

Cytoprotective and enhanced anti-inflammatory activities of liposomal piroxicam formulation in lipopolysaccharide-stimulated RAW 264.7 macrophages

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Background: Liposomal drug delivery systems, a promising lipid-based nanoparticle technology, have been known to play significant roles in improving the safety and efficacy of an encapsulated drug.

Methods: Liposomes, prepared using an optimized proliposome method, were used in the present work to encapsulate piroxicam, a widely prescribed nonsteroidal anti-inflammatory drug. The cytotoxic effects as well as the *in vitro* efficacy in regulation of inflammatory responses by free-form piroxicam and liposome-encapsulated piroxicam were evaluated using a lipopolysaccharide-sensitive macrophage cell line, RAW 264.7.

Results: Cells treated with liposome-encapsulated piroxicam demonstrated higher cell viabilities than those treated with free-form piroxicam. In addition, the liposomal piroxicam formulation resulted in statistically stronger inhibition of pro-inflammatory mediators (ie, nitric oxide, tumor necrosis factor- α , interleukin-1 β , and prostaglandin E₂) than piroxicam at an equivalent dose. The liposome-encapsulated piroxicam also caused statistically significant production of interleukin-10, an anti-inflammatory cytokine.

Conclusion: This study affirms the potential of a liposomal piroxicam formulation in reducing cytotoxicity and enhancing anti-inflammatory responses *in vitro*.

Keywords: liposomes, nitric oxide, cytokines, prostaglandin E₂, interleukin-1 β , piroxicam

Introduction

Macrophages play a critical role in the regulation of inflammation and immune responses that protect the host against microbial invasion as well as tissue injury.^{1,2} Lipopolysaccharide (LPS), a well-studied component from the outer membrane of Gram-negative bacteria, is widely considered one of the most potent activators of macrophages.^{3,4} During an inflammatory process, activated macrophages initiate a diverse series of functional responses such as the production of nitric oxide (NO) and cytokines (eg, tumor necrosis factor [TNF], interleukin [IL], and growth factors) as well as the activation of phospholipase A₂, which produces lipid metabolites of arachidonic acid (eg, prostaglandin [PG] and leukotrienes).⁵⁻⁷ Recognition of bacterial LPS endotoxin and subsequent intracellular signal transduction cascades in macrophages are key in eliminating invading pathogens and deleterious stimuli.^{8,9}

Nevertheless, an excessive or continuous production of inflammatory mediators has been linked to the development of various acute and chronic human diseases, including septic shock, hemorrhagic shock, atherosclerosis, rheumatoid arthritis, ulcerative

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colitis, multiple sclerosis, hepatitis, pulmonary fibrosis, and cancer. Hence, an adequate inhibition of these mediators is essential in suppressing enduring inflammatory process and preventing inflammation-driven diseases.^{10,11} In the present work, the *in vitro* efficacy of different drug formulations (ie, free form and liposome based) in regulating inflammatory responses by inhibition of different inflammatory mediators was assessed using a sensitive LPS-stimulated macrophage model.

Liposomal encapsulation technology has been demonstrated in various studies to ameliorate the therapeutic indices and pharmacological activities of conventional drug formulations.^{12,13} In addition, the lipid-based nanoparticle drug carrier is becoming increasingly popular due to its successes in altering the biopharmacological properties of entrapped hydrophobic drugs (eg, improving the drug's solubility, dissolution kinetics, and bioavailability).^{14–16} Hence, utilization of this promising liposomal delivery system to enhance the pharmacological properties exhibited by piroxicam in a cellular model of inflammation is truly worthwhile.

Piroxicam, an oxycam derivative, is among the most frequently prescribed nonsteroidal anti-inflammatory drugs (NSAIDs) for the management of inflammation in various musculoskeletal diseases.^{17,18} As with other NSAIDs, piroxicam's mechanism of action is not completely understood. It is generally agreed that its therapeutic properties are primarily derived from the decreased formation of PG precursors.^{18,19} Owing to its large market potential, a feasible strategy for maximizing the drug's benefits to patient health while minimizing its toxic side effects is, therefore, highly desired.

Materials and methods

Materials

Pro-lipo™ Duo was obtained from Lucas Meyer Cosmetics (Champlan, France). Piroxicam, dimethyl sulfoxide (DMSO), LPS from *Escherichia coli* (serotype 055:B5, phenol extract), and phosphate-buffered saline were purchased from Sigma-Aldrich (St Louis, MO, USA). The RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and penicillin-streptomycin solution were purchased from Thermo Fisher Scientific (Waltham, MA, USA), while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from EMD Millipore (Billerica, MA, USA). Griess reagent was purchased from Merck (Darmstadt, Germany).

