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RESEARCH ARTICLE

ANTI-INFLAMMATORY POTENTIAL OF ARTIFICIAL MICROCAPSULES CONTAINING THALIDOMIDE FOR USE IN TREATING CROHN'S DISEASE

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ABSTRACT

Crohn's disease is a chronic inflammatory bowel disease associated with an abnormal immune response in the gastrointestinal tract. Several studies demonstrate that thalidomide could be effective in the treatment of refractory Crohn's disease. However, its widespread use has been limited because of potential side effects. In the present study, we investigated the inhibitory activity of alginate-poly-L-lysine-alginate (APA) microcapsules containing thalidomide on Lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophage cells and on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced Crohn's disease. Results showed that APA microcapsules containing thalidomide inhibited the release of pro-inflammatory cytokines in cell supernatant following LPS activation. Moreover, treatment with microencapsulated thalidomide decreased the level of TNF-α, IL-6 and IL-1β by 49.3%, 62.3% and 54.6% respectively in TNBS-treated mice. The present project validates the efficiency of APA microcapsules in providing a targeted delivery of thalidomide for treating chronic conditions such as Crohn's disease.

Keywords: Crohns disease (CD), Lipopolysaccharide (LPS), Nitric Oxide (NO), Tumor necrosis factor-α (TNF- α); Interleukin-6 (IL-6); Interleukin-1B (IL-1B).

INTRODUCTION

Crohn's disease is an auto-immune disease characterized by an exaggerated Th1- mediated immune response that affects any part of the gastrointestinal tract from the mouth to the anus. Symptoms include diarrhea, abdominal pain, and weight loss. Although thalidomide exhibits anti-inflammatory properties useful for the treatment of Crohn's disease, it presents several harmful side effects such as fatigue, drowsiness and constipation.[1] Hence, appropriate delivery systems should be used in order to limit the side effects associated with thalidomide. In this study, we examine the potential of APA microcapsules containing thalidomide in reducing intestinal inflammation both in-vitro and in-vivo. LPS-activated RAW 264.7 mouse macrophage cells are commonly used to mimic intestinal inflammation. [2, 3] LPS-induced inflammation is characterized by an increase in inflammatory cytokines, and other crucial mediators of inflammation such as nitric oxide (NO). [4, 5] The inflammatory cytokines investigated in the present study are TNF-α, IL-6 and IL-1β. TNF-α is a pro-inflammatory cytokine that plays a major role in the inflammation related to Crohn's disease.[6] In addition, acute inflammation is characterized by an increased level of IL-1β leading to cell or tissue damage.[7] Finally, IL-6 is a critical pro-inflammatory cytokine that is a primordial mediator of chemokine production and leukocyte apoptosis. [8] An animal model of intestinal inflammation is also used to mimic Crohn's disease. The model is based on the intrarectal administration of TNBS to Balb/c mice. This chemically-induced model has shown to trigger an inflammatory response similar to that in human Crohn's disease. [9]

MATERIAL AND METHODS

Chemicals and laboratory equipment

The Research IER-20 cell encapsulator was purchased from Inotech Biosystems International. The chemicals thalidomide, alginic acid, poly–L-lysine (hydrobromide) and dimethyl sulfoxide were purchased by Sigma-Aldrich Canada. The MTS Reagent Powder was purchased from Promega. Cells were incubated in a Sanyo MCO-18M Oxygen/Carbon Dioxide Incubator and stored in a Sanyo MDF-U50V -86 degrees Celsius Freezer, supplied by SANYO Canada. A Lomo Biological Inverted Microscope BIOLAMP, supplied by LOMO America, was used for microscopic cellular observations. ELISA testing was done on Mouse ELISA Ready-SET-Go! supplied by eBioscience. A Nitric Oxide Colorimetric Assay Kit from Biovision was used for calculating the amount of nitric oxide in cell supernatants. A Bio-Tek uQuant Universal Microplate Spectrophotometer from Fisher Scientific was used for ELISA plate analysis. Finally, cell centrifugation was performed on a NAPCO 2028R Centrifuge, supplied by Precision.

