 µg·50 µL−1. Group (i) received PBS as the only treatment; Group (ii) received sulfamethoxazole/trimethoprim alone; Group (iii) received sulfamethoxazole/trimethoprim plus 50 µL of "empty" PLGA nanoparticles; Group (iv) received 20 µg·50 µL−1 of "free" P10; and Groups (v) (vi) (vii) (viii) and (ix) received 1 µg, 5 µg, 10 µg, 20 µg, and (ix) received 40 µg·50 µL−1, respectively, of P10 entrapped within PLGA. Thirty days following the fungal challenge, the treatment regimens were initiated and maintained. The The same process, approach, and ratio of PLA: PGA (50:50) that were utilized to generate the nanoparticles coupled with P10 were also used to prepare the empty PLGA nanoparticles that were given to group (iii). This resulted in the same final concentration in both preparations with and without P10. Using a particle size analyzer (Zetasizer, Malvern, UK), laser light scattering was used to measure the particle size and size distribution. The size distribution was examined between 1 and 1000 nm, and the mean diameter for every sample was determined. After measuring the PLGA particles at different doses with and without peptide P10, the average diameter was found to be 410 ± 4.9 nm for the empty particle and 430 ± 5.1 nm for the loaded one. Animals received daily from groups (ii) to (ix) injections of trimethoprim/sulfamethoxazole (15 mg kg -1 and 3 mg kg -1, respectively). Over the course of four weeks, mice belonging to groups (iv) to (ix) were given four doses of peptide P10 once a week as follows: Twenty 'free' or entrapped within PLGA at concentrations of 1 g, 5 g, 10 g, 20 g, and 40 g/50 µL−1 or 20 µg·50 µL−1. Complete Freund's adjuvant was given to "free" P10 in the hind paw, while P10 trapped in different concentrations of PLGA was given intraperitoneally (i.p.) without adjuvant.

Fungus load measurement: The residual fungal burden in the lungs, liver, and spleen was used to evaluate the combination treatment effects. The lungs, liver, and spleen were aseptically removed and weighed after the animals from all experimental groups were killed by cervical rupture 30 and 90 days after the start of therapy After cleaning and homogenizing the organs in sterile PBS at pH 7.4, 100 µL of the homogenates was cultured in BHI agar supplemented with 4% horse serum, 5% P. brasiliensis 192 culture filtrate, 10,000 IU penicillin (Cultilab, Brazil), and 10,000 mg·L−1 streptomycin (Cultilab, Brazil) in duplicate. A procedure that has been reported previously was used to create the Pb192 culture filtrate (Singer-Vermes *et al*., 1992). To calculate the CFU·g−1 of tissue, the plates were incubated at 37°C and the colony-forming units (CFUs) were counted 10 days after plating.

Assays for cytokines: We measured interleukins-4 (IL-4), -10 (IL-10) and -12 (IL-12) in lung homogenates along with IFN-γ to evaluate cytokine production. The lung pieces were extracted aseptically, weighed, and then homogenized in sterile PBS supplemented with a protease inhibitor (Roche, USA). The cytokines were identified by means of BD Biosciences – Pharmingen, San Diego, CA, USA – sells commercial ELISA kits.

Analytical statistics: We utilized version 15 of the Statistical Package for Social Sciences (SPSS) to analyze our data. The means ± standard error are used to express all results. To examine intergroup differences, a one-way analysis of variance (anova) with Tukey's post-test was used. Using the Mann-Whitney test, differences between paired groups were examined. Significant P-values were those that were less than 0.05.

Materials: Trimethoprim was acquired from Ducto (Bac-sulfitrin, Ducto) and sulfamethoxazole from Sigma (St. Louis, MO, USA). Sigma (St. Louis, MO, USA) provided the poly-lactic acid (PLA) and poly-glycolic acid (PGA) needed to make the nanoparticles. The P10 peptide was donated by Dr. Maria A. Juliano of the Department of Biophysics at the Federal University of São Paulo and was synthesized using the 9-fluoroenylmethoxy-carbonyl method (Huang *et al*., 1993). The British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al*., 2008) is followed for naming drugs and molecular targets.

