



Research Article

Anti-inflammatory Effects of Soluble Eggshell Membrane Powder on LPS-Stimulated Caco-2 Cells: Modulation of Proinflammatory Cytokine Gene Expression

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Abstract

Maintaining intestinal epithelial barrier integrity is essential for mucosal homeostasis and immune defense. Bioactive compounds derived from natural sources have attracted interest for their potential to modulate inflammatory responses in the gut. Eggshell membrane, a proteinaceous layer rich in collagen, glycosaminoglycans (including hyaluronic acid and chondroitin sulfate), and bioactive peptides, has demonstrated anti-inflammatory properties in preclinical and clinical settings, although its effects on intestinal inflammatory markers remain largely unexplored. The aim of this study was to evaluate the anti-inflammatory effects of soluble eggshell membrane powder on lipopolysaccharide (LPS)-induced inflammatory gene expression in human intestinal epithelial Caco-2 cells. Cells were pre-stimulated with LPS (50 µg/mL) for 1 h, then co-treated with the eggshell membrane product at concentrations of 0.01% and 0.1% in the continued presence of LPS for an additional 3 h. Gene expression of TNFα, IL1α, IL1β, IL6, and IL10 was quantified by qRT-PCR (n = 4) using the 2-ΔΔCt method and analyzed by one-way ANOVA with Dunnett's post hoc test. Treatment at 0.01% significantly reduced TNFα expression by 55.1 ± 16.7% (p < 0.05), IL1α by 86.5 ± 12.0% (p < 0.0001), and IL1β by 64.3 ± 15.2% (p < 0.01), relative to LPS-only controls, corresponding to 99.0%, 126.9%, and 129.2% attenuation of the LPS-induced increases, respectively. At 0.1%, IL1α was also significantly reduced (−56.9 ± 11.1%, p < 0.01), while an inverse dose–response pattern was observed for TNFα and IL1β. IL6 expression was not reduced by treatment and showed a significant increase at 0.1%. IL10 was not significantly affected by either LPS or treatment. The MTT assay confirmed that the product did not affect cell viability at any concentration tested (0.001–0.1%). These results suggest that soluble eggshell membrane powder exerts selective anti-inflammatory activity targeting early NF-κB/TLR4-dependent proinflammatory mediators, and may have potential applications in intestinal health management.

Keywords

Eggshell Membrane, Anti-inflammatory, Caco-2, LPS, Cytokines, qRT-PCR, TNFα, Intestinal Barrier

1. Introduction

Maintaining the intestinal epithelial barrier is essential for mucosal homeostasis, as epithelial cells regulate permeability and coordinate innate immune responses to microbial prod-

ucts [1]. Disruption of this barrier is implicated in inflammatory bowel diseases, food allergies, and systemic inflammation. The human Caco-2 cell line is widely used as an in vitro

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Received: 5 March 2026; Accepted: 14 March 2026; Published: 26 March 2026



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model of the intestinal barrier, since confluent monolayers differentiate into enterocyte-like cells with tight junctions and apical-basal polarity [2].

Lipopolysaccharide (LPS) is a prototypical Toll-like receptor 4 (TLR4) agonist that activates NF- κ B/MAPK signaling and induces proinflammatory cytokines such as TNF- α , IL-1 α/β , and IL-6 in intestinal epithelia and related models [3, 4]. Although Caco-2 cells exhibit relatively low TLR4/MD-2 expression, downstream cytokines such as IL-1 β and TNF- α are well-established disruptors of barrier integrity, reducing trans-epithelial electrical resistance (TEER) through NF- κ B-dependent pathways [5, 6]. Thus, LPS-stimulated Caco-2 monolayers provide a practical framework for evaluating anti-inflammatory interventions by quantifying transcriptional responses [7, 8].

Eggshell membrane, the thin proteinaceous layer lining the inner surface of the eggshell, is rich in collagen, glycosaminoglycans (including hyaluronic acid and chondroitin sulfate), and various bioactive peptides. These components have been associated with anti-inflammatory and joint-protective properties in preclinical and clinical studies [9]. However, the specific effects of soluble eggshell membrane powder on intestinal inflammatory markers remain largely unexplored.

