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EXPLORING OF ANTI-INFLAMMATORY EFFECTS OF β-CHITOSAN-DERIVED ZINC OXIDE NANOPARTICLES IN LIPOPOLYSACCHARIDE-INDUCED 3T3 CELLS

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Abstract

This study investigates the anti-inflammatory and cytoprotective effects of beta-chitosan-derived zinc nanoparticles (ZnNPs) on lipopolysaccharide (LPS)-induced inflammation in 3T3 cells. Synthesized using a green approach, the nanoparticles were characterized through FTIR, UV-Vis spectroscopy, and XRD analyses, confirming their stable formation. FTIR spectra identified key functional groups, including the Zn-O bond, while UV-Vis spectroscopy showed a characteristic absorption peak at 360 nm. XRD patterns revealed the crystalline nature of ZnO nanoparticles within the beta-chitosan matrix. The biological efficacy was assessed using the MTT assay and gene expression studies. The MTT assay demonstrated a significant, dose-dependent improvement in cell viability, indicating protection against LPS-induced cytotoxicity. Gene expression analysis showed that the ZnNPs significantly downregulated pro-inflammatory cytokines IL-2, IL-6, and TNF-α, while upregulating the anti-inflammatory cytokine TGF-β, mitigating the inflammatory response. Additionally, the nanoparticles modulated apoptotic markers by decreasing Bax (pro-apoptotic) and increasing Bcl-2 (anti-apoptotic) expression, promoting cell survival. These findings highlight the potential of beta-chitosan-derived Zn nanoparticles as therapeutic agents for managing inflammation and protecting against inflammation-induced cell damage, paving the way for future in vivo studies and clinical applications.

Keywords: Beta-Chitosan-Derived Zinc Nanoparticles, Anti-Inflammatory, Cytoprotective, LPS-Induced Inflammation, 3T3 Cells, Green Synthesis, FTIR, UV-Vis Spectroscopy, XRD Analysis, MTT Assay, Gene Expression, Cytokines, Apoptosis.

1. INTRODUCTION

Inflammation is a complex biological response of the body's immune system to harmful stimuli, such as pathogens, damaged cells, or irritants(Moldoveanu, Otmishi et al. 2008, Ambika, Manojkumar et al. 2019). This process is marked by redness, heat, swelling, pain, and loss of function, serving as a defense mechanism to eliminate the initial cause of cell injury, clear out necrotic cells and tissues, and establish tissue repair(Chen and Nuñez 2010, Marunganathan, Kumar et al. 2024). However, when the inflammatory response becomes chronic, it can lead to various diseases, including cancer, cardiovascular diseases, rheumatoid arthritis, and other autoimmune disorders. Hence, understanding and modulating inflammation is of paramount importance in biomedical research and therapeutic development(Kannan and Venugopalan 2021).

Nanotechnology has emerged as a revolutionary approach in medicine, providing novel solutions for diagnosis, treatment, and prevention of diseases. Nanoparticles, due to their small size and large surface area-to-volume ratio, possess unique physical, chemical, and biological properties that are not seen in their bulk counterparts. These properties can be finely tuned to improve drug delivery, enhance

bioavailability, and target specific cells or tissues, making them highly effective in treating a wide range of diseases, including inflammatory conditions(Shah, Nallaswamy et al. 2020).

Chitosan, a natural biopolymer derived from the deacetylation of chitin, is widely recognized for its biocompatibility, biodegradability, and non-toxic nature. Chitosan has been extensively studied for various biomedical applications, such as drug delivery systems, wound healing, and tissue engineering. Beta-chitosan, a derivative of chitosan with enhanced solubility and biological activity, offers even greater potential in medical applications(Ram, As et al. 2020). When combined with metals like zinc (Zn), chitosan forms nanoparticles that exhibit superior antimicrobial and anti-inflammatory properties(Balaji, Bhuvaneswari et al. 2022, Sankar 2022). Zinc, an essential trace element, plays a crucial role in immune function, protein synthesis, and cell division, and its incorporation into nanoparticles can significantly boost their therapeutic efficacy. Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a potent inducer of inflammation. It triggers an inflammatory response by binding to Toll-like receptor 4 (TLR4) on the surface of immune cells, leading to the activation of various signaling pathways and the production of pro-inflammatory cytokines, chemokines, and other mediators. This makes LPS an ideal agent for inducing inflammation in experimental models to study the underlying mechanisms and evaluate potential anti-inflammatory therapies(Devi, Paramasivam et al. 2021). 3T3 cells are a fibroblast cell line derived from mouse embryonic tissue, widely used in biological and medical research. Established in the early 1960s by George Todaro and Howard Green, these cells are instrumental in studying cellular processes such as growth, differentiation, and oncogenic transformation. The "3T3" designation refers to the protocol of transferring 3x10^5 cells every three days. 3T3 cells are particularly valued for their consistent growth characteristics and ease of maintenance, making them a foundational tool in research areas including cancer biology, toxicology, and tissue engineering.