Preparation of APA microcapsules containing thalidomide

Alginic acid was added to deionized water to make a 1.5% alginate solution. (±)- Thalidomide ((±)-2-(2,6-Dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione) was dissolved in deionized water at a concentration of 0.035 mg/ml by stirring and heating for 24 hours and added to the alginate solution. Alginic acid was additionally added to maintain a 1.5% concentration. APA beads were then formed by running the above solution through an Inotech encapsulator pump using a 300 µm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage to 1.48 kV. Formed beads were collected in a prepared 0.1M calcium chloride solution to avoid cell aggregation. The beads were then washed with deionized water and soaked

in a 0.1% poly-l-lysine bath for 10 minutes. Beads were washed again and soaked in 0.15% alginate solution for 15 minutes. Final washing was done with water and beads were transferred into calcium chloride for storage. The capsules were visually evaluated for uniformity and integrity through a Lomo light microscope with 200X magnification.

Macrophage cell culturing

Mouse RAW 264.7 macrophage cells were purchased from the American Type Culture Collection (ATCC) and cultured according to standard procedures using Dulbecco's Modified Eagles's Medium (DMEM). The cells were incubated in a 37 ˚ Celsius and 5% CO2 environment in a Sanyo MCO-18M Oxygen/Carbon Dioxide incubator.

Cell viability assay

The aim of this study is to evaluate the effect of microencapsulated thalidomide on cell viability. Mitochondrial reduction of MTS into aqueous soluble formazan was used as an indicator of cell viability. [10, 11] The cells were cultured in a 96-well plate at a concentration of 5 × 104 cells/well. The plate was incubated for 24 hours at 37°C in a humidified, 5% CO2 atmosphere. After aspirating the culture media, the wells were separately treated with empty APA microcapsules, APA microcapsules containing thalidomide (0.1g), thalidomide (0.7 mg/ml) and LPS (10 µg/ml). A control group consisting of no treatment was also included in the study. After incubating the plate for 24 hours at 37°C in a humidified, 5% CO2 atmosphere, the cells were resuspended in 100 µL medium and cell viability was analyzed by MTS assay. Procedures were performed as described by the manufacturer's protocol. Briefly, 20 µL of MTS/PMS solution was added to 100 µL of culture medium. After incubating the plate for 1 hour at 37 degrees in a humidified, 5% CO2 atmosphere, the absorbance was read at 490 nm using an ELISA plate reader. The amount of formazan product measured spectrophotometrically is directly proportional to the number of living cells in culture. The percentage cell viability is illustrated in Figure 1.

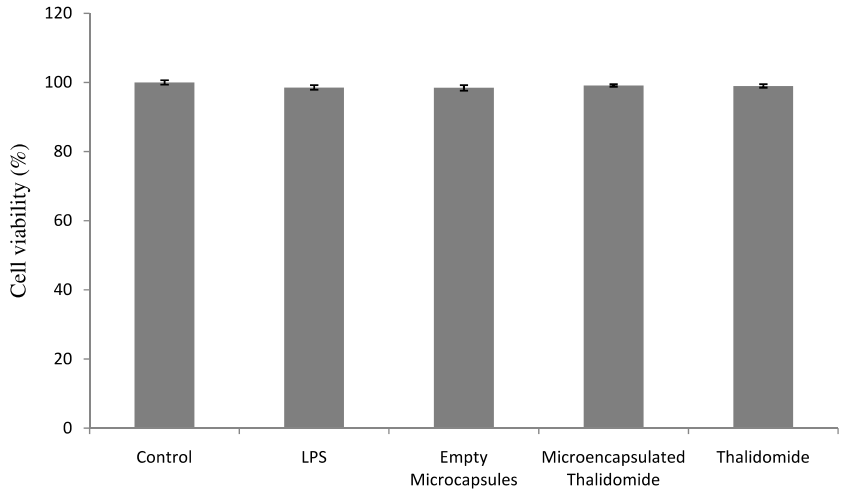

Figure 1: Effect of LPS (10 µg/ml), empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on RAW 264.7 cell viability. Each column represents the percentage cell viability compared to the control group. Values are expressed as the mean + S.D.