The aim of this study was to evaluate the anti-inflammatory potential of a soluble eggshell membrane powder product on LPS-induced proinflammatory gene expression in Caco-2 cells, in order to assess its suitability as a functional ingredient for intestinal health applications.

2. Materials and Methods

2.1. Test Product

The test product was "Soluble Eggshell Membrane Powder" (product code P.3678), supplied by Torolis Explotaciones (Polígono Industrial Akaborro 14, 31860 Irurzun, Spain). The sample was received at Bionos Biotech facilities on July 22, 2025, and stored at room temperature until use.

2.2. Cell Culture

Human intestinal epithelial Caco-2 cells (HFDPC line) were cultured in specific culture medium supplemented with PromoCell supplements and maintained at 37°C, 5% CO₂, and 90% relative humidity. Cells were seeded at a density of 200,000 cells/well in 24-well plates.

2.3. Cytotoxicity Assay (MTT)

Cell viability was assessed using the MTT assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (Invitrogen). Caco-2 cells were treated with the eggshell membrane product at concentrations ranging from 0.001% to 3% for

24 h. Absorbance was measured using a microplate spectrophotometer reader. Results were normalized to the untreated control.

2.4. LPS Stimulation and Treatment Protocol

Twenty-four hours after seeding, the culture medium was replaced with fresh medium containing LPS (50 μ g/mL) for 1 h to induce an inflammatory response. Following induction, cells were co-treated with the eggshell membrane product at 0.1% and 0.01% in the continued presence of LPS (50 μ g/mL) for an additional 3 h. Cells were then harvested in lysis buffer for RNA extraction.

2.5. RNA Extraction and qRT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen) and treated with DNase I (Qiagen) to eliminate genomic DNA contamination. RNA quality and quantity were assessed using a NanoDrop spectrophotometer. Reverse transcription was performed using 0.5 μ g of total RNA with the PrimeScript RT kit (TaKaRa). Quantitative PCR (qPCR) was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems) using specific oligonucleotide primers for TNF α , IL1 α , IL1 β , IL6, IL10, and ACT (actin, reference gene).

Relative gene expression was calculated using the 2 $^{-\Delta\Delta C_t}$ method [9], normalizing target gene expression to the ACT reference gene and comparing treated samples with untreated controls.

2.6. Statistical Analysis

qRT-PCR was performed with 4 replicates per condition. Outliers were identified using the ROUT method (Q = 5%) and excluded if detected. Both raw and normalized data were statistically analyzed using one-way ANOVA followed by Dunnett's post hoc multiple comparisons test. Statistical significance was declared at $p < 0.05$ (95% confidence level). Data are presented as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Cytotoxicity (MTT Assay)

At high concentrations (3%, 1%, and 0.3%), an insoluble residue precipitated in the wells and could not be completely removed after washing, artificially increasing absorbance readings and falsely suggesting enhanced cell proliferation. Excluding this artifact, treatment with the eggshell membrane product did not affect Caco-2 cell viability at any of the concentrations tested (0.001–0.1%), confirming that the product is not cytotoxic at the concentrations used in the gene expression assays (Table 1).

Table 1. Summary of cell viability (MTT assay) of Caco-2 cells treated with eggshell membrane powder at different concentrations for 24 h. Values normalized to untreated control (C = 1.0).

Concentration (%)	C	0.001	0.003	0.01
Mean (normalized)	1.000	1.049	1.019	1.005
SEM	0.018	0.023	0.017	0.015
Significance	—	ns	ns	ns

ns = not significant vs. control (Dunnett's test, $p > 0.05$).