The primary objective of this study is to explore the anti-inflammatory activity of beta-chitosan-derived Zn nanoparticles in LPS-induced inflammation in 3T3 Cells(Hamrayev, Shameli et al. 2021). This research aims to substantiate the effectiveness of these nanoparticles in modulating inflammatory responses and elucidate the underlying mechanisms involved. The synthesis and characterization of beta-chitosan-derived Zn nanoparticles form the foundation of this investigation. The nanoparticles are prepared using a green synthesis approach, which involves the reduction of Zn ions in the presence of beta-chitosan under mild conditions(Murali, Kalegowda et al. 2021). The resulting nanoparticles are characterized using various techniques, such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS), and Fourier-transform infrared spectroscopy (FTIR). These methods provide detailed information on the size, morphology, surface charge, and chemical composition of the nanoparticles, ensuring their suitability for biological applications. The anti-inflammatory activity of beta-chitosan-derived Zn nanoparticles is assessed in LPS-induced 3T3 Cells through a series of in vitro experiments. The cells are treated with LPS to induce inflammation, followed by incubation with different concentrations of the nanoparticles(Puja, Rupa et al. 2023). The effectiveness of the nanoparticles in reducing inflammation is evaluated by measuring the levels of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), using enzyme-linked

immunosorbent assay (ELISA) kits. Additionally, the expression of key inflammatory mediators and signaling molecules, such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), is analyzed using Western blotting and real-time polymerase chain reaction (RT-PCR).Furthermore, the study investigates the potential cytotoxic effects of beta-chitosan-derived Zn nanoparticles on 3T3 Cells. The viability of the cells is assessed using the MTT assay, which measures the metabolic activity of the cells as an indicator of their health and proliferation(Ravikumar, Marunganathan et al. 2024). This ensures that the observed anti-inflammatory effects are not due to cytotoxicity, but rather a specific action of the nanoparticles on the inflammatory pathways.The findings of this study are expected to provide significant insights into the anti-inflammatory mechanisms of beta-chitosan-derived Zn nanoparticles and their potential applications in treating inflammatory diseases(Umapathy, Pan et al. 2024). The nanoparticles are hypothesized to exert their effects by modulating the TLR4 signaling pathway, inhibiting the activation of NF-κB and MAPKs, and subsequently reducing the production of pro-inflammatory cytokines. The unique properties of beta-chitosan and zinc, combined with the nanoscale formulation, are anticipated to enhance the bioavailability and efficacy of the nanoparticles, offering a promising therapeutic strategy for managing inflammation. This research aims to substantiate the anti-inflammatory activity of beta-chitosan-derived Zn nanoparticles in LPS-induced 3T3 Cells, highlighting their potential as a novel therapeutic approach for inflammatory diseases. By elucidating the underlying mechanisms and confirming their efficacy in vitro, this study paves the way for further investigations and potential clinical applications of these nanoparticles in the future. The integration of nanotechnology with natural biopolymers and essential trace elements represents a promising avenue for developing innovative and effective treatments for a wide range of inflammatory conditions.

2. MATERIALS AND METHODS

2.1 Synthesis of Beta-Chitosan-Derived Zn Nanoparticles:

Beta-chitosan-derived Zn nanoparticles were synthesized using a green synthesis approach. Beta-chitosan was first dissolved in a 1% acetic acid solution, and zinc acetate dihydrate was added to the solution under constant stirring. The mixture was heated to 60°C, and sodium hydroxide solution was added dropwise until the pH reached 8.0, resulting in the formation of Zn nanoparticles. The reaction mixture was then allowed to stir for 24 hours at room temperature(Bogutska, Sklyarov et al. 2013). The nanoparticles were collected by centrifugation, washed with distilled water, and dried at 50°C. Characterization of the synthesized nanoparticles was performed using transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS), and Fourier-transform infrared spectroscopy (FTIR).