Outcomes

Fungal load in animals receiving treatment: utilizing P10 peptide encapsulated in PLGA in combination with sulfamethoxazole/ trimethoprim, we tested the combined therapy's effectiveness in vivo utilizing antifungal drugs on BALB/c mice infected with P. brasiliensis, isolate Pb18. Following 30 days of infection, the treatment plans were initiated, and they were assessed 30, and 90 days later. The animals' lungs, liver, and spleen were examined for fungus loads. Thirty days following intratracheal (i.t.) infection, no discernible amount of fungi was found in the liver and a very small amount in the spleen. This outcome was anticipated because the animal's liver or spleen infection can only be identified in its early stages a mycosis model. On the other hand, the infection's primary target was the lungs. In the lungs of the control group, which was given simply PBS, a significant amount of fungal cells were found 30 and 90 days after the treatment started. After 90 days, the group that received only sulfamethoxazole/trimethoprim treatment had a large number of fungal cells, similar to the group that did not receive treatment, but the infection was under control during the first 30 days of treatment Using 20 µg of "free" P10 emulsified in Freund's adjuvant, a reduction in CFUs in comparison with untreated animals was demonstrated (Marques *et al*., 2006), reversing the relapse shown in the group that received only sulfamethoxazole/trimethoprim treatment. Based on previous findings, the dosage of 20 µg for the 'free' form of P10 peptide was selected (Taborda *et al*., 1998; Taborda *et al*., 2004). Mice were reported to be protected against P. brasiliensis infection by lymphoproliferative and cell-mediated immune responses under these conditions. There was usually a notable level of protection in the groups receiving the combined therapy of P10 entrapped in PLGA and sulfamethoxazole/ trimethoprim. Following thirty days of therapy, 1–10 µg·50 µL−1 of P10 encapsulated in PLGA was effective as an adjuvant to chemotherapy, lowering by at least 20 times the dose of the peptide required to lower the fungal load and prevent illness relapse. The mice given sulfamethoxazole/trimethoprim alone did not exhibit a

significant additive activity when used as adjuvants to chemotherapy, but the groups that received 20 or 40 µg·50 µL−1 of P10 entrapped within PLGA did (data not shown). After 30 days, the P10-free PLGA nanoparticles demonstrated a protective effect, demonstrating their usefulness as an adjuvant to chemotherapy on their own. However, after 90 days of treatment, the recurrence that was noted with sulfamethoxazole/trimethoprim treatment was not prevented by these "empty" nanoparticles. Therefore, following treatment with empty nanoparticles, the fungal burden recovery was higher than following any of the loaded PLGA nanoparticles (containing 1, 5 or 10 µg·50 µL−1 of P10 peptide)