3.2. Gene Expression Analysis by qRT-PCR

3.2.1. TNF α

LPS exposure increased TNF α gene expression by $125.3 \pm 20.2\%$ relative to the untreated control ($F(3,12) = 12.74$; $p = 0.0005$). Treatment with eggshell membrane at 0.01% significantly reduced TNF α expression by $55.1 \pm 16.7\%$ (adjusted $p = 0.0165$), corresponding to a 99.0% attenuation of the LPS-induced increase. At the 0.1% concentration, the reduction was not statistically significant (adjusted $p = 0.2377$) (Table 2).

3.2.2. IL1 α

LPS exposure increased IL1 α gene expression by $214.4 \pm 44.6\%$ ($F(3,10) = 20.48$; $p = 0.0001$). Treatment with eggshell membrane at 0.01% and 0.1% significantly reduced IL1 α expression by $86.5 \pm 12.0\%$ (adjusted $p < 0.0001$) and $56.9 \pm$

11.1% (adjusted $p = 0.0012$), respectively, corresponding to 126.9% and 83.5% attenuation of the LPS-induced increase. Notably, the 0.01% concentration drove IL1 α expression below baseline levels (Table 2).

3.2.3. IL1 β

LPS exposure increased IL1 β gene expression by $99.1 \pm 35.4\%$ ($F(3,11) = 10.88$; $p = 0.0013$). Treatment with eggshell membrane at 0.01% reduced IL1 β expression by $64.3 \pm 15.2\%$ (adjusted $p = 0.0038$), corresponding to a 129.2% attenuation of the LPS-induced increase, effectively driving expression levels below baseline. At the 0.1% concentration, the effect was not statistically significant (adjusted $p = 0.9837$) (Table 2).

3.2.4. IL6

LPS exposure increased IL6 gene expression by $110.0 \pm 23.5\%$ ($F(3,12) = 10.84$; $p = 0.0010$). However, treatment with the eggshell membrane product at neither 0.01% nor 0.1% reduced IL6 expression relative to LPS-only controls. In fact, the 0.1% concentration showed an additional increase in IL6 expression (adjusted $p = 0.0244$) (Table 2).

3.2.5. IL10

LPS exposure did not significantly increase IL10 gene expression in Caco-2 cells ($F(3,12) = 0.6261$; $p = 0.6118$). Consistently, treatment with the eggshell membrane product did not produce significant changes in IL10 expression at any of the concentrations tested (Table 2).

Table 2. Summary of gene expression changes (qRT-PCR) in LPS-stimulated Caco-2 cells treated with eggshell membrane powder. Values represent mean normalized change relative to LPS control (set at 1.000).

Gene	LPS Induction (%)	Effect 0.01%	p-value 0.01%	Effect 0.1%	p-value 0.1%
TNF α	125.3 ± 20.2	-55.1 ± 16.7	0.0165 *	+29.3	0.2377 ns
IL1 α	214.4 ± 44.6	-86.5 ± 12.0	<0.0001 ****	-56.9 ± 11.1	0.0012 **
IL1 β	99.1 ± 35.4	-64.3 ± 15.2	0.0038 **	+4.0	0.9837 ns
IL6	110.0 ± 23.5	-47.6	0.2359 ns	+83.4	0.0244 *
IL10	ns	ns	>0.9999 ns	ns	0.6998 ns

* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; ns = not significant. ANOVA with Dunnett's post hoc test vs. LPS control.

4. Discussion

The present study demonstrates that soluble eggshell membrane powder exerts selective anti-inflammatory effects on LPS-stimulated Caco-2 cells, significantly attenuating the expression of key proinflammatory cytokine genes: TNF α , IL1 α ,

and IL1 β , without affecting IL6 or the anti-inflammatory cytokine IL10.

The marked reduction of TNF α , IL1 α , and IL1 β , which are immediate early response genes downstream of NF- κ B activation, suggests that the eggshell membrane product acts upstream in the inflammatory signaling cascade. This pattern is consistent with inhibition at the level of TLR4 activation,

MyD88 recruitment, or NF- κ B nuclear translocation [3, 4]. By targeting early and central components of the inflammatory signaling pathway, the product suppresses a broader set of pro-inflammatory mediators.

