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EFFECT OF BETA- SITOSTEROL ON EXPRESSION OF TGF- β, IGF-1 AND TNF-α mRNA IN LPS INDUCED FIBROBLAST CELL LINE- A MOLECULAR STUDY

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Abstract

Introduction

The lipopolysaccharides(LPS) found in the gram negative bacteria is one of the important factors for the inflammation of pulp and periapical tissues. LPS is reported to increase inflammation by increasing TNF-α and downregulates growth factors like TGF-β and IGF-1. Phytosterols have a prominent anti-inflammatory function and wound healing capacity. The Aim of the study was to determine the effect of Beta Sitosterol on the expression of TGF-β, IGF-1, and TNF-α mRNA in LPS treated fibroblast cell lines.

Materials and Method

The 3T3 fibroblast cell line was cultured in Dulbecco's Eagle modified medium (DMEM) in 5 culture plates. Cells in 4 culture plates were incubated for 1 hour with LPS (100 ng/mL) following this the cells in 3 culture plates were treated with 10, 20 and 50μM Beta Sitosterol respectively for 24 hours and the cells were harvested. The gene specific primers were used and real time RT-PCR analysis was performed for quantification of TGF-β, IGF-1, and TNF-α mRNA.

Results

LPS induced fibroblast cultures showed an increase in the expression of TNF-α mRNA and reduced expression of TGF-β and IGF-1 mRNA. Treatment with Beta Sitosterol reduced the expression of TNF-α mRNA and increased the expression of TGF-β and IGF-1 mRNA in LPS induced fibroblast cell culture.

Conclusion
Beta sitosterol increased the expression of growth factors and downregulated the inflammatory factors thus potentiating its use in inflammatory conditions. It can be effectively used as intracanal medicament or pulp capping agent. Nevertheless, there is a need for controlled trials to establish its effectiveness clinically.

Keywords: - Transforming growth factor-beta (TGF-β), Tumour Necrosis Factor–α (TNF-α), lipopolysaccharide (LPS), Insulin Growth Factor (IGF-1), Beta Sitosterol, Fibroblast, health, medical

Introduction

Fibroblasts are differentiated from mesenchymal cells. These fibroblasts produce extracellular matrix proteins that are usually in a state of dormancy in non pathological conditions. Highly specialised myofibroblasts confer a major role in wound healing. However, these cells are pathogenic in case of chronic inflammatory conditions like cancer and fibrosis. In case of injury, myofibroblasts can be activated through the TGF-β signalling pathway to procedure certain ECM proteins. These proteins are critical for health, resolution of inflammation and wound healing(1).

Root canals of necrotic teeth commonly contain gram negative bacteria, the cell wall of these bacteria contain endotoxins which are lipopolysaccharide (LPS) complexes. LPS are potent inflammatory agents (2,3),(4). Tumour Necrosis Factor–α (TNF-α) is an inflammatory cytokine. It causes vasodilation and recruitment of lymphocytes(5). LPS also is known to have negative effects on growth factors. Transforming growth factor-beta (TGF-β) is a highly pleiotropic cytokine which causes cell proliferation, chemotaxis, cell differentiation and apoptosis (2).

Plant extracts are used to treat various ailments around the world and are rich in nutrition. Phytosterols are steroid like organic molecules obtained from plants with numerous benefits. Beta-sitosterol (BS) is a plant sterol similar to cholesterol in its chemical structure(6,7). It is a natural micronutrient found in different parts such as leaves, rhizomes, fruit and tissue cultures of higher plants(8,9),(10,11). This β-sitosterol, an active phytosterol, is rich in natural products and foods including fruits, vegetables, berries, nuts, vegetable oils and stem bark of Solanum surattense. Traditionally BS is used for the treatment of respiratory diseases, gonorrhoea, rheumatism, fever, asthma and diabetes (17, 18). β-sitosterol also has cholesterol-lowering, anti-inflammatory, non-alcoholic fatty liver disease prevention, anticancer and antioxidant properties(19,20,21,22,23). Hence the present study was done to determine the effect of Beta Sitosterol on the expression of TGF-β, IGF-1, and TNF-α mRNA in LPS induced fibroblast cell lines.

Materials and Methods

Chemical and Reagents

All the chemicals used in this study were extra pure and analytical grade. Swiss Albino mouse fibroblast cell line (3T3) was obtained from NCCS Pune, India. The culture of the 3T3 cells were carried out in 5% CO2 and 95% humidified air at 37∘C. The Dulbecco's Eagle modified medium (DMEM) along with calf serum(10%), HEPES buffer (25 mM) and 1% penicillin(100 U/mL)-streptomycin (100 μg/mL) was used to maintain the cells. After 2 days the cells were differentiated for 48 hours in Dulbecco's Eagle modified medium constituting 3-isobutyl-1- methylxanthine(0.5 mM), 10% Fetal bovine serum (FBS), insulin (μg/mL) and dexamethasone(0.25 μM). Following this the incubation of cells was done in 10% FBS/DMEM for 72 h. Once in 2 days, the FBS/DMEM was replaced with fresh medium. The cells were cultured in 5 culture plates.

After 8 days of differentiation, cells in 4 culture plates were incubated for 1 hour with LPS (100 ng/mL) following this the cells in 3 culture plates were treated with 10, 20 and 50μM Beta Sitosterol respectively for 24 hours. The cells were harvested and stored at −80∘ C.

Real-Time-PCR analysis

The real time RT-PCR analysis was performed using gene specific primers. cDNA was synthesised from total RNA isolated from 3T3-L1 cells using the first strand synthesis kit (Qiagen, Germany). The protocol to be used for real-time PCR is as follows: The PCR mixture consists of 5 µl of cDNA sample (1:10 dilution