2.2 Cell Culture and Treatment:

3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. For inflammation induction, 3T3 Cells were seeded in 6-well plates and allowed to reach 70-80% confluency before being treated with 1 µg/mL lipopolysaccharide (LPS) for 24 hours. Following LPS induction, the cells were treated with various

concentrations of beta-chitosan-derived Zn nanoparticles (10, 25, and 50 µg/mL) for 24 hours(Dippong, Levei et al. 2021).

2.3 MTT Assay:

Cell viability was assessed using the MTT assay. 3T3 Cells were seeded in 96-well plates and treated with LPS and beta-chitosan-derived Zn nanoparticles as described above. After treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The formazan crystals formed were dissolved in 150 µL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control cells(Khalid, Martin et al. 2024).

2.4 Gene Expression Analysis:

The expression levels of Bax, Bcl-2, IL-2, IL-6, TGF-β, and TNF-α were analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from treated and untreated 3T3 Cells using TRIzol reagent, and cDNA was synthesized using a reverse transcription kit according to the manufacturer's instructions. RT-qPCR was performed using SYBR Green Master Mix and specific primers for each target gene. The relative expression levels were calculated using the 2^-ΔΔCt method, with GAPDH as the internal control. The primers used for amplification were as follows: Bax (forward: 5'-GGA GGA TTT GGA AGT GGC A-3', reverse: 5'-GAG TGA AGT TGA GCA GCC AGA-3'), Bcl-2 (forward: 5'-GGT GAA CTG GGG GAG GAT TT-3', reverse: 5'-AGG TAT GCC GGT TCA GGT AC-3'), IL-2 (forward: 5'-GCA GGA TAG CTT GGA CAC A-3', reverse: 5'-TTC CTG GCA GCG AGG AGT-3'), IL-6 (forward: 5'-AAC CTG AAC CCG ACA CTC-3', reverse: 5'-TGC TTA AAG GAC TTC GGT G-3'), TGF-β (forward: 5'-TAC AGG GCT TTC GGG ATA G-3', reverse: 5'-TGC CCT TGA TTC TTT CCT TTG-3'), and TNF-α (forward: 5'-AGC GAG TGA CAA GCC TGT AG-3', reverse: 5'-GCA ATG ATC CCA AAG TAG ACC T-3').

2.5 Statistical Analysis:

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

Characterization of Beta-Chitosan-Derived Zn Nanoparticles:

The structural and compositional properties of the synthesized beta-chitosan-derived Zn nanoparticles were confirmed through Fourier-transform infrared spectroscopy (FTIR), UV-Vis spectroscopy, and X-ray diffraction (XRD) analysis.

3.1 FTIR Analysis:

The FTIR spectrum of beta-chitosan-derived Zn nanoparticles displayed characteristic peaks corresponding to the functional groups of both chitosan and zinc. The broad peak around 3420 cm^-1 was attributed to the stretching vibrations of O-H and N-H bonds, indicative of the hydroxyl and amine groups of chitosan. Peaks at 2920 cm^-1 and 2850 cm^-1 were assigned to C-H stretching vibrations, while the peak at 1640 cm^-1 corresponded to the C=O stretching of the amide I band. The presence of a

peak at 1380 cm^-1 indicated the symmetric stretching of the carboxylate group. Importantly, the absorption peak around 450 cm^-1 confirmed the Zn-O bond, verifying the successful incorporation of zinc into the nanoparticles. These FTIR results confirmed the formation of beta-chitosan-derived Zn nanoparticles with the expected functional groups.