Preparation of liposome samples

Pro-lipo Duo, a commercially available proliposome mixture, was used to prepare piroxicam-loaded and blank liposomal samples in accordance with an optimized procedure previously described.²⁰ Briefly, stock piroxicam solution (60 mg/mL DMSO) was added into Pro-lipo Duo and stirred moderately (125 ± 25 rpm) for 1 hour. Concentrated piroxicam-loaded liposomal suspension was formed by the dropwise addition of distilled water (dH₂O). This liposomal suspension was hydrated by 10 hours of continuous stirring at room temperature before being further diluted with dH₂O and stirred continuously for another 30 minutes. The ratio of stock piroxicam solution to Pro-lipo to dH₂O (hydration) to dH₂O (dilution) was 1:5:9:25 w/w/w/w. Blank liposomes were prepared following the same procedure, except that DMSO was used instead of stock piroxicam solution. All freshly prepared samples were diluted to the required drug concentration and volume before use.

Characterization

The drug entrapment and size profiles of the prepared liposomal samples were determined using high-performance liquid chromatography (Jones™ HPLC Genesis® C18 column, Biotage, Uppsala, Sweden) and photon correlation spectroscopy (Zetasizer Nano S, Malvern Instruments, Malvern, UK), respectively, as previously reported.^{20,21} Duplicate samples for analysis were prepared from each of the three individual batches of liposomal samples ($n = 6$). The morphological observation of blank liposomes and liposome-encapsulated piroxicam was done using a Philips CM12 transmission electron microscope (Amsterdam, The Netherlands).

Cell culture and treatment

RAW 264.7 macrophages were cultured in phenol red-free DMEM with high glucose (4500 mg/L) and L-Glutamine (4 mM/L) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (10,000 U/mL), and streptomycin (10,000 µg/mL). The cells were maintained in a humidified incubator containing 5% CO₂ at 37°C. For all experiments, cells were grown to 80%–90% confluence, and subjected to no more than 20 cell passages. Cells were scraped out from the plastic culture flasks then centrifuged at 110g at 4°C for 10 minutes. The medium was then removed and the cells were suspended with fresh DMEM containing the same supplements. The concentration was adjusted to 2×10^6 cells/mL and cell viability was always more than 80%, as determined using a standard trypan blue cell-counting technique. Cells were dispensed (50 µL) into wells

of tissue culture-grade 96-well plates (ie, 1×10^5 cells/well) and incubated for 2 hours at 37°C in 5% CO_2 atmosphere to attach the cells. Unattached cells were discarded gently after 2 hours. The attached cells were then stimulated with $10 \mu\text{g}/\text{mL}$ *E. coli* LPS in the presence or absence of the treatment sample (ie, piroxicam or liposome-encapsulated piroxicam) at a final volume of $800 \mu\text{L}/\text{well}$. The final concentration of DMSO in each well, including in the non-stimulated and non-treated control cells, was maintained at 0.67%. Cells were then incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere.

Measurement of cell viability

Following overnight incubation with treatment samples, cell viability was assessed by MTT assay after the removal of spent media from the 96-well plates. Cleavage of the MTT ring only occurs in the active mitochondria of living cells, hence, this assay was based on the ability of viable cells to reduce MTT from a pale yellow water-soluble dye to a dark blue insoluble formazan product.²² In brief, MTT was first dissolved in phosphate-buffered saline at $5 \text{ mg}/\text{mL}$. Thereafter, the stock MTT solution was filtered to sterilize and remove any insoluble residue before being kept away from direct light exposure. Stock MTT solution ($10 \mu\text{L}$ per $100 \mu\text{L}$ medium) was added to all wells of the assay plates, and these were incubated at 37°C . After 4 hours, the medium was removed and the remaining dark blue MTT crystals in each well were fully dissolved by the addition of $100 \mu\text{L}$ DMSO. Absorbance of each well was measured using a microplate reader (Infinte M200, Tecan, Grödig, Austria) at a test wavelength of 570 nm . Cell viability was determined as the relative reduction of optical density, which correlates with the amount of viable cells in relation to the control cells.

Measurement of NO/nitrite production

The level of nitrite (NO_2) in cell-free culture supernatants, which reflects intracellular NO synthase activity, was determined by Griess reaction.²³ Briefly, $100 \mu\text{L}$ of supernatant was collected from each of the stimulated and treated cell cultures. These supernatants were mixed with an equal volume of Griess reagent in a 96-well plate and incubated at room temperature for 10 minutes. This was followed by spectrophotometric measurements using a microplate reader at a test wavelength of 550 nm . The NO_2 concentrations in the supernatants were determined through a comparison with a sodium nitrite standard curve. Percentage inhibition was calculated based on the ability of the different

treatment samples to inhibit NO_2 below the level produced by control cells.