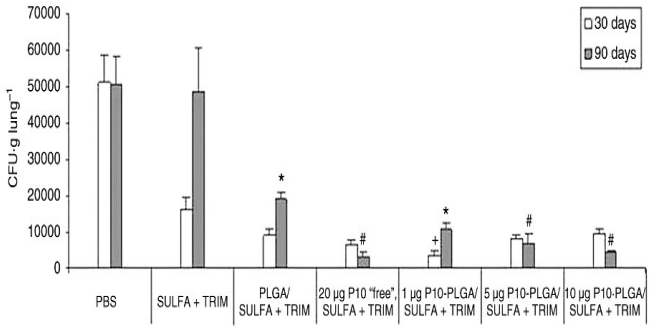

Figure 2. Fungal burden recovery assessed by colony-forming units [CFU (g·lung·tissue)−1] in mice infected with P. brasiliensis Pb18 and subjected to a combined therapy of sulfamethoxazole/trimethoprim (Sulfa + Trim; 15 mg·kg−1 and 3 mg·kg−1 respectively) and either 20 µg·50 µL−1 P10 peptide solubilized in Freund's adjuvant ('free') or P10 peptide (1, 5 or 10 µg·50 µL−1) entrapped within PLGA. Each bar represents the average CFU (g·tissue)−1 with standard deviations. After 30 days of treatment, 1 µg·50 µL−1 of P10-PLGA/Sulfa + Trim yielded the best response (lowest fungal CFU recovery) of all groups (+P < 0.05). After 90 days of treatment, no significant differences were found between the responses of the 'free' P10 (20 µg·50 µL−1) and the P10-PLGA (5–10 µg·50 µL−1)-treated groups (marked with #). At day 90, a significantly lower number of fungal cells were recovered from mice treated with 1 µg·50 µL−1 P10-PLGA compared with the PLGA alone treated group (*P < 0.05). PLGA, poly(lactic acid-glycolic acid).

Production of cytokines produced by P10-PLGA: The levels of IFN-γ, IL-4, IL-10, and IL-12 in lung tissue homogenates from the animals receiving the combination therapy of sulfamethoxazole/trimethoprim and peptide P10 were monitored to assess cytokine production. the generation of IFN-γ and IL-12, two type 1 cytokines. After 30 and 90 days of treatment, the groups that received 20 µg of P10 emulsified in Freund's adjuvant or 1 µg of P10 entrapped within PLGA showed increased levels of IFN-γ compared to the corresponding controls. An essential Th1 cytokine that provides defense against P. brasiliensis infection is IFN-γ. There was no comparable change in IL-12 levels. Table 1 additionally displays alterations in the cytokines (IL-4 and IL-10) more typical of Th2 responses. Following thirty and ninety days of After beginning therapy, there was a significant drop in IL-10 in the group that got sulfamethoxazole/trimethoprim and 20 µg·50 µL−1 of 'free' P10 emulsified in Freund's adjuvant, which confirmed previous findings. After 30 days and less after 90 days, P10 trapped in PLGA at a dose of 1µg also decreased IL-10 levels. There was no discernible drop in the levels of IL-4, and the decrease in IL-10 with increased P10 quantities in PLGA was less pronounced.

DISCUSSION

In the current study, we investigated the antifungal properties and protective effects of a therapy for experimental PCM that included the immunostimulatory peptide P10 encapsulated in PLGA-dimercaptosuccinic acid (DMSA) with sulfamethoxazole/ trimethoprim. Using a mouse model of chronic PCM, the effectiveness of treatment and cytokine production were investigated. Low-cost sulphonamide treatments like trimethoxazole/trimethoprim have been used as first line therapy for non-disseminated PCM cases (Lortholary *et al*., 1999; Travassos *et al*., 2008b). While this treatment is suggested for long-term use, there have been reports of fungal resistance and relapsing disease (Travassos *et al*., 2008a). Enhancing chemotherapy for fungal illnesses may be possible through the development of immunotherapies that employ peptides as adjuvant agents. The application of According to Traba *et al*. (1998), P10 peptide has demonstrated efficacy in mitigating the fungal load in mice harboring P. brasiliensis infections, hence inducing a proficient cellular immune response against the fungus. In the mouse model, P10's association with sulfamethoxazole/trimethoprim was beneficial in treating PCM and preventing the mycosis's time-dependent relapse (Marques *et al*., 2006). One potential drawback of utilizing free peptides in vivo could be their low metabolic stability and need for adjuvants that are prohibited for usage in humans.

It has been demonstrated that a multiple antigenic peptide (MAP) construct with four shortened P10 branches is protective even in the absence of extra adjuvants. Nevertheless, the process of creating MAPs chemically is intricate and prone to mistakes. during the procedure, making it challenging to purify and characterize them (Taborda *et al*., 2004). Therefore, there is still a need to design easier and more effective delivery strategies for immunostimulating peptides. In the current study, sulfamethoxazole/trimethoprim was employed in combination with PLGA-DMSA acid nanoparticles carrying P10 in levels ranging from 1 µg·50 µL−1 to 40 µg·50 µL−1. The encapsulation of these molecules using PLGA-based formulations was intended to help in drug administration by lowering the frequency of injections and shielding the peptide from in vivo degradation (Amaral *et al*., 2009). Because PLGA nanoparticles have a low rate of co-polymer breakdown and a continuous release of the peptide, they are suitable vehicles for delivering the peptide and would be more effective in rousing immune cells. The According to findings from other studies (Mittal *et al*., 2007; Amaral *et al*., 2009), the 50:50 ratio of PLA and PGA utilized in this kind of drug delivery system demonstrated a sustained release of the active component over an average period of 72 hours.