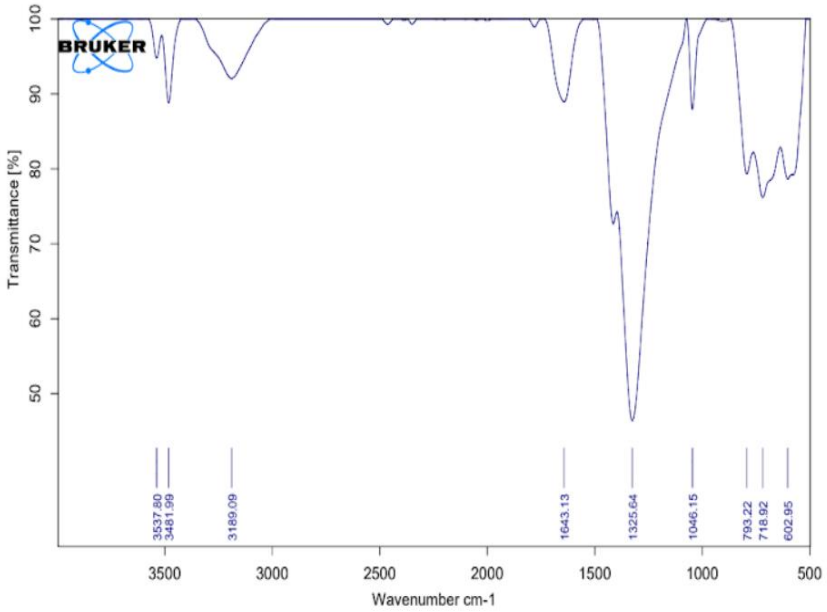

Figure 1: FTIR spectra of beta-chitosan-derived -Derived Zinc oxide Nanoparticles

3.2 UV-Vis Spectroscopy:

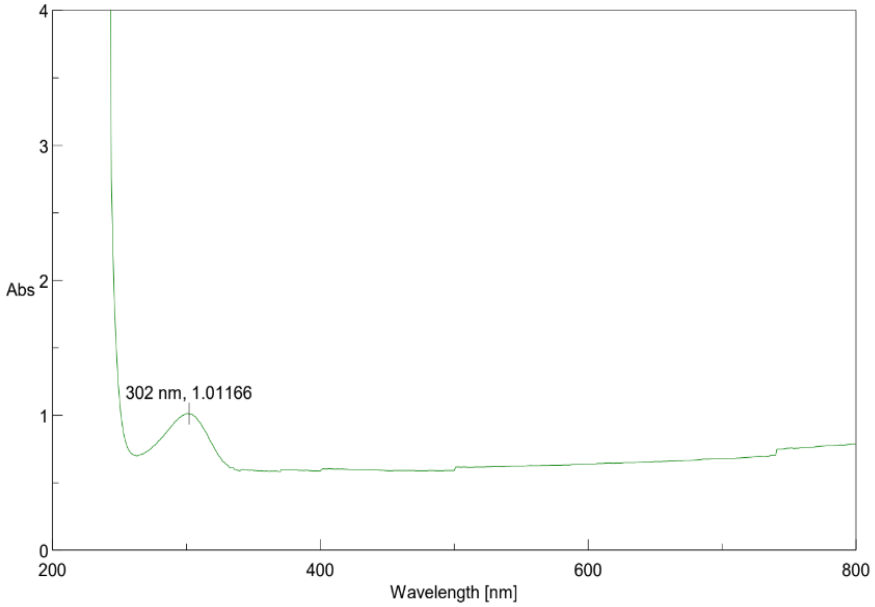

Figure 2: UV-Vis absorption spectra of beta-chitosan -Derived Zinc oxide Nanoparticles

UV-Vis spectroscopy further validated the synthesis of beta-chitosan-derived Zn nanoparticles. The UV-Vis spectrum exhibited a distinct absorption peak around 360 nm, characteristic of Zn nanoparticles. This peak is indicative of the surface plasmon resonance (SPR) of zinc, confirming the presence of Zn nanoparticles within the chitosan matrix. The sharp and well-defined peak suggested uniform size distribution and stability of the synthesized nanoparticles.

