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The Immune Response Profile of Activated Macrophages Following Lunasin Treatment: Lunasin as a Potential Anti-Inflammatory Medicament

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Abstract

Introduction: Inflammation plays an important role in the pathogenesis of the Pulp-Periapical (PP) disease. Inflammatory cytokines production is regulated through Nuclear Factor Kappa B (NF-κB) pathway. Lunasin is a 43-amino acid peptide containing a unique Arg-Gly-Asp (RGD) cell adhesion motif isolated from soybean. It has been reported that lunasin inhibits the production of inflammatory mediators by suppressing the NF-κB pathway in murine Macrophages (MQ). This study aimed to investigate the effect of lunasin on the production of pro- and anti-inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-10) involved in PP disease, and compare it to that of ibuprofen.

Methods: THP-1 cells were plated in wells and differentiated into MQ using Phorbol-12-Myristate-13-Acetate (PMA). These wells were then divided into 13 different groups of LPS and non-LPS-activated MQ that were then treated with different concentrations of lunasin, negative control powder and ibuprofen. Levels of inflammatory cytokines including IL-1β, IL-6, TNF-α and IL-10 were measured using Cytometric Bead Array. Data were analyzed using Kruskal-Wallis and Mann-Whitney U-tests.

Results: 100 μM lunasin significantly reduced the production of IL-1β, IL-6, TNF-α and enhanced the production of IL-10 compared to LPS-activated MQ (P=0.001). However, lower concentrations of lunasin did not significantly alter the cytokines' levels compared to LPS-activated MQ (P>0.05). Ibuprofen did not significantly affect the levels of cytokines in LPS-activated MQ (P>0.05).

Conclusions: Considering the suppressive effect of lunasin on the production of cytokines responsible for inflammation and bone resorption, the use of lunasin as a potential anti-inflammatory agent can be evaluated in future studies.

Keywords: Lunasin; Soybean; Inflammation; Cytokines; Endodontics

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Introduction

The significant role of inflammatory reactions has been confirmed in the initiation and progression of Pulp-Periapical (PP) disease [1,2]. These inflammatory reactions are controlled by variable mediators such as cytokines [3]. Pro- and anti-inflammatory cytokines are produced by different types of cells such as macrophages and T lymphocytes [3]. IL-1β, IL-6 and TNF-α are among the pro-inflammatory cytokines that are involved in PP disease [4]. They are known to stimulate and activate Macrophages (MQ), leukocytes and promote bone resorption [4]. On the other hand, IL-4 and IL-10 are potent anti-inflammatory cytokines with immunosuppressive properties that can inhibit the progression of inflammatory reactions and bone resorption in the periapical region [4].

Macrophages are one of the major components in inflammatory reactions. They control the inflammatory pathway by regulating the production of pro- and anti-inflammatory mediators through transcription factor nuclear factor-kappa B (NF-κB) [3]. NF-κB is a family of transcription factors that can be activated by external stimuli such as bacterial Lip Polysaccharide (LPS) [5]. Activation of NF-κB induces the expression of genes involved in inflammatory responses including IL-1β, TNF-α, IL-2, IL-6, IL-8, and IL-12 [5-7]. Therefore, it may be postulated that inducers and inhibitors of NF-κB transcriptional activity could affect the progression of inflammatory reactions