Induction of inflammatory cytokines by LPS

Lipopolysacharide (LPS) was used to stimulate RAW 264.7 macrophage cells. It was shown that a concentration of 10 µg/ml of LPS can significantly induce the macrophage cells to produce more pro-inflammatory cytokines such as TNF-α[12, 13]. 0.25 ml of media solution containing 380 000 RAW 264.7 cells were added to 0.25 ml of LPS (10 µg/ml) within a Falcon Brand 24-well Flat Bottom Tissue Culture Plate. The experiment was divided into four different groups. The first group consisted of cells treated with LPS alone. The second group consisted of cells treated with LPS and 0.15 g of APA microcapsules containing thalidomide solution (0.7 mg/ml). In the third group, 0.15 g of empty microcapsules was added to LPS-induced cells. The last group consisted of cells treated with LPS and free thalidomide in solution (0.7 mg/ml). The amount of inflammatory cytokines and nitric oxide was then measured from cell supernatant collected at five separate time points: 0h, 1h, 3h, 9h, 16h, 24h and 48h, after incubation in a standard 5% CO2 environment, at 37 degree.

Cytokine production in cell supernatant

The amount of inflammatory cytokines produced in cell supernatant was measured using commercially available ELISA Kit for TNF-α, IL-6 and IL-1β. Briefly, replicate serial dilutions of the antigen standard and experimental samples were prepared. 50 µl of Assay Buffer was then added into each well of the 8-well ELISA strips. After transferring 50 µl of samples and standards to the appropriate wells, the plate was gently shaken for 10 seconds and allowed to incubate for 2 hours at room temperature. The ELISA plate was then manually washed with 1x washing buffer. This process was repeated twice. Then, 100 µl of Detection Antibody solution was added to each well. After incubating the plate for 1 hour at room temperature, the ELISA wells were washed again as

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described above. 100 µl of Avidin-HRP solution was then added to all wells, and the plate was incubated for 30 minutes at room temperature. After 4 washing steps, 100 µl of Development Solution was added to each well and the plate was incubated for 15 min at room temperature, in the dark. Finally, 100 µl of Stop solution was added and the absorbance was read at 450 nm and 570 nm.

Measurement of nitric oxide from cell supernatant

NO production was assayed by measuring nitrite (a stable degradation product of NO) in supernatant of cultured RAW 264.7 cells. [14] Nitric oxide plays a crucial role in inflammation and can be measured from cell supernatant. [15, 16] Nitric Oxide Colorimetric Assay was performed as described by the manufacter's protocol. 5 µl of the Nitrate Reductase mixture and enzyme cofactor was added to each well containing cell supernatant diluted in Assay buffer. The plate was then covered and incubated at room temperature for 1h in order to convert nitrate to nitrite. The enhancer (5 µL) was then added to each well and was let incubated for 10 min. The last step uses Griess reagents to convert nitrite to a deep purple azo-compound. The absorbance was read at 540 nm using an ELISA plate reader.

Induction of inflammation using TNBS

Male Balb/c mice, 6 weeks old and weighing 23-26 g, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Lyman Duff animal center of Mcgill University. TNBS (120 mg/kg/bodyweight) dissolved in 30% ethanol [10, 17] was used to induce intestinal inflammation in this strain of mice. This is a well-characterized model that has been shown to resemble human Crohn's disease.[10] The technique involves the use of a rubber catheter inserted 3-4 cm via the anus.18 Prior to TNBS injection, the mice were slightly anesthetized with isoflurane gas. The mice were then kept in vertical position for 30s to prevent leakage of TNBS. After TNBS injection, the mice were monitored daily for survival and body mass. All animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

Treatment protocol

The animal use protocol was approved by the Animal Care Committee of McGill University. In order to investigate the therapeutic effects of APA microcapsules containing thalidomide, mice were divided randomly into five groups. Control group (n=5, receiving 30% ethanol only and no treatment) and TNBS group (n=5, receiving TNBS and no treatment) were included in this study. The treated mice were divided into three distinct groups (n=10) that consist of daily gavaging the animals with empty APA microcapsules, APA microcapsules containing thalidomide (100 mg/kg/bodyweight) and thalidomide (100 mg/kg/bodyweight) for two weeks five days following TNBS injection.

Assessment of inflammatory markers

In order to investigate the therapeutic effects of microencapsulated thalidomide, the level of TNF-α, IL-6, IL-1β and NO was measured from blood samples. These cytokines participate in the inflammation associated with Crohn's disease.[19, 20] Blood sample was collected from mice at weekly interval via the tail vein. The serum was then separated from the blood sample by centrifugation at 36,000 rpm for 8 min. The concentration of pro-inflammatory cytokines was measured in serum using ELISA analysis (eBioscience) and the level of NO was quantified using Nitric Oxide Colorimetric Assay (Biovision).