Inflammatory cytokine immunoassay

To determine the effects of treatment samples on the production of inflammatory cytokines (ie, $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-10), cells were cultured and treated as described in the previous section. Supernatants collected from wells of a 96-well plate were assayed using specific enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

Prostaglandin E_2 (PGE_2) immunoassay

The concentration of PGE_2 produced from endogenous arachidonic acid in the macrophage cell cultures, which were initially treated with different treatment samples, was measured. The collected cell culture supernatants were quantified by specific ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Statistical analyses

Data reported are presented as the mean \pm standard error of the mean of triplicate cultures of three independent experiments ($n = 9$). Statistical significance was determined using analysis of variance followed by Dunnett's multiple comparison test among groups, and Student's *t*-test for comparison between means of two groups. *P* values < 0.05 were considered indicative of significance. All statistical analyses were carried out using SPSS software (v 16.0; IBM, Armonk, NY, USA).

Results

Characterization of liposomal samples

Table 1 shows the entrapment and size profiles of the liposomal samples used in the experimental work reported here. The use of a previously optimized proliposome method for piroxicam encapsulation successfully resulted in drug-loaded liposomes with satisfactory drug entrapment profiles. All samples (ie, blank and drug-loaded liposomes) had sufficiently small particle sizes in the nanometer range and exhibited a relatively narrow size distribution. These spontaneously formed liposomal sample particles were spherical and were seen to have concentric lamellae under transmission electron microscope (Figure 1).

MTT assay

Treatment-induced cytotoxic responses in RAW 264.7 macrophages, expressed as cell viability, are presented in Table 2.

Table 1 Entrapment and size profiles of liposomal samples

| Liposomal sample | Entrapment profile | | Size profile | |
|----------------------------|---|--------------------------|-----------------------|----------------------|
| | Entrapment capacity (μg piroxicam/g Pro-lipo™) | Percent entrapped (%) | Particle size (nm) | Polydispersity index |
| Piroxicam-loaded liposomes | 805.5 ± 43.9 | 15.2 ± 1.2 | 362.6 ± 15.4 | 0.453 ± 0.009 |
| Blank liposomes | N/A | N/A | 376.3 ± 7.8 | 0.455 ± 0.005 |

Note: Values shown are mean \pm standard error of the mean ($n = 6$).

Abbreviations: μg , micrograms; N/A, not applicable; nm, nanometers.

The cell viability of the basal group (ie, non-treated and non-stimulated cells) was designated 100%, indicating no cytotoxicity. Data obtained show that 24-hour exposure of non-LPS-stimulated cells to different treatment samples, except the highest dose of piroxicam (ie, 0.4 mg/mL), did not cause significant decrement in cell viability. Contrarily, simultaneous LPS-stimulation resulted in significant cytotoxic activities in cells treated with piroxicam and liposome-encapsulated

piroxicam at drug dosages of 0.1, 0.2, and 0.4 mg/mL. Only the highest dose of piroxicam showed a significant difference in cell viability when statistically compared with respective control groups. Further analyses revealed that there was a significant difference between 0.4 mg/mL piroxicam and its equivalent drug dosage of liposome-encapsulated piroxicam in non-LPS-stimulated cells. This indicates that the latter exhibited a lower cytotoxic effect than the former.