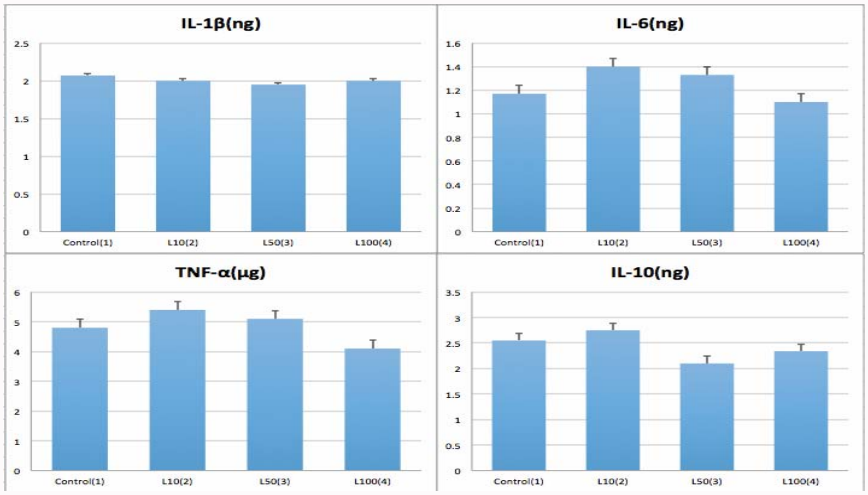

Figure 1: The effect of different concentrations of lunasin on the mean level of cytokines. Each value represents the mean of three replicates and standard deviation in error bars. Control(1): MQ, L10(2): MQ+10 μM lunasin, MQ+50 μM Lunasin, and MQ+100 μM lunasin.

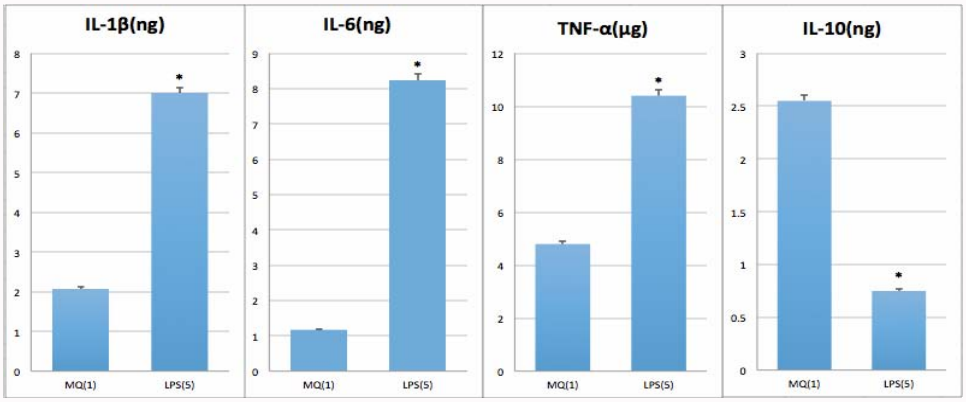

Figure 2: The effect of different concentrations of lunasin on the mean level of cytokines. Each value represents the mean of three replicates and standard deviation in error bars. *Statistically different compared to MQ(1) (P<0.001). MQ(1): MQ and LPS(2): MQ+LPS.

in PP disease.

The tripeptide Arg-Gly-Asp (RGD) is a unique sequence of amino acids that can inhibit the activation of the NF-κB pathway and suppress the release of inflammatory cytokines from activated human macrophages [8,9]. Lunasin is a 43-amino acid peptide containing an RGD sequence isolated from soybean [10]. It has been shown that peptides containing lunasin inhibit the production of inflammatory mediators [11]. De Mejia et al. [11] reported that lunasin significantly inhibits inflammation through suppressing the NF-κB pathway in murine MQ cell line and reducing the transcription of genes with pro-inflammatory properties. However, it was not shown if lunasin is capable of influencing the transcription of genes with anti-inflammatory traits.

Ibuprofen is one of the most commonly used Non-Steroidal Anti-Inflammatory Drugs (NSAID) for managing endodontic pain [12]. However, various cardiovascular and gastrointestinal side effects have been reported with ibuprofen [13]. To the best of our knowledge, no study has investigated and compared the anti-inflammatory effect of lunasin on human monocytic cell line with ibuprofen in the field of endodontics. Considering that major pro- and anti-inflammatory cytokines are regulated through the NF-κB pathway, altering this pathway might affect the pathogenesis of PP disease [14]. The present study aimed to assess and compare the anti-inflammatory effect of lunasin with ibuprofen on LPS-activated MQ.

Materials and Methods

In the present *in-vitro* study, THP-1 monocytic cell line was originally obtained from ATCC and regularly maintained in the Weinberg lab (Case Western Reserve University). Cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) containing 10% heat-inactivated FCS, 10 mM HEPES, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin; they were maintained at 37°C under 5% CO_2. Cells were split when the density reached around 1×10^6 cells/mL. All reagents and medium were purchased from Gibco BRL (Gaithersburg, MD). PMA and LPS were purchased from Sigma (St. Louis, MO).