Statistical analysis

Values are expressed as mean ± SD. Study was considered a randomized balanced design. Statistical comparisons between various biomarkers were carried out by repeated measures analysis of variance (ANOVA). Statistical comparisons between various treatment groups were carried out by using the general linear model (GLM). Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Cell viability assay

Experiments were designed to determine the non-cytotoxicity effect of microencapsulated thalidomide.

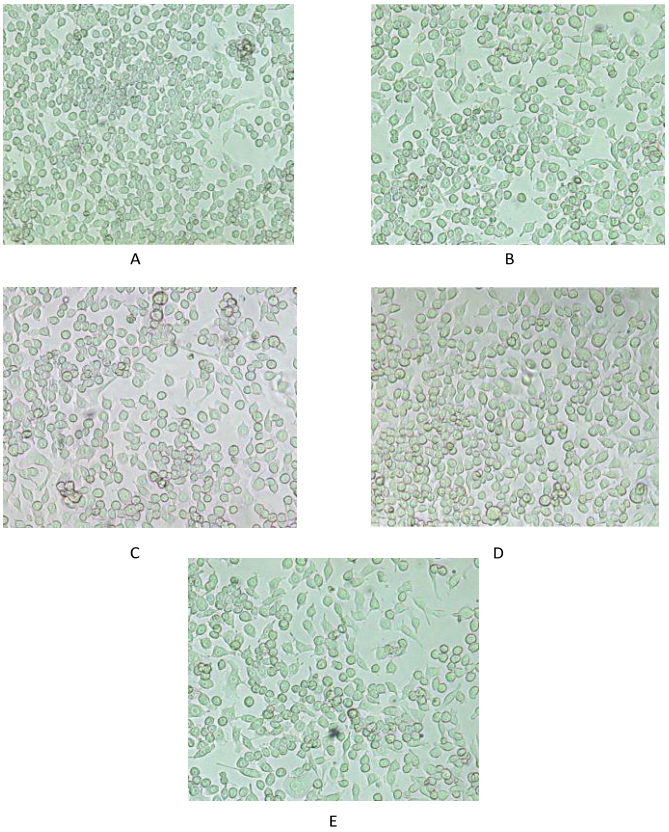

Figure 2: *Comparison photomicrographs of RAW 264.7 macrophage cells treated for 24 hours at 37°C with: (A) Control group, (B) Empty microcapsules, (C) Microencapsulated thalidomide (0.7 mg/ml), (D) Thalidomide solution (0.7 mg/ml) and (E) LPS (10 µg/ml).*

MTS assay clearly suggests that the viability of RAW 264.7 macrophage cells remains intact after treating with APA microcapsules containing Thalidomide at a concentration of 0.7 mg/ml. As illustrated in Figure 1, the percentage viability of macrophage cells compared to the control group was 98.43 ± 0.79 %, 99.11 ± 0.36%, 98.99 ±

0.5 % and 98.55 ± 0.67 % after treating with empty APA microcapsules, microencapsulated thalidomide, free thalidomide and LPS respectively. Cells were then observed in the microscope under x200 magnification and no cell damage was observed (Figure 2). The RAW 264.7 macrophage cells retained their ability to proliferate in Dulbecco's modified Eagle's medium while maintaining identical shape.

Cytokine production in cell supernatant

The amount of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β was measured from the supernatant after incubation of cells with 10 µg/ml of LPS. In the LPS control group, the initial concentration of TNF-α, IL-1β and IL-6 was 238.3 ± 5.8 pg/ml, 4.3 ± 0.1 pg/ml and 235 ± 10 pg/ml respectively. Activating RAW 264.7 cells with LPS significantly increased the production of pro-inflammatory cytokines. After 48h of incubation in LPS, the levels of TNF-α, IL-1β and IL-6 in cell supernatant reached a concentration of 911.7 ± 35.1 pg/ml, 10.2 ± 0.1 pg/ml and 451.7 ± 30.6 pg/ml respectively. Treating the cells with empty APA microcapsules did not significantly alter the expression profile of these inflammatory cytokines. However, free thalidomide was able to suppress the production of TNF-α, IL-1β and IL-6 immediately 3h following LPS induction. A delayed inhibition of cytokine production was observed when treating the cells with APA microcapsules containing thalidomide. The concentration of TNF-α, IL- 1β and IL-6 measured after 9h of incubation with microencapsulated thalidomide was 625 ± 26.5 pg/ml, 8.13 ± 0.21 pg/ml and 345 ± 10 pg/ml respectively. However, after 48 hours of incubation, the concentration of TNF-α, IL-1β and IL-6 decreased to 415 ± 65.6, 5.4 ± 0.3 and 228.3 ± 25.2 (Figure 3, 4 and 5).