Due to its great stability, quick uptake by endocytosis, ability to target particular organs, and availability in a wide variety of PLA and PGA combinations, nanoparticulated PLGA has been employed as a drug delivery system for a while (Dhiman *et al*., 2000). Drug regulatory organizations, including the Food and Drug Administration, have approved the use of this drug delivery method since it does not pose any toxicity on its own (Khang *et al*., 2003; Gabler *et al*., 2007). In vitro experiments with Various cell lines demonstrated that nanoparticulated PLGA was not harmful (Khandare and Minko, 2006; Gomes *et al*., 2008). When taken alone, PLGA does have the benefit of triggering both mild inflammation and cytotoxic T-cell responses, which may contribute to its adjuvant properties (Jiang *et al*., 2005). The amount of peptide P10 required in our study to lower the fungus burden in the sick animals and prevent illness relapse was decreased by integrating it into PLGA. During the first 30 days of treatment, a little amount (1 µg·50 µL−1) of P10 in nanoparticles had the same immunotherapeutic impact as 20 µg·50 µL−1 of "free" P10 emulsified in Freund's adjuvant. But following 90 days of therapy, the PLGA-encapsulated peptide's greatest protective impact was demonstrated by the groups that were given 5 or 10 µg·50 µL−1 of P10 in PLGA, allowing the peptide to be reduced by at least four times while maintaining the ability to prevent infection recurrence. The efficacy of the lower dosages of P10-PLGA may be explained by the trapping of P10 within PLGA nanoparticles, which is likely to successfully shield it from in vivo enzymatic degradation, according to a prior publication (Johansen *et al*., 2000). The enhancement of immunomodulatory effects may be due to PLGA's interaction with antigen-presenting cells (Jaganathan *et al*., 2005).

Additionally, the use of PLGA for peptide controlled release removes the requirement for an adjuvant, which is advantageous because there are only a few adjuvants approved for usage in humangovernance (Jiang *et al*., 2005). According to earlier research (Marques *et al*., 2006), the protection evoked by 20 µg·50 µL−1 of "free" P10 in Freund's adjuvant as well as P10-PLGA at 1 µg·50 µL−1 most likely dependent on the induction of the high levels of IFN-γ. Even after 90 days of therapy, the 10 µg P10-PLGA treatment had high

IL-10 and low IFN-γ cytokine values, but it was still able to have a decent protective impact. This might be explained by the group's low IFN-γ level being compensated for by the maintenance of high levels of IL-12 and low production of IL-10 at 90 days, which was nearly two times lower than that of the PBS and Sulfa + Trim-treated groups . The majority of patients with the According to Oliveira *et al*. (2002), severe forms of PCM exhibit a polarized Th2 response, producing IL-4 and IL-10. On the other hand, people who produce IFN-γ and have a polarized Th1 response usually have a better ability to treat P. brasiliensis infections (Oliveira *et al*., 2002; Calich *et al*., 2008).

CONCLUSION

As peptide P10 produces a Th1-like immune response capable of controlling fungal infection, using PLGA as a carrier for P10 in conjunction with sulfamethoxazole/trimethoprim represents a viable alternative to treat mycosis. The cellular immunity-stimulating P10 peptide can boost the therapeutic efficacy of sulfamethoxazole/ trimethoprim, a cheap medication. Peptide P10's encapsulation in PLGA obviated the requirement for an adjuvant and cut the amount of the peptide required for a therapeutic response by up to 20 times. Furthermore, when using PLGA nanoparticles for human medicine, there should be a decrease in expenses and an improvement in antifungal protection.

Conflicts of interest: None to declare.

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