Lunasin extract and a negative control powder (does not contain any bioactive lunasin) were obtained from Reliv (Chesterfield, MO) in powder form. According to the manufacturer's instructions, suspending 400 mg of the lunasin in 20 mL of complete culture medium will result in 200 μM bioactive lunasin. The resultant solution was centrifuged for 5 min at 10k RPM; this was repeated 3 times. The supernatants were transferred into fresh tubes each time. The final stock solution was filter-sterilized with 0.2 μM filter and contained 100 μM lunasin. Accordingly, lunasin in 10 μM, 50 μM and 100 μM were diluted from 200 μM with complete medium.

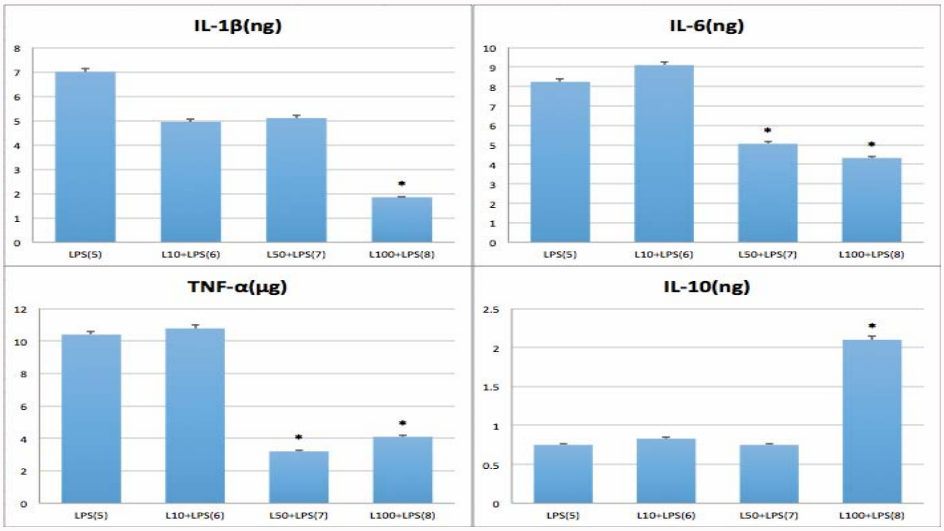

Figure 3: The effect of different concentrations of lunasin on the mean level of cytokines. Each value represents the mean of three replicates and standard deviation in error bars. *Statistically different compared to LPS(5)(P<0.001). LPS(5): MQ + LPS, L10+LPS(6): MQ+LPS+10 μM lunasin, L50+LPS(7): MQ+LPS+50 μM lunasin, and L100+LPS(8): MQ+LPS+100 μM lunasin.