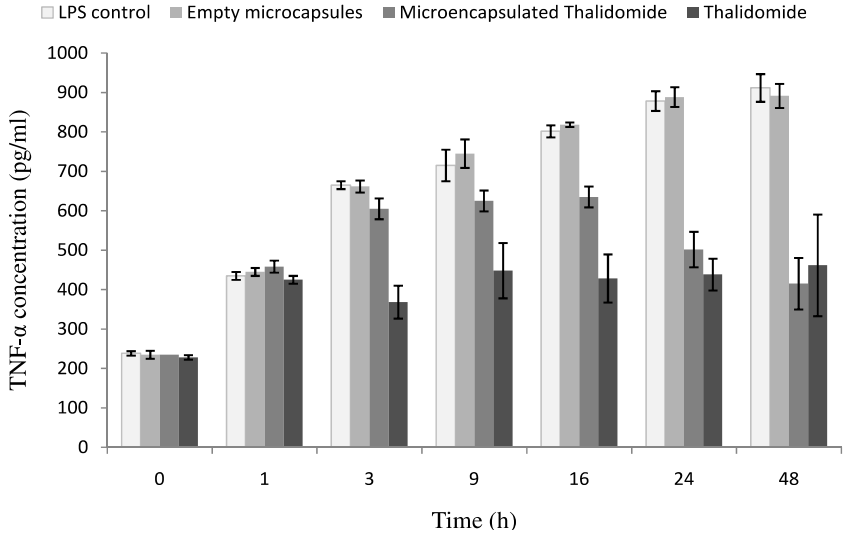

Figure 3: *Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on TNF-α concentration in cell supernatant from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.*

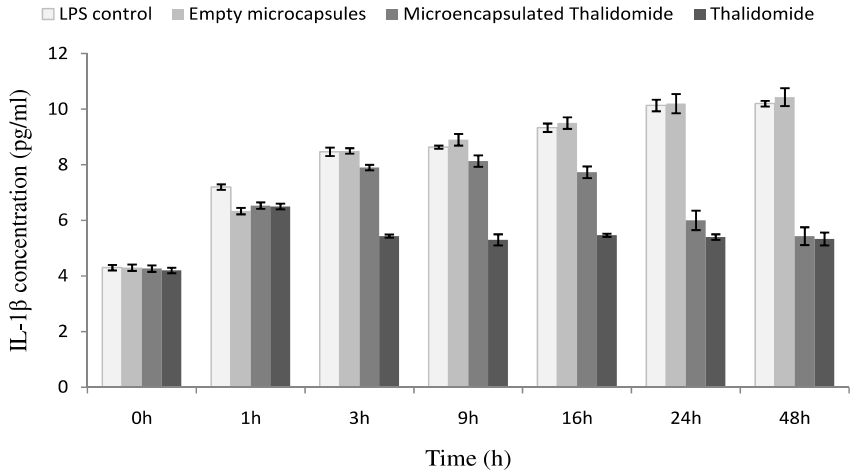

Figure 4: *Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on IL-1β concentration in cell supernatant from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.*

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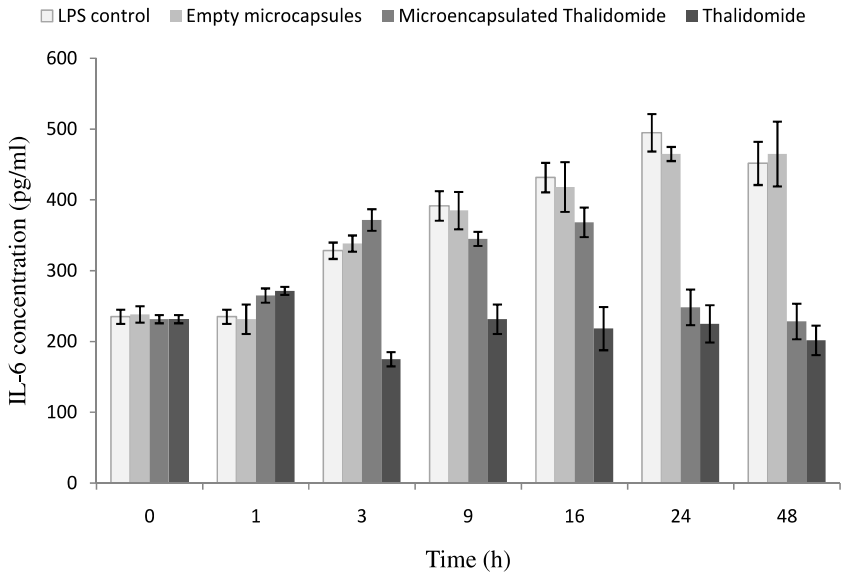