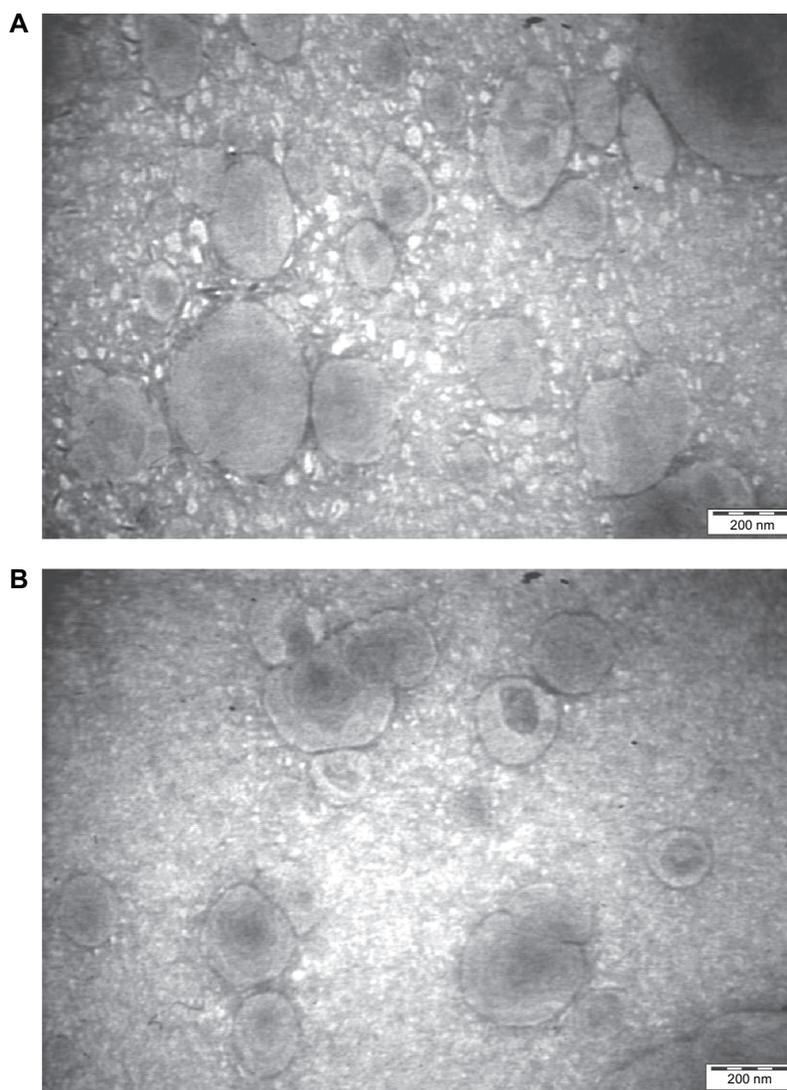


Figure 1 Transmission electron microscope photographs of (A) blank liposomes and (B) piroxicam-loaded liposomes.

Table 2 Effects of different treatments on the cell viability of RAW 264.7 macrophages

| Treatment group | Drug dosage (mg/mL) | Cell viability (%) | |
|---------------------------------|---------------------|-----------------------------|-----------------------------|
| | | Non-stimulated cells | LPS-stimulated cells |
| Basal | 0.0 | 100.00 ± 2.34 | N/A |
| Piroxicam | 0.0 (control) | 98.87 ± 1.43 | 94.84 ± 2.00 |
| | 0.1 | 98.12 ± 2.08 | 92.17 ± 1.67 ^a |
| | 0.2 | 94.10 ± 1.93 | 91.98 ± 1.87 ^a |
| | 0.4 | 91.62 ± 1.23 ^{a,b} | 86.75 ± 1.53 ^{a,b} |
| | | | |
| Liposome-encapsulated piroxicam | 0.0 | 99.14 ± 1.97 | 96.22 ± 1.75 |
| | 0.1 | 98.59 ± 2.00 | 92.78 ± 2.05 ^a |
| | 0.2 | 96.66 ± 1.81 | 92.04 ± 1.33 ^a |
| | 0.4 | 95.98 ± 1.53 ^c | 90.22 ± 1.95 ^a |
| | | | |

Notes: Values shown are mean ± standard error of the mean. ^aSignificant difference ($P < 0.05$) when compared to basal; ^bsignificant difference ($P < 0.05$) when compared to control (piroxicam; 0 mg/mL); ^csignificant difference ($P < 0.05$) when compared to group with equivalent dosage of piroxicam.

Abbreviations: LPS, lipopolysaccharide; mg/mL, milligrams per milliliter.

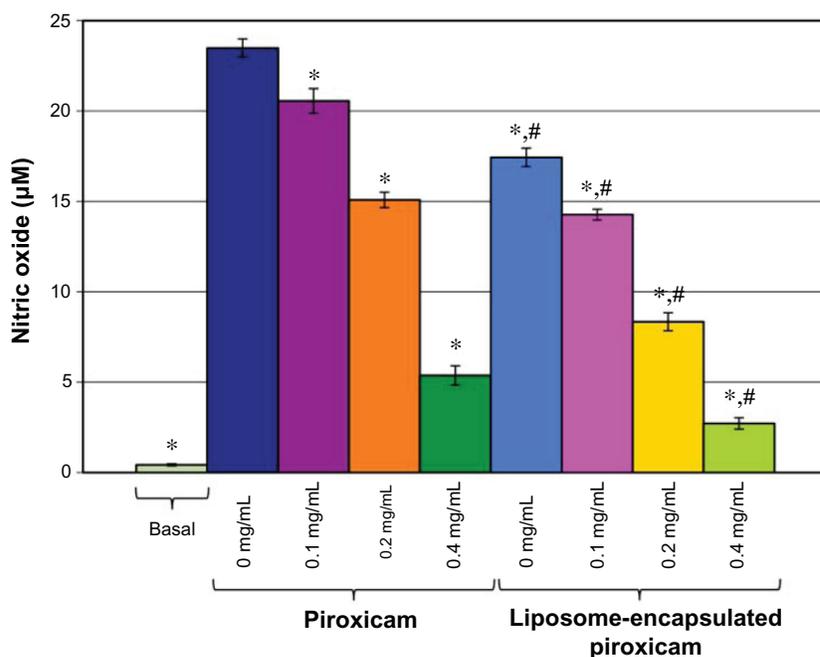
NO assay

The effect of the different treatments on NO production in RAW 264.7 macrophages is presented in Figure 2. All treatment groups in the present study, regardless of drug dosage or liposomal encapsulation, exhibited significantly less LPS-induced NO production when compared with the control group. In addition, statistical analyses also show that liposome-encapsulated piroxicam exhibited significantly greater NO reduction when compared with its non-encapsulated form at all equivalent drug dosages. The percentage of inhibition at 0.1, 0.2, and 0.4 mg/mL was increased by 26.75%, 28.70%, and 11.32%, respectively.