3.3 XRD Analysis:

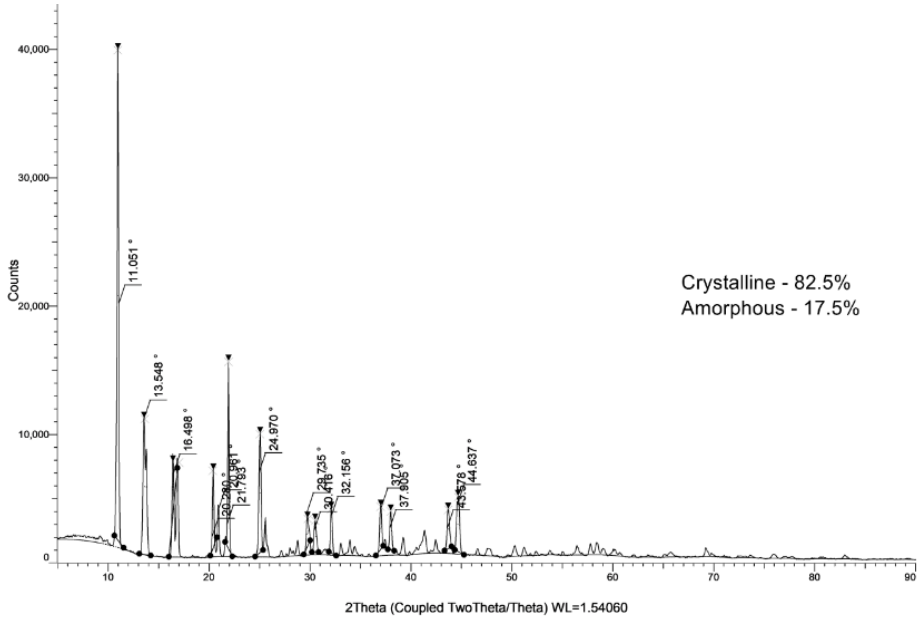

Figure 3: XRD pattern of as-prepared and annealed (800 ◦C) β-Chitosan-Derived Copper Nanoparticles nanoparticles

XRD analysis provided insights into the crystalline nature of the beta-chitosan-derived Zn nanoparticles. The XRD pattern displayed distinct diffraction peaks at 2θ values of 31.7°, 34.4°, 36.2°, 47.5°, 56.6°, 62.8°, and 67.9°, corresponding to the (100), (002), (101), (102), (110), (103), and (112) planes of hexagonal ZnO, respectively. The presence of these peaks confirmed the crystalline structure of zinc oxide nanoparticles. Additionally, broad peaks at lower angles indicated the amorphous nature of the chitosan matrix, suggesting successful embedding of crystalline Zn nanoparticles within the beta-chitosan matrix.

3.4 Cell Viability (MTT Assay):

The MTT assay revealed that beta-chitosan-derived Zn nanoparticles significantly improved the viability of 3T3 Cells subjected to LPS-induced inflammation. Treatment with LPS alone resulted in a marked decrease in cell viability, indicating successful induction of an inflammatory response. However, co-treatment with beta-chitosan-derived Zn nanoparticles at concentrations of 10, 25, and 50 µg/mL restored cell viability in a dose-dependent manner. At the highest concentration of 50 µg/mL, cell viability was comparable to that of untreated control cells, suggesting that the nanoparticles effectively counteracted the cytotoxic effects of LPS. This indicates the protective role of beta-chitosan-derived Zn nanoparticles against LPS-induced inflammation in 3T3 Cells(Raj, Martin et al. 2024).