Figure 5: *Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on IL-6 concentration in cell supernatant from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.*

Nitric oxide production in cell supernatant

The concentration of nitric oxide in cell supernatant was measured to assess the extent of inflammation. Figure 6 illustrates the level of nitric oxide released after stimulation of RAW 264.7 macrophage cells with Lipopolysaccharides (LPS). It was shown that treating the cells with LPS alone (control group) for 48h increased the concentration of NO from 1.5 ± 0.2 µM to 42.6 ± 0.6 µM. Treating the LPS-activated cells with empty APA microcapsules did not significantly alter the production of NO compared to the control group. However, microencapsulated thalidomide and free thalidomide (0.7 mg/ml) lowered the concentration of NO to 25.9 ± 0.6 µM to 22.6 ± 0.7 µM respectively after 48h of incubation.

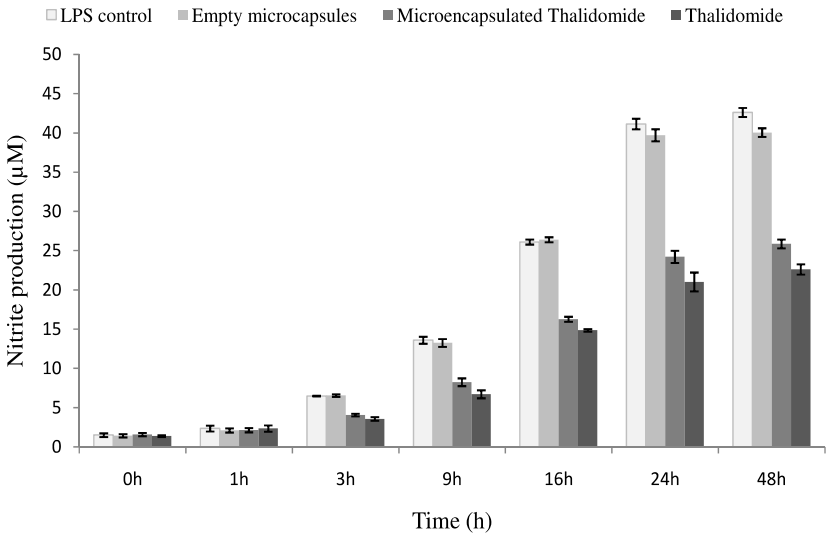

Figure 6: *Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on nitrite production from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.*

TNF-α, IL-6 and IL-1β level in serum

The concentration of TNF-α, IL-6 and IL-1β is measured in blood serum from Balb/c mice following TNBS administration (Figure 7). ELISA analysis of cytokine levels confirmed that experimental mice treated with TNBS have a higher level of TNF-α, IL-6 and IL-1β compared to the control group. It was shown that APA microcapsules containing thalidomide (100 mg/kg/bodyweight) caused a marked decreased in the level of pro-inflammatory cytokines. Treating the mice with empty microcapsules did not alter the concentration of cytokines following TNBS administration. Moreover, using APA microcapsules as a delivery carrier for thalidomide have proven to be much more successful in

lowering TNF-α, IL-6 and IL-1β compared to free thalidomide. Treating the 60 mice with thalidomide (100 mg/kg/bodyweight) for a period of two weeks decreased the level of TNF-α, IL-6 and IL-1β from 2557.1 ± 22.5 pg/ml, 7561.8 ± 103.4 pg/ml, 2388.4 ± 64.2 pg/ml to 1295.8 ± 34.1 pg/ml, 2852.5 ± 90.6 pg/ml and 1084.2 ± 38.6 pg/ml respectively. However, treating the mice with microencapsulated thalidomide (100 mg/kg/bodyweight) for two weeks decreased the level of TNF-α, IL-6 and IL-1β to 803.25 ± 44.9 pg/ml, 1883 ± 124.9 pg/ml and 1165.5 ± 31.3 pg/ml respectively.