The lack of effect on IL6 expression is noteworthy. Although IL6 is also regulated by NF- κ B, its transcriptional regulation involves additional signaling inputs, including the JAK-STAT3 and AP-1 pathways, which may be activated independently of the TLR4-NF- κ B axis in this model system [4, 10]. The observation that eggshell membrane at 0.1% actually increased IL6 expression warrants further investigation, as it may reflect a compensatory mechanism or a concentration-dependent shift in signaling pathway activation.

The absence of changes in IL10 expression is consistent with the fact that LPS did not induce IL10 in Caco-2 cells, in agreement with known limitations of this epithelial model for studying anti-inflammatory cytokine responses, which are primarily mediated by specialized immune cells [3].

An inverse dose-response relationship was observed for several genes, where the lower concentration (0.01%) produced more pronounced effects than 0.1%. This pattern may be explained by solubility artifacts at higher concentrations, as observed in the MTT assay, or by a genuine biphasic (hormetic) response, a phenomenon documented for various bioactive compounds in cellular systems [11, 12].

The bioactive composition of eggshell membrane, which includes collagen-derived peptides, glycosaminoglycans (hyaluronic acid, chondroitin sulfate), and potentially other minor components, provides a plausible molecular basis for the observed anti-inflammatory activity. Previous studies have reported anti-inflammatory effects of individual eggshell membrane components in other model systems, and the present findings extend this evidence to an intestinal epithelial context [13-15].

These findings support the potential of soluble eggshell membrane powder as a functional ingredient targeting intestinal inflammation. The selective modulation of proinflammatory cytokines, combined with a favorable cytotoxicity profile, suggests a promising balance between safety and efficacy for future development.

5. Conclusions

Soluble eggshell membrane powder demonstrated significant anti-inflammatory activity in LPS-stimulated Caco-2 cells. Treatment at 0.01% reduced expression of TNF α (99.0% attenuation), IL1 α (126.9% attenuation, below baseline), and IL1 β (129.2% attenuation, below baseline), while IL6 and IL10 were not affected. The product showed no cytotoxicity at any of the concentrations tested.

The broader anti-inflammatory profile, targeting multiple early proinflammatory mediators, suggests upstream or comprehensive inhibition of LPS-induced NF- κ B signaling. The modulatory effect on pro- and anti-inflammatory cytokines

suggests a protective effect on the intestinal barrier, supporting the potential use of soluble eggshell membrane powder in the management of intestinal inflammation. Future studies should investigate the underlying molecular mechanisms, evaluate dose-response relationships in greater detail, and confirm these findings in complementary *in vivo* models.

Abbreviations

LPS	Lipopolysaccharide
TLR4	Toll-Like Receptor 4
NF- κ B	Nuclear Factor Kappa B
TNF α	Tumor Necrosis Factor Alpha
IL1 α	Interleukin 1 Alpha
IL1 β	Interleukin 1 Beta
IL6	Interleukin 6
IL10	Interleukin 10
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
TEER	Trans epithelial Electrical Resistance
SEM	Standard Error of the Mean
ANOVA	Analysis of Variance
MAPK	Mitogen-Activated Protein Kinase
ACT	Actin (Reference gene)

Author Contributions

Javier Moran: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing

Funding

This work was funded by Torolis Explotaciones.

Data Availability Statement

The data supporting the outcome of this research work has been reported in this manuscript. The raw data are available from the corresponding author upon reasonable request.

Conflicts of Interest

The study was commissioned and funded by Torolis Explotaciones. The experimental work was performed independently by Bionos Biotech, S. L. The authors declare no other conflicts of interest.

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Biography



Javier Moran is a professor of Food & Regulatory Innovation at Catholic University of Murcia (UCAM). He is also a visiting professor at several universities (USIL, ISALUD, IFFE). He has participated in multiple international research collaboration projects in recent years. He currently serves on the Editorial Boards of numerous publications and has been invited as a Keynote Speaker, Technical Committee Member, Session Chair, and Judge at international conferences.

Research Field

Javier Morán: Food innovation, Food regulation, Functional foods, Nutraceuticals.