In-vitro investigations of baclofen-loaded PLGA nanoparticles**

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Nanoparticle (NP)-based targeted drug delivery is a promising approach for tackling various health conditions where multiple side effects arise in conventional treatment. We have previously developed baclofen (Bcf)-loaded PLGA NPs for combating neuropathic pain. In the present work, levels of pro-inflammatory (TNF-α, IFN-γ, IL-6) and anti-inflammatory (IL-4 and IL-10) cytokines were studied in RAW 246.7 murine macrophage cells using the enzyme-linked immunosorbent assay (ELISA) technique. It was observed that Bcf-PLGA-NPs were able to suppress levels of TNF-α, IFN-γ and IL-6 while aqueous Bcf was effective in upregulation of IL-4 (anti-inflammatory cytokine). No significant effect was observed in IL-10, neither with the drug-loaded NPs nor with the aqueous drug. These findings suggest Bcf-PLGA-NPs act as potent pro-inflammatory cytokine inhibitors, which could be beneficial for alleviating inflammation-induced neuropathic pain conditions.

Keywords: ELISA, inflammation, poly(lactic-co-glycolic acid)

1. Introduction

Inflammation is one of the most important bodily processes. It arises because of tissue damage, injury, pathogenic infection or any other similar body stress. It constitutes a stress-resistant defence mechanism for combating foreign pathogenic invaders. The inflammatory reactions are widely modulated by various types of body cells such as monocytes, macrophages, mast cells etc. The monocytes and macrophages are the most important defence molecules, they are responsible

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for the secretion of various cytokines, chemokines and inflammatory mediators such as interferons, tumour necrosis factors, interleukins, prostaglandins (PGE_2) etc. [1–3].

Cytokines, low molecular weight proteins, are important signaling molecules that regulate the immune defence mechanism. Macrophages and lymphocytes are the major cytokine-producing cells. The nature of cytokine production depends upon the environment in which the macrophages are found: they produce pro-inflammatory cytokines (IL-6, TNF-α) in an inflammatory microenvironment and anti-inflammatory cytokines (IL-4) in an anti-inflammatory microenvironment. It has been reported that macrophages recognize crystals or nanoparticles through cell surface receptors, which induce an immune response [4–5].

It must be noted that for development of inflammatory responses, mitogen-activated protein kinase (MAPK) pathways play an important role. Activation of these pathways is initiated by a sequential phosphorylation process, which leads to cascade activation of the extracellular signal-regulated kinase (ERK) pathway, and the ERK1/2 substrate travels to the nucleus to activate various transcription factors such as Elk-1, c-Fos and the c-Jun N-terminal kinase pathway, which along with AP-1 (activated protein 1) lead to activation of inflammatory responses via RANTES ("regulated on activation, normal T cell expressed and secreted") and GM-CSF (granulocyte-macrophage colony-stimulating factor) [6]. Also, activated ERK1/2 via the MEK pathway (MAPK/ERK kinase pathways combined) in the cytosol along with ribosomal S6 kinase (RSK) activate CREB (cAMP response element binding protein), Fos and Elk-1, which leads to increased cell proliferation and differentiation [7–9].

Baclofen (Bcf) is an anti-spasmodic drug used as a first line of treatment for various conditions such trigeminal neuralgia. Poly(lactic-co-glycolic acid) (PLGA) is a copolymer of lactic acid and glycolic acid. It is USFDA approved, biodegradable and biocompatible [10]. In our previous work, Baclofen-loaded PLGA nanoparticles (Bcf-PLGA-NPs) were effective for combating neuropathic pain conditions; when loaded onto nanoparticles the drug showed higher brain uptake than the simple aqueous form [10]. In the present work, Bcf-PLGA-NPs were evaluated and assessed for immunomodulating properties on murine RAW macrophage 246.7 cell line. ELISA-based evaluation of various cytokines was undertaken for interferon-γ (INF-γ), tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6) and interleukin-10 (IL-10).

2. Methodological approach

2.1 Cell culture conditions

RAW 246.7 murine macrophage cells (TIB-71) were purchased from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle's Medium supplemented with fetal bovine serum (10%) along with penicillin (100 U/mL) and streptomycin (100 μg/mL) purchased from Sigma–Aldrich. Cells were propagated and maintained in a humidified incubator at 37 °C and 5% CO_2. Upon reaching subconfluency, cells were treated with bacterial lipopolysaccharides (LPS from *Escherichia coli* O11:B4, Sigma–Aldrich) at a concentration of 100 ng/mL for 6 h. After stimulation with LPS, cells were further treated with previously prepared 50 μg/mL Bcf-PLGA-NPs, along with aqueous Bcf and placebo (unloaded) nanoparticles for 24 h [10]. Cell supernatants were collected from each treatment group and centrifuged at 2000 *g* in a

refrigerated centrifuge (C-24plus, REMI, India) for 10 min at 4 °C, after which supernatant was collected and stored at –80 °C until further analysis [11–12].