Production of inflammatory cytokines

The concentrations of TNF- α and IL-1 β (indicative of pro-inflammatory cytokine activity), as well as IL-10 (indicative of anti-inflammatory activity) in the treated RAW 264.7 macrophages are illustrated in Figures 3–5, respectively.

Results show that concurrent piroxicam treatment with these cells significantly inhibited the production of both TNF- α and IL-1 β at higher dosages (ie, 0.2 and 0.4 mg/mL). In contrast, liposome-encapsulated piroxicam significantly inhibited the LPS-induced rise of both pro-inflammatory cytokines at a dosage as low as 0.1 mg/mL. Further statistical analyses confirmed that liposomal piroxicam formulations were

**Figure 2** Effects of different treatment upon nitric oxide (NO) production.

Notes: Values shown are mean ± standard error of the mean ($n = 9$ /group). *Significant difference ($P < 0.05$) when compared to control (piroxicam; 0 mg/mL); #significant difference ($P < 0.05$) when compared to group with equivalent dosage of piroxicam.

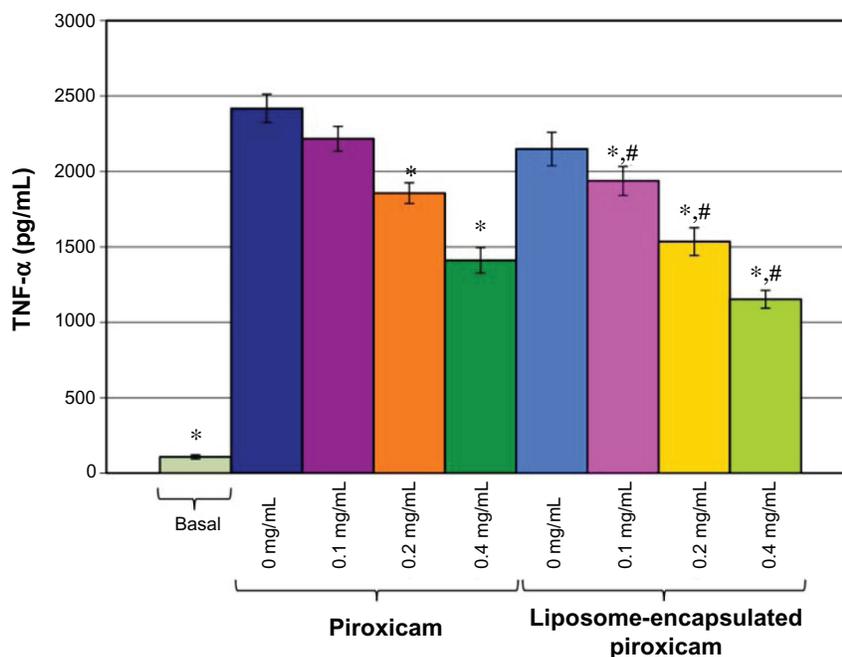


Figure 3 Effects of different treatment upon tumor necrosis factor-alpha (TNF- α) production.

Notes: Values shown are mean \pm standard error of the mean ($n = 9/\text{group}$). *Significant difference ($P < 0.05$) when compared to control (piroxicam; 0 mg/mL); #significant difference ($P < 0.05$) when compared to group with equivalent dosage of piroxicam.

significantly more effective in inhibiting pro-inflammation cytokines than their equivalent piroxicam doses. The percentage of TNF- α inhibition was increased by 11.58%, 13.29%, and 10.69% at drug doses of 0.1, 0.2, and 0.4 mg/mL, respectively. The highest augmentation of IL-1 β inhibition, which resulted

from liposomal encapsulation, was exhibited at a drug dose of 0.2 mg/mL. The inhibition increased by as much as 26.66%. Liposome-encapsulated piroxicam, at the highest tested dosage (ie, 0.4 mg/mL) also successfully reversed the LPS-induced rise of IL-1 β in the stimulated macrophages.