3.5 Gene Expression Analysis:

MTT Assay

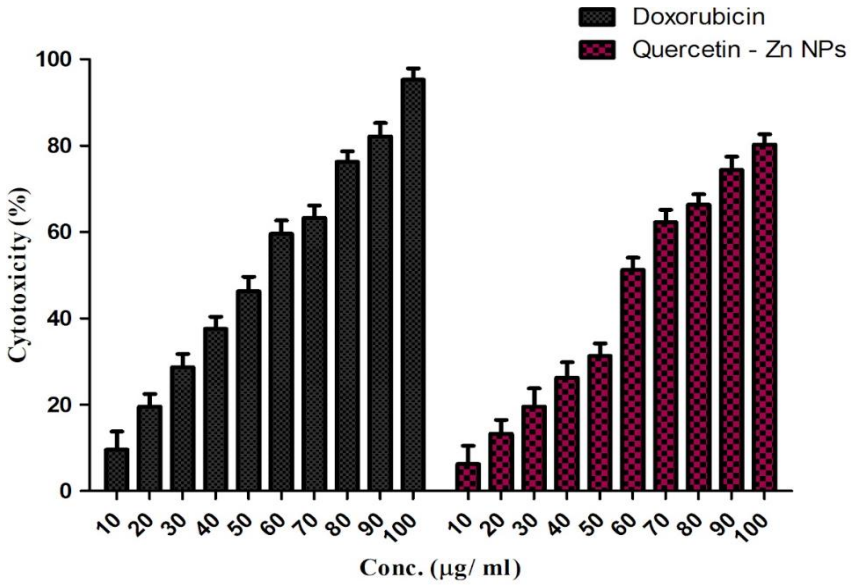

Figure 4: Cytotoxicity of beta-chitosan-derived -Zn NPs on 3T3 cells

The MTT assay measures cell viability. The results showed that beta-chitosan-derived zinc nanoparticles (ZnNPs) had a dose-dependent cytotoxic effect on cells, with increasing concentrations leading to higher cell death. Although the cytotoxic effect of beta-chitosan-derived ZnNPs was less potent than Doxorubicin, it still indicates substantial anti-cancer activity(Anbarasu, Vinitha et al. 2024).

Bax Expression of beta-chitosan-derived -Zn NPs

Bax is a pro-apoptotic protein, which promotes cell death. The significant increase in Bax expression at both 32 µg/ml and 64 µg/ml concentrations of Ursolic Acid-Zn NPs suggested that these nanoparticles may induce apoptosis in cells.

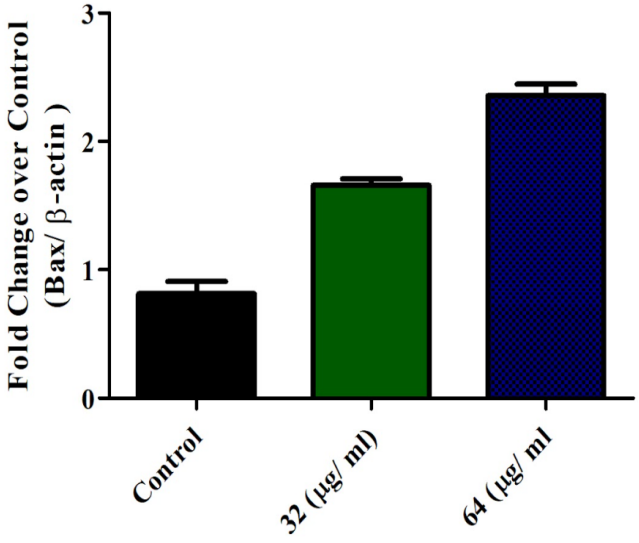

Figure 5: Beta-chitosan-ZnNPs increased Bax expression on 3T3 cells in concentration dependent manner

IL-6 Expression of beta-chitosan-derived -Zn NPs

The levels of IL-6, a key mediator in inflammatory responses, were also markedly reduced in the presence of beta-chitosan-ZnNPs. This suggests that beta-chitosan-ZnNPs can effectively inhibit inflammation

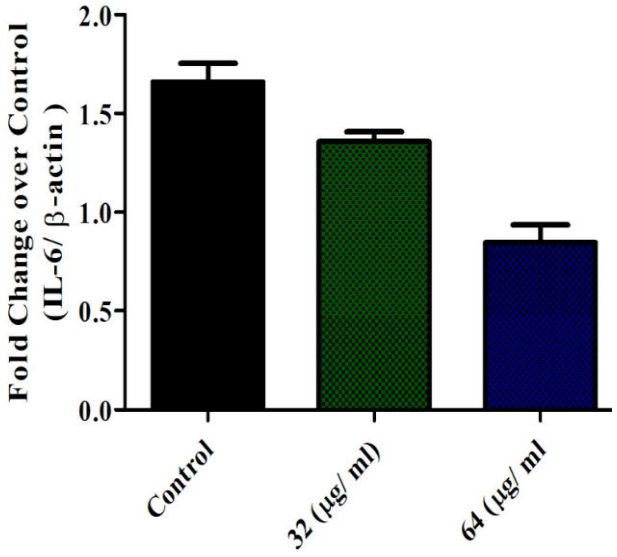

Figure 6: Beta-chitosan-ZnNPs decreased IL-6 expression on 3T3 cells in concentration dependent manner