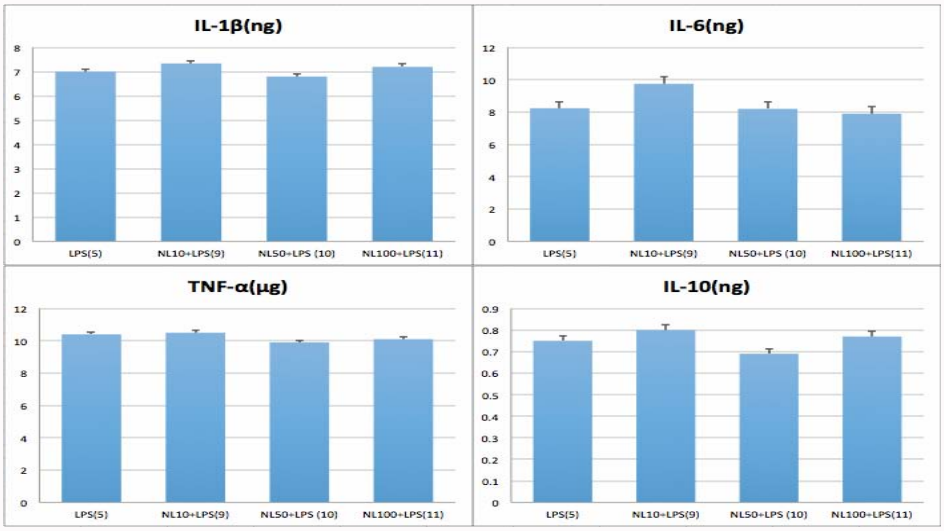

Figure 4: The effect of different concentrations of negative lunasin on the mean level of cytokines. Each value represents the mean of three replicates and standard deviation in error bars. LPS(5): MQ + LPS, NL10+LPS(9): MQ+LPS+10μM negative lunasin, NL50+LPS(10): MQ+LPS+50μM negative lunasin, and NL100+LPS(11): MQ+LPS+100μM negative lunasin.

Escherichia coli LPS was used at a concentration of 10 ng/mL. Pure ibuprofen powder was purchased from Sigma Chemical Company (St. Louis, MO) and prepared at a final concentration of 40 μg/mL.

THP-1 cells were placed on two 48-well plates at a density of 7.5 × 10^6 cells/well. Cells in all wells were then differentiated into MQ by using 10 ng/mL Phorbol-12-Myristate-13-Acetate (PMA) for 48 hours. The differentiation of THP-1 cells was confirmed under light microscope. Then the supernatants were removed and replaced with serum-free fresh media (1 mL/well) in all wells.

Wells, containing MQ, were divided into 13 groups. They were treated with different reagents based on the following protocol:

1. MQ (Control group)
2. MQ + 10 μM lunasin
3. MQ + 50 μM lunasin
4. MQ + 100 μM lunasin
5. MQ + LPS (LPS-activated MQ)
6. MQ + LPS + 10 μM lunasin
7. MQ + LPS + 50 μM lunasin
8. MQ + LPS + 100 μM lunasin
9. MQ + LPS + 10 μM negative control powder
10. MQ + LPS + 50 μM negative control powder
11. MQ + LPS + 100 μM negative control powder
12. MQ + Ibuprofen
13. MQ + LPS + Ibuprofen

Three replicates were done for each group. After 24 hours treatment, the cultured supernatants were collected, centrifuged and

stored at -70°C. Levels of cytokines (IL-1β, IL-6, TNF-α and IL-10) were then determined using a Human Inflammatory Cytokine CBA Kit (BD Biosciences, CA, USA) and commercial flowcytomix software (FF Software, BMS–FFS; Bender Med Systems, Vienna, Austria) according to the manufacturer's instructions. Kruskal–Wallis and Mann-Whitney U-tests for nonparametric comparisons were used to determine statistical differences between the groups (α=0.05). Data analysis was performed using SPSS (ver. 21).

Results

IL-8 and IL-12 were excluded because they were below the level of detection. The mean levels of cytokines in different groups are shown in Figures 1-5. As shown in Figure 1, lunasin treatment of MQ did not alter the level of cytokines tested, which suggests that lunasin has no effect on the base level of inflammatory cytokines (P>0.05).

LPS treatment of MQ significantly increased the levels of pro-inflammatory cytokines of IL-1β, TNF-α, and IL-6 (P<0.001) and decreased the level of IL-10 anti-inflammatory cytokine (P=0.025) (Figure 2).

Figure 3 showed the effect of lunasin treatment on the production of pro- and anti inflammatory cytokines. Production of IL-1β, IL-6 and TNF-α, were significantly reduced by 100 μM lunasin treatment in LPS activated MQ compared to LPS activated MQ without lunasin (P<0.001). 50 μM lunasin significantly reduced levels of IL-6 and TNF-α, however, not IL-1β. 10 μM lunasin did not significantly reduce the production of pro-inflammatory cytokines (P>0.05). Furthermore, 100 μM lunasin treatments was the only group to show a significant increase the level of IL-10 in LPS activated MQ (P<0.001).

Negative Lunasin showed no significant effect on the level of inflammatory cytokines in the LPS activated MQ groups (P>0.05) (Figure 4).

Figure 5 shows the effect of Ibuprofen (Ibu) on the production of inflammatory cytokines. Ibu treatment of LPS activated MQ did not alter the production of inflammatory cytokines compared to LPS activated MQ group (P>0.05).