2.2 Cytokine analysis using ELISA

For analysing various levels of cytokines, the enzyme-linked immunosorbent assay (ELISA) technique was used, with kits procured from Krishgen Biosystems, Mumbai, India. Analyses were done for TNF-α, IFN-γ, IL-4, IL-6 and IL-10; the kit manufacturer's protocol was followed throughout. Briefly, sample and standard supernatants of various treatment groups were applied to the ELISA plates (100 μL/well). Wells were then washed four times using wash buffer (1X) and then incubated with the detection antibody for 2 h. After incubation, wells were again washed 4 times using wash buffer and incubated using 100 μL streptavidin–HRP conjugate for 1 h at 25 °C. Wells were then washed by soaking in wash buffer for 30 s multiple times, 100 μL of tetramethylbenzidine (TMB) solution as the enzyme substrate was added, and incubated in the dark for 15 min. 100 μL of stop solution was added to terminate the reaction and optical absorbance determined at 450 nm using an ELISA microplate reader (ECIL, India) [13].

3. Results and discussion

The macrophage cells were induced with bacterial LPS, which is an essential component of Gram-negative bacteria. These LPS molecules are actively recognized by Toll-like receptors (TLR-4), which in turn activate the innate immunity and secretion of various pro-inflammatory cytokines in macrophage cells [14].

Cytokine analysis was undertaken to evaluate the modulation of both pro-inflammatory (TNF-α, IL-6 and IFN-γ) and anti-inflammatory (IL-4, IL-10) cytokines. Bcf-loaded PLGA NPs showed promising results in the case of IFN-γ (Fig. 1): these NPs were able to significantly lower levels of IFN-γ compared to the aqueous drug solution. IFN-γ is basically secreted by CD4 and CD8 T lymphocytes and natural killer (NK) cells. It operates via two functional mechanisms: first, along with TNF-α, forming a cross-link with the TNFR1 receptor, activating macrophages; the other mechanism involves induction of expression of the major histocompatibility complex (MHC), which promotes differentiation of CD4 to T helper cells, leading to excess of secretion of IFN-γ, IL-2 and TNF-β [15]. The downregulation of this IFN-γ by Bcf-NPs demonstrates the facilitation of the action of Bcf as a potential pro-inflammatory cytokine inhibitor, thus helping in suppression of inflammation.

Secretion of TNF-α, a pro-inflammatory cytokine, is upregulated upon LPS induction. In addition, upon co-activation with IL-6 there is an immediate local and systemic inflammation response [16]. Bcf-PLGA-NPs were only able to produce borderline effects in the suppression of TNF-α compared to aqueous Bcf (Fig. 2). A similar lack of effect was observed with IL-10, an anti-inflammatory cytokine (Fig. 3). IL-10 is reported to have antagonistic functions, inhibiting the production of IFN-γ, TNF-α and IL-12. An effective immunomodulating formulation should lower the level of pro-inflammatory cytokines and/or upregulate the secretions of anti-inflammatory cytokines. However, it was observed that the Bcf-PLGA-NPs did not show any promising effects on IL-10 secretion levels, which remained almost the same in both aqueous drug and nanoparticles form.

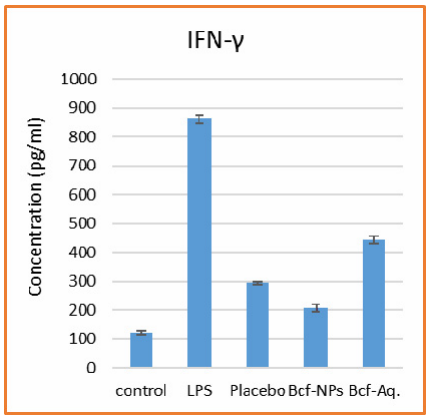

Figure 1. IFN-γ levels from untreated RAW macrophages (control), after bacterial lipopolysaccharide (LPS) treatment, with unloaded NPs (placebo), Bcf-NPs and Bcf in aqueous solution.

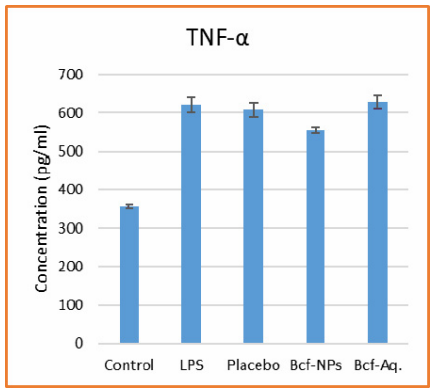

Figure 2. TNF-α levels from untreated RAW macrophages (control), after bacterial lipopolysaccharide (LPS) treatment, with unloaded NPs (placebo), Bcf-NPs and Bcf in aqueous solution.