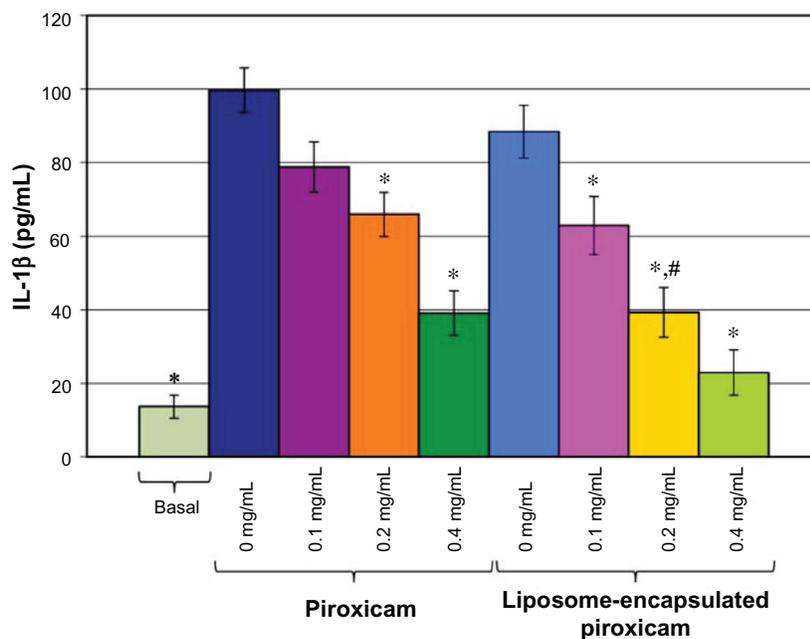


Figure 4 Effects of different treatment on interleukin (IL)-1 β production.

Notes: Values shown are mean \pm standard error of the mean ($n = 9/\text{group}$). *Significant difference ($P < 0.05$) when compared to control (piroxicam; 0 mg/mL); #significant difference ($P < 0.05$) when compared to group with equivalent dosage of piroxicam.

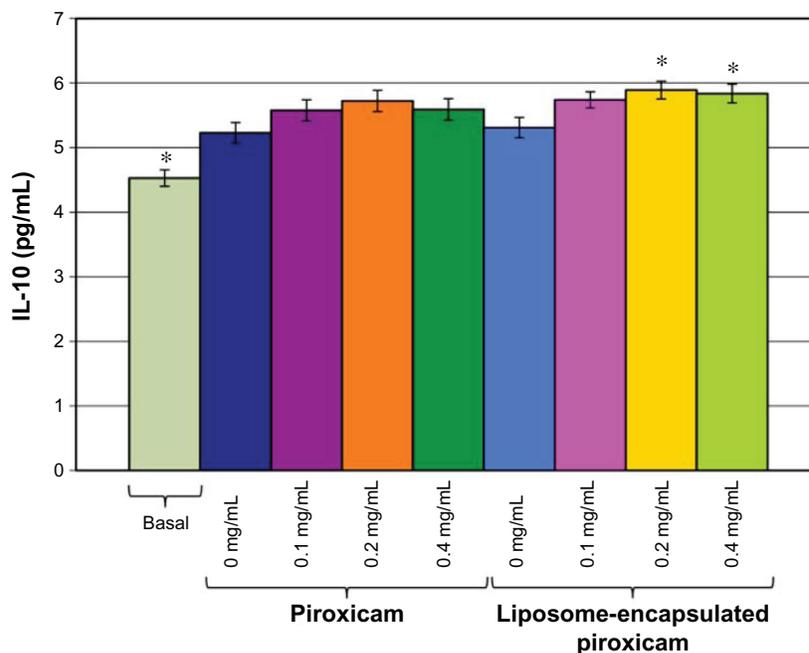


Figure 5 Effects of different treatment on interleukin (IL)-10 production.

Notes: Values shown are mean \pm standard error of the mean ($n = 9/\text{group}$). *Significant difference ($P < 0.05$) when compared to control (piroxicam; 0 mg/mL); #significant difference ($P < 0.05$) when compared to group with equivalent dosage of piroxicam.

In the case of anti-inflammatory cytokine activity, treatments potentiated a significant rise in IL-10 concentration in the RAW 264.7 macrophages. Further, significantly higher cytokine concentrations compared with the control group were exclusively exhibited by liposome-encapsulated piroxicam at 0.2 and 0.4 mg/mL. The IL-10 concentrations when the liposome-encapsulated piroxicam concentration was 0.2 and 0.4 mg/mL were 3.21% and 4.70%, respectively, higher than their equivalent dosages of piroxicam.

PGE₂ biosynthesis

The production of PGE₂, a well-known inflammatory mediator regulated by piroxicam, is presented in Figure 6. Results show a dose-dependent inhibition of PGE₂ biosynthesis by both non-encapsulated and liposome-encapsulated forms of piroxicam at 0.1, 0.2, and 0.4 mg/mL. Statistical analyses also revealed that the LPS-induced rise in PGE₂ was successfully reversed on concurrent treatment with piroxicam at 0.4 mg/mL, as well as the liposome-encapsulated drug at 0.2 and 0.4 mg/mL, with inhibition between 93.98% and 98.94%. Further analysis revealed that the PGE₂ inhibitory activities of liposome-encapsulated piroxicam at 0.2 and 0.4 mg/mL were significantly greater than their equivalent drug dosages of piroxicam. The percentage of inhibition was increased by as much as 18.91% for 0.2 mg/mL.

Discussion

Macrophages are particularly important for both innate and adaptive immunity due to their crucial role in many inflammatory processes.^{24,25} These cells have been implicated in many disease states, including inflammation, infection, atherosclerosis, diabetes, lysosomal storage disease, lupus, and cancer.²⁶ In this research, RAW 264.7 (which is a macrophage-like, Abelson murine leukemia virus-transformed cell line derived from BALB/c mice) was selected to study the events that regulate the function of diseased cells.^{3,26} Using this well-established cellular model of inflammation, the main aim of the present experimental work was to evaluate the potential of a liposomal drug-encapsulation strategy in improving the safety and anti-inflammatory properties of piroxicam.