Discussion

Inflammatory reactions, which are initiated following the host's immune response to infection of the root canal system, are necessary for the development of Pulp-Periapical (PP) disease [15]. These inflammatory responses are characterized by marked elevation of inflammatory cells including different subsets of T-lymphocytes and macrophages [16]. These cells are associated with pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α, which are involved in the initiation and progression of PP disease [4]. Conversely, IL-10 exhibits strong anti-inflammatory properties and is produced by a different subset of T-cells including Th1, Th2, and T-reg cells [17].

In the present study, we assessed the anti-inflammatory activity of lunasin was assessed on the human monocytic cell line (THP-1). Primary tissue MQ cannot be easily expanded in *ex vivo* models. Therefore, THP-1 cells are used to model macrophage (MQ) function since primary tissue macrophages cannot be readily expanded in *ex vivo* models [18]. Monocytic cell lines have obvious advantages in terms of ease of acquisition. Various THP-1 differentiation protocols such as PMA treatment have been suggested to simulate human macrophage behavior, including cell adhesion, morphology, and the expression of surface markers [18]. In this study, 10 ng/mL PMA was used for THP-1 cell differentiation. This resulted in differentiated macrophages that respond well to secondary weak stimuli such as LPS without being overwhelmed by the over expression of genes involved in the production of inflammatory cytokines [18].

We assessed the effect of lunasin on the inflammatory profile of MQ was assessed in 10 μM, 50 μM and 100 μM concentrations. These concentrations were used according to previous findings, which showed that after 24 hour incubation with 10 μM to 100 μM lunasin there was no change in cell viability. However, lunasin at higher concentrations can slightly inhibit the cell proliferation [19].

To evaluate the anti-inflammatory properties of lunasin, levels of IL-1β, IL-6, TNF-α, and IL-10 were assessed. These inflammatory cytokines were selected based on their prominent role in the pathogenesis [17]. Based on the results, 100 μM lunasin treatment significantly reduced the levels of all pro-inflammatory cytokines in LPS-activated MQ. 50 μM lunasin treatment significantly reduced the levels of IL-6 and TNF-α; however, no significant change was seen with IL-1β. On the contrary, it was demonstrated that 10 μM lunasin was not capable of reducing the levels of any of the pro-inflammatory cytokines following LPS treatment. These results are consistent with previous studies, which showed that lunasin exerts an anti-inflammatory effect in concentrations higher than 10 μM [19]. These findings might confirm the inhibitory effect of lunasin on the NF-κB pathway that is associated with suppressing the inflammatory reactions in pulpal and periapical regions.

In the present study, the anti-inflammatory effect of lunasin was compared to Ibuprofen (Ibu). Ibu is one of the most widely used Non-Steroidal Anti-Inflammatory Drugs (NSAID) for managing endodontic pain [12]. Activation of nociceptors surrounding the tooth following release of inflammatory mediators is the major cause of pain [20].

Ibuprofen is effective in controlling endodontic pain through blocking the Cyclooxygenase-1 (COX-1) and -2 (COX-2) enzymes [21]. However, little is known regarding the effect of oral administration of this drug on the synthesis of inflammatory cytokines. Tugendreich et al. [22] showed that the administration of NSAIDs in rats resulted in the stimulation of gene expression similar to that observed when rats were exposed to LPS. Also it has been shown that ibuprofen administration fails to affect the inflammatory cytokines levels in exudates of chronic periapical lesions [23]. We reported that 40 μg Ibu treatment of LPS activated MQ did not result in reduction of pro inflammatory cytokines. The 40 μg concentration was used based on the bioavailability of 600 mg Ibu at the target site [24]. Our results are in agreement with other studies that reported Ibu shows no effect on cytokine synthesis induced by LPS like IL-1β, IL-6 and TNF-α [25]. Ibu is administered as mixtures of S- and R-enantiomer. Studies have demonstrated that S-enantiomer inhibits the cyclooxygenases and R-enantiomer inhibits the activation of transcription factor NF-κB [26]. There is a complex interplay between NF-κB and COX/prostaglandin pathways in inflammatory reactions [27]. The results of our study showed that Ibu might not effectively inhibit the NF-κB pathway. Considering the importance of inhibiting pro-inflammatory cytokines, it could be speculated that Ibu mixture can be adjusted based on equal inhibitors of NF-κB and COX/prostaglandin pathways for adequate pain and inflammation control.