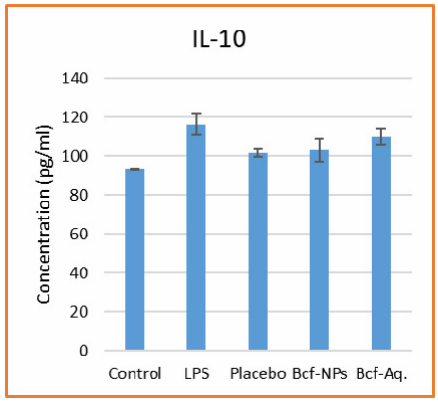

Figure 3. IL-10 levels from untreated RAW macrophages (control), after bacterial lipopolysaccharide (LPS) treatment, with unloaded NPs (placebo), Bcf-NPs and Bcf in aqueous solution.

IL-6, another important cytokine, enhances the mononuclear cell accumulation of various T cells, B cells, NK cells and monocytes during inflammation. It stimulates B cell maturation (B cell differentiation to mature immunoglobulin (Ig)-secreting plasma cells) [17]. During conditions such as neuropathic pain, arthritis, ankylosing spondylitis etc. levels of pro-inflammatory cytokines (including IL-6) are increased, which leads to high tissue damage. Bcf-PLGA-NPs significantly decreased levels of IL-6 compared to aqueous Bcf (Fig. 4). Hence the drug loaded onto the NPs provides a promising alternative in combating pro-inflammatory cytokines where the existing drug therapy showed only limited effects.

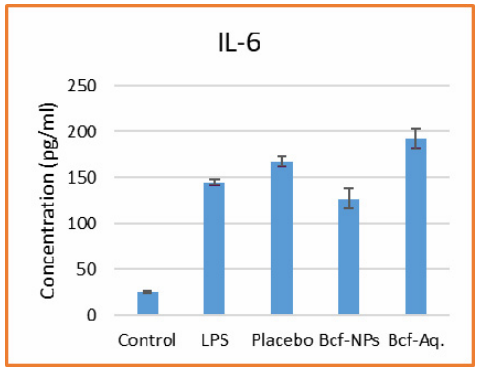

Figure 4. IL-6 levels from untreated RAW macrophages (control), after bacterial lipopolysaccharide (LPS) treatment, with unloaded NPs (placebo), Bcf-NPs and Bcf in aqueous solution.

Contrary results were seen in the case of IL-4, which is reported to be an anti-inflammatory cytokine produced by lymphoid cells such as T cells and mast cells (Fig. 5). IL-4 serves a dual purpose, negatively regulating the secretion of pro-inflammatory cytokines alongside promoting type 2 helper cell differentiation (which in turn increases secretion of IL-4, IL-5, IL-10 and IL-13) and elevating the levels of immunoglobulin E (which stimulates type I hypersensitivity reactions) [18]. In this case, the Bcf-PLGA-NPs were less effective than aqueous baclofen, which was able to increase the level of IL-4.

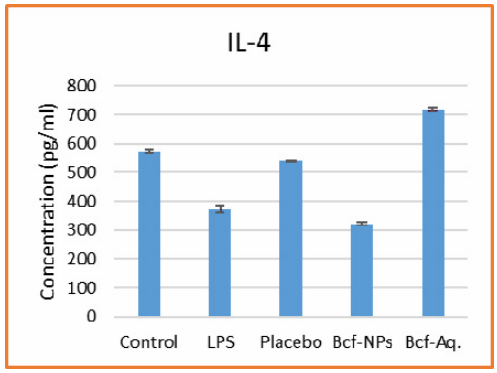

Figure 5. IL-4 levels from untreated RAW macrophages (control), after bacterial lipopolysaccharide (LPS) treatment, with unloaded NPs (placebo), Bcf-NPs and Bcf in aqueous solution.

4. Conclusions

Bcf-loaded PLGA NPs had beneficial effects on pro-inflammatory cytokines, i.e. decreasing their levels. Hence, the loaded NPs should effectively counter inflammatory conditions. In contrast, the drug-loaded NPs were less effective in beneficial upregulation of the anti-inflammatory cytokine IL-4 than the aqueous drug. Small or insignificant differences were seen in the cases of TNF-α and IL-10, which are pro- and anti-inflammatory, respectively. In some cases at least, the NPs are effective carriers of the drug.

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