Similar to other NSAIDs, piroxicam has been associated with diverse in vivo drug-induced toxicities.¹⁸ The inherent cytotoxic effects of different treatment on the cellular model used in the present study were predetermined and compared using MTT assay. High percentages of cell viability ($\geq 86.75\%$) were observed for all treatment groups in the present work, indicating that the treatments induced negligible cytotoxic effects on RAW 264.7 macrophages. Data analyses indicate that blank liposomes were not toxic under the present experimental conditions. The results also show that piroxicam, whether non-encapsulated or in liposome-encapsulated form, synergistically acted with

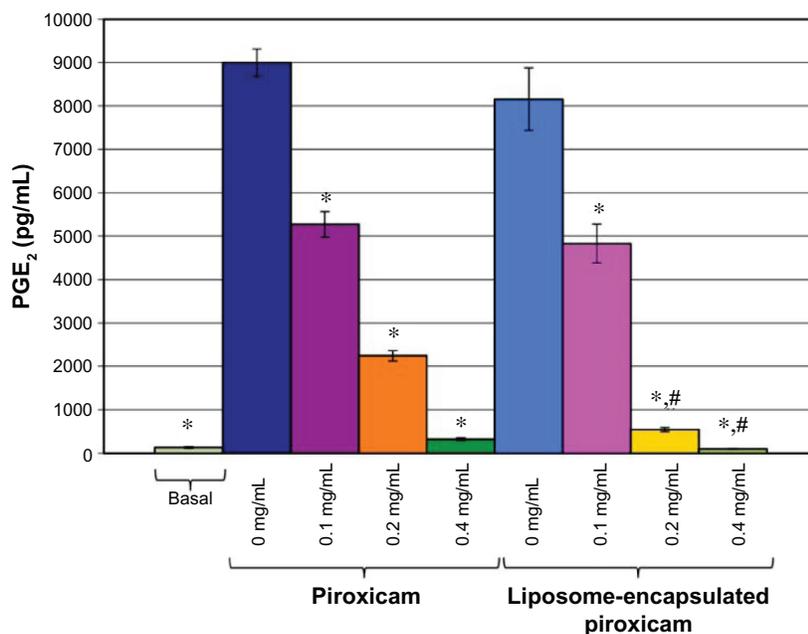


Figure 6 Effects of different treatment on prostaglandin E₂ (PGE₂) biosynthesis.

Notes: Values shown are mean \pm standard error of the mean ($n = 9/\text{group}$). *Significant difference ($P < 0.05$) when compared to control (piroxicam; 0 mg/mL); #significant difference ($P < 0.05$) when compared to group with equivalent dosage of piroxicam.

bacterial LPS endotoxin to cause a significant reduction in cell viability in the macrophages. Nevertheless, at all equivalent dosages, there was no statistically significant difference between piroxicam and liposome-encapsulated piroxicam. Interestingly, liposomal encapsulation of the drug resulted in relatively higher percentages of cell viability, particularly at high drug dosages in non-stimulated cells. These findings suggest that the strategy of encapsulating the drug within liposomes possesses a protective effect in reducing the toxicity of piroxicam. More work (eg, acute and chronic in vivo toxicity testing) is required to further support this claim.

The effects of endotoxin-induced injury on macrophages are extensive. They include a decreased capacity to produce antigen, as well as altered production of key mediators such as free radicals, reactive oxygen species, cytokines, and bioactive lipids.^{8,17,24} Nowadays, the treatment for inflammatory diseases is largely based on interrupting the action or synthesis of critical mediators that drive the host's response to injury. Various therapeutic agents, including NSAIDs, steroids, and antihistamines, have been developed for this purpose.¹ In the present work, the efficacy of different piroxicam formulations in modulating the production of inflammatory mediators was evaluated using LPS-stimulated cells. Data obtained from the assays undertaken in this study prove that bacterial LPS endotoxin triggered significant production of NO,

inflammatory cytokines (ie, TNF- α and IL-1 β), and PGE₂ in RAW 264.7 macrophages.