We also showed the efficacy of lunasin in increasing the production

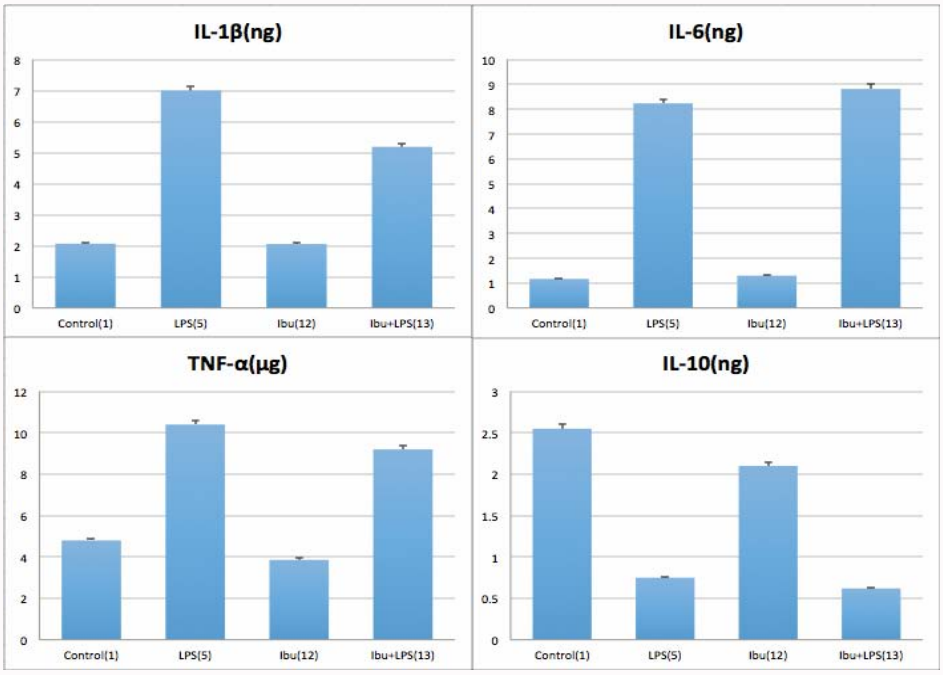

Figure 5: The effect of ibuprofen on the mean levels of cytokines. Each value represents the mean of three replicates and standard deviation in error bars. Control (1): MQ, LPS(5): MQ+LPS, Ibu(12): MQ+Ibuprofen, and Ibu+LPS(13): MQ+LPS+Ibuprofen.

of anti inflammatory cytokine (IL-10). LPS treatment of MQ resulted in marked suppression of IL-10 production as it was confirmed in our study [28]. IL-10 is a key anti-inflammatory cytokine and the main inhibitor of cytokine synthesis and macrophage activity. It also inhibits the production of the pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, in various cell types [29]. We reported that treatment of LPS activated MQ with 100 µM lunasin could significantly elevate the level of IL-10. However, Ibu treatment of LPS activated MQ showed no effect on the levels of IL-10. This finding might indicate the superiority of lunasin over Ibu regarding the suppression of inflammatory responses.

Based on the results of our study, lunasin showed a significant anti-inflammatory effect by suppressing the production of pro-inflammatory cytokines and increasing anti-inflammatory cytokines. It was also confirmed that ibuprofen could not reduce the pro-inflammatory cytokines involved in PP disease. Nevertheless, ibuprofen is still the main medication used for controlling pain and inflammation. Various side effects of ibuprofen including cardiovascular, gastrointestinal bleeding and ulceration might necessitate the need for an alternate medicine with a local mode of delivery [13]. Considering the significant capability of lunasin in inhibiting the production of cytokines involved in inflammatory reactions and bone resorption (IL-1β, IL-6 and TNF-α) compared to Ibu, application of lunasin in endodontics to control inflammation and bone resorption can be evaluated in future studies.

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