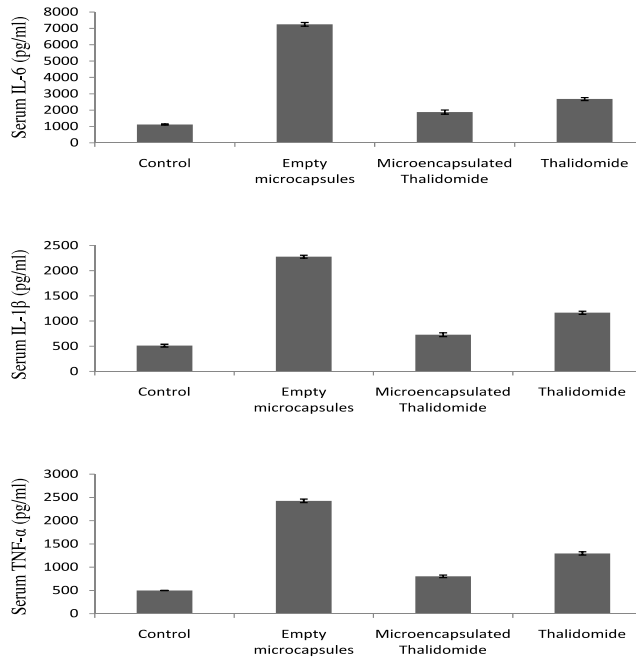

Figure 7: *Effect of empty microcapsules, microencapsulated thalidomide (100 mg/kg/bodyweight) and thalidomide (100 mg/kg/bodyweight) on serum IL-6, IL-1β and TNF-α level following TNBS injection in Balb/c mice. The level of cytokines in the serum was measured after two-weeks treatment. Values are shown as mean + S.D. of mice for each group.*

CONCLUSION

Results demonstrate that APA microcapsules containing thalidomide exert inhibitory effects on the secretion of NO, TNF-α, IL-6 and IL-1β from RAW 264.7 cell supernatant. The latter inflammatory markers are key mediators of host defense and inflammatory response associated with Crohn's disease. [21, 22] Moreover, it was shown that treating the cells with microencapsulated thalidomide resulted in a delayed inhibition of inflammatory markers of several hours compared to the cells treated with thalidomide alone. This suggests that APA membrane enables a slow release of thalidomide, thus increasing total delivery time. This important characteristic of APA microcapsule could be useful in the treatment of Crohn's disease where local delivery of the encapsulated drug to affected sites of the gastrointestinal tract is needed.[23] Furthermore, results demonstrate that treating the cells with empty APA microcapsules, APA microcapsules containing thalidomide, thalidomide solution (0.7mg/ml) and LPS (10 µg/ml) did not affect cell viability. Cell still maintain their ability to grow and proliferate in culture and the percentage of viable cells observed after 24h of incubation with the treatment was close to 100%. However, it was shown that treating RAW 264.7 cells with LPS at a concentration of 10 µg/ml caused a change in cell morphology and size. More specifically, the majority of the macrophage cells lost their circular shape and became more elongated after stimulation with LPS. Overall the results showed that artificial cell containing thalidomide could significantly suppress the formation of NO, TNF-α, IL-6 and IL-1β from RAW 264.7 cell supernatant after LPS stimulation. MTS assay confirmed the fact that treating the cells with APA microcapsules containing thalidomide does not affect their viability and ability to grow in cultured medium. Indeed, this characteristic of APA microcapsules makes it an ideal carrier for thalidomide delivery since the proposed therapy can significantly lower the production of pro-inflammatory cytokines while preserving the intestinal tract integrity. Our results also provide the in-vivo evidence that microencapsulated thalidomide exert anti-inflammatory properties using a murine model of Crohn's disease. Following induction of intestinal inflammation by TNBS injection, the level of pro- inflammatory cytokines TNF-α, IL-1β and IL-6 in blood serum significantly increased. Treating the mice with APA microcapsules containing thalidomide caused a marked decrease in the level of TNF-α, IL-1β and IL-6. Understanding the inhibitory effect of artificial cells containing thalidomide in the production of inflammatory markers from macrophage cells will help contribute to the development of novel therapies for Crohn's disease and other immune-mediated disorders.

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