NO, a highly reactive free radical produced from L-arginine by NO synthase (NOS), is well known for its involvement in diverse physiological and pathological processes.^{8,27} Under normal physiological conditions, the production of this short-lived molecular messenger is regulated by constitutive isoforms of NOS (ie, neuronal and endothelial NOS). This minute quantity of NO plays a beneficial role as a smooth muscle vasodilator; a neurotransmitter; and in nonspecific immune responses to infection, host defense, and cytotoxicity.²⁸⁻³⁰ However, on exposure to specific stimulants such as bacterial LPS endotoxin, inflammatory cytokines, or calcium ionophores, an enzyme known as "inducible NOS" results in prolonged and high-output NO production. Excessive production of NO can be harmful and result in inflammation, intracellular oxidative stress, and autoimmune diseases. Thus, the degree of inducible NOS or total NO suppression provides a measure to assess the efficacy of a particular treatment in inhibiting an inflammatory process.³¹⁻³⁴ In the present study, the data obtained during NO assay indicate that all treatment groups exhibited good NO-inhibitory activities in LPS-stimulated macrophages. Further statistical analyses revealed that each group of liposome-encapsulated piroxicam possessed significantly stronger NO inhibition than their respective equivalent dosage of piroxicam. Since the improved inhibitory activities were not due to cytotoxic

effects, as indicated by cell viability analyses, this study has proven that liposomal encapsulation technology enhances NO-induced inflammatory processes.

During inflammation, a distinct cytokine cascade unfolds. Copious amounts of TNF- α are released as a response to the invasive stimuli. In turn, the over-production of TNF- α stimulates the release of various inflammatory mediators such as IL-1 β , IL-6, NO, and PGE₂. These modulate important cellular events such as gene expression, DNA damage, and cellular proliferation, which lead to aggravation and progression of various diseases.^{24,35} Thus, cellular manipulation for synthesis of cytokines (eg, TNF α and IL-1 β) is important in regulating inflammatory responses. Results from our study demonstrate that liposome-encapsulated piroxicam, at lower drug dosages, is sufficient to inhibit the formation of TNF- α and IL-1 β . Further data analyses also support the fact that liposomal encapsulation strategy is effective in improving the downregulation of pro-inflammatory cytokines without needing to increase the drug dosage.

In contrast to the pro-inflammatory cytokines, in the present work, IL-10 formation was dose-dependently increased by liposome-encapsulated piroxicam. IL-10 is an anti-inflammatory cytokine produced by monocytes and lymphocytes. It has been reported to inhibit the production of pro-inflammatory cytokines including TNF- α . Additionally, IL-10 might exert its anti-inflammatory action by inducing the formation of the IL-1 receptor antagonist.^{36,37} Thus, the present experimental findings suggest that the liposomes resulted in attenuation of the pro-inflammatory/anti-inflammatory cytokines ratio, which in turn contributed to the improved anti-inflammatory efficacy of the liposome-encapsulated drug samples.

Apart from modulating NO and cytokine release, various anti-inflammatory drugs including piroxicam are also able to inhibit PG synthesis. PGs, in particular PGE₂, are molecules with potent inflammatory and vasodilatory functions. These important prostanoids are involved in a wide variety of physiological and pathological processes.^{6,25} There are two isoforms of cyclooxygenase (COX), the key enzyme for the conversion of arachidonic acids to PGs. COX-1 is constitutively expressed in many normal tissues, whereas COX-2 is an inducible enzyme involved primarily in the regulation of inflammatory and immunological events. Expression of COX-2 is significantly upregulated by inflammatory stimuli (eg, bacterial LPS endotoxin, growth factors, cytokines, oncogenes, and carcinogens), which produces PGs, predominantly PGE₂, that contribute to pain and swelling in trauma and inflammatory and malignant diseases.^{11,29,32} Thus, reduction

in the level of COX-mediated PGE₂ synthesis is an effective strategy to inhibit inflammation as well as carcinogenesis.

Compared with that of macrophages treated with LPS alone, in our study, both piroxicam and liposome-encapsulated piroxicam resulted in the significant inhibition of PGE₂ synthesis. However, the dose-dependent inhibitory activities were significantly stronger in macrophages treated with liposome-encapsulated drug samples. Results show that, on treatment with moderate and high dosages of liposome-encapsulated piroxicam, PGE₂ accumulation in macrophages was significantly lower than with the respective equivalent dosages of free piroxicam. This signifies that the modulation of PGE₂ secretion during an inflammatory process was more effective using the liposome-encapsulated drug.

Conclusion

The present findings reveal that both piroxicam and liposome-encapsulated piroxicam exhibit different degrees of cytotoxic and inflammatory responses in RAW 264.7 macrophages. In summary, the cell viability study shows that liposomes protect macrophages from drug-induced and LPS-induced cytotoxicity. Results also demonstrate that the liposome-encapsulated piroxicam had stronger in vitro anti-inflammatory activities than the non-encapsulated form of piroxicam. Significantly reduced production of major pro-inflammatory mediators such as NO, TNF- α , IL-1 β , and PGE₂ was observed in macrophages treated with these liposome-encapsulated samples. Additionally, using liposomal drug-encapsulation technology, a lower drug dose was sufficient to inhibit the production of TNF- α and IL-1 β in LPS-stimulated macrophages. Finally, the present work indicates that the production of the anti-inflammatory cytokine IL-10 may also contribute to the improved therapeutic effects of liposome-encapsulated piroxicam.

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Disclosure

The authors report no conflicts of interest in this work.

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