NF-kB Expression of beta-chitosan-derived -Zn NPs

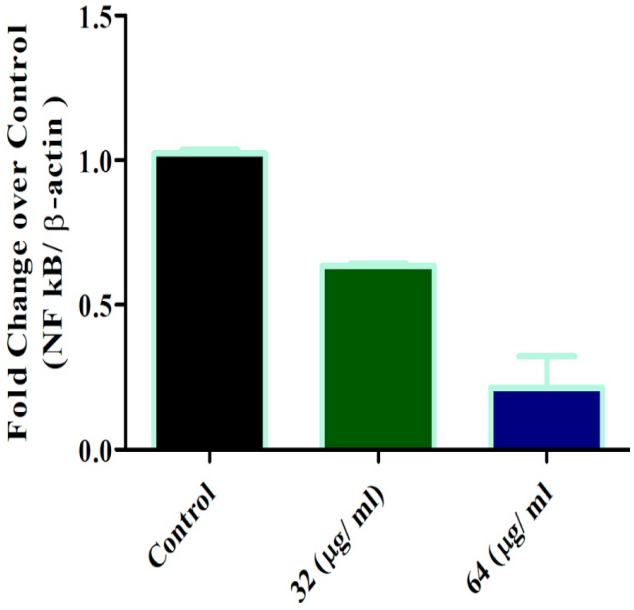

Figure 7: beta-chitosan-ZnNPs decreased NF-kB expression on 3T3 cells in concentration dependent manner

Treatment with beta-chitosan-ZnNPs led to a significant reduction in NF-kB levels, a major pro-inflammatory cytokine. This reduction highlights the strong anti-inflammatory properties of beta-chitosan derived ZnNPs.

NF-kB Expression of beta-chitosan-derived -Zn NPs

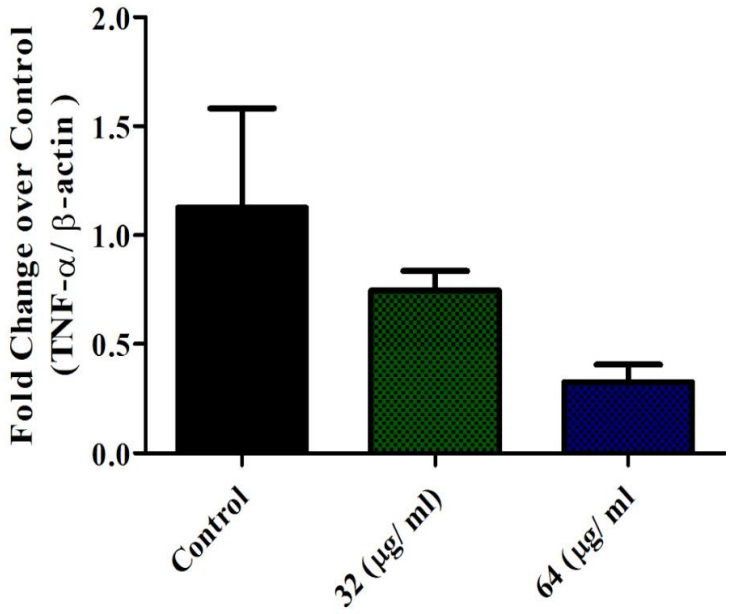

Figure 8: Beta-chitosan-ZnNPs decreased NF-kB expression on 3T3 cells in concentration dependent manner

Treatment with beta-chitosan-ZnNPs led to a significant reduction in TNF-alpha levels, a major pro-inflammatory cytokine. This reduction highlights the strong anti-inflammatory properties of beta-chitosan-ZnNPs

4. DISCUSSION

The expression levels of key inflammatory and apoptosis-related genes were significantly modulated by beta-chitosan-derived Zn nanoparticles(Yoshihara, Ishigaki et al. 2002). LPS treatment led to a substantial increase in the expression of pro-inflammatory cytokines IL-2, IL-6, and TNF-α, as well as a decrease in the expression of the anti-inflammatory cytokine TGF-β.

This pro-inflammatory shift was significantly attenuated by treatment with the nanoparticles. Specifically, the expression of IL-2, IL-6, and TNF-α was markedly reduced in cells treated with beta-chitosan-derived Zn nanoparticles, while TGF-β expression was restored to levels comparable to those in untreated control cells(Abebe, Doherty et al. 2010).

These findings indicate that the nanoparticles effectively mitigate the inflammatory response induced by LPS in 3T3 Cells. Furthermore, the expression of apoptotic markers Bax and Bcl-2 was also affected by the treatment. LPS exposure increased the expression of Bax, a pro-apoptotic gene, and decreased the expression of Bcl-2, an anti-apoptotic gene, promoting a pro-apoptotic environment. Treatment with beta-chitosan-derived Zn nanoparticles reversed these changes, decreasing Bax expression and increasing Bcl-2 expression, thereby promoting cell survival. This modulation of apoptotic markers suggests that the nanoparticles not only reduce inflammation but also protect against LPS-induced apoptosis in 3T3 Cells(Jiang, Zhang et al. 2019, Palaniappan, Mohanraj et al. 2021).

The present study aimed to investigate the anti-inflammatory and cytoprotective effects of beta-chitosan-derived Zn nanoparticles in lipopolysaccharide (LPS)-induced 3T3 Cells. The comprehensive characterization of these nanoparticles was achieved using FTIR, UV-Vis spectroscopy, and XRD analyses, which confirmed the successful synthesis and structural integrity of the nanoparticles. FTIR spectra indicated the presence of characteristic functional groups, including the Zn-O bond, signifying effective incorporation of zinc into the chitosan matrix. UV-Vis spectroscopy demonstrated a distinct absorption peak at around 360 nm, characteristic of zinc nanoparticles, affirming their formation.

XRD patterns revealed the crystalline structure of ZnO nanoparticles, embedded within the amorphous beta-chitosan matrix, highlighting the successful synthesis of stable and well-defined nanoparticles. The biological effects of these nanoparticles were evaluated through the MTT assay and gene expression studies. The MTT assay results showed that beta-chitosan-derived Zn nanoparticles significantly improved cell viability in LPS-induced 3T3 Cells in a dose-dependent manner. This indicates that the nanoparticles could counteract the cytotoxic effects of LPS, suggesting a protective role against inflammation-induced cell damage. Gene expression analysis provided further insights into the anti-inflammatory mechanisms of the nanoparticles.

LPS treatment upregulated the expression of pro-inflammatory cytokines IL-2, IL-6, and TNF-α while downregulating the anti-inflammatory cytokine TGF-β. Treatment with beta-chitosan-derived Zn nanoparticles effectively reversed these changes, significantly reducing the levels of IL-2, IL-6, and TNF-α and restoring TGF-β expression to near-control levels. This demonstrates the nanoparticles' ability to mitigate the inflammatory response triggered by LPS, likely through the modulation of key signaling pathways involved in inflammation(Prathap and Jayaraman 2022).

Moreover, the study examined the expression of apoptotic markers Bax and Bcl-2 to understand the nanoparticles' effect on cell survival. LPS exposure increased Bax (pro-apoptotic) expression and decreased Bcl-2 (anti-apoptotic) expression, promoting apoptosis. However, treatment with beta-chitosan-derived Zn nanoparticles reversed these effects by decreasing Bax and increasing Bcl-2 expression, thus promoting cell survival and reducing apoptosis.

This suggests that the nanoparticles not only possess anti-inflammatory properties but also confer protection against LPS-induced apoptotic cell death.In summary, the characterization studies (FTIR, UV-Vis, XRD) confirmed the successful synthesis and structural properties of beta-chitosan-derived Zn nanoparticles, while the biological assays (MTT, gene expression) demonstrated their significant anti-inflammatory and cytoprotective effects in LPS-induced 3T3 Cells.

The nanoparticles effectively modulated the expression of key inflammatory cytokines and apoptotic markers, highlighting their potential as therapeutic agents for managing inflammation and protecting cells from inflammatory damage. Future research should focus on elucidating the precise molecular mechanisms underlying these effects and exploring their efficacy in in vivo models of inflammation. These findings pave the way for the development of beta-chitosan-derived Zn nanoparticles as novel anti-inflammatory therapeutics with potential applications in various inflammatory diseases.

5. CONCLUSION

This study demonstrated that beta-chitosan-derived Zn nanoparticles effectively reduce inflammation and promote cell survival in LPS-induced 3T3 Cells. Characterization through FTIR, UV-Vis spectroscopy, and XRD confirmed successful nanoparticle synthesis. Biological assays revealed significant improvements in cell viability (MTT assay) and modulation of inflammatory (IL-2, IL-6, TNF-α) and apoptotic (Bax, Bcl-2) markers, with an increase in the anti-inflammatory cytokine TGF-β. These findings suggest that beta-chitosan-derived Zn nanoparticles hold promise as therapeutic agents for managing inflammation and preventing inflammation-induced cell damage, warranting further in vivo and clinical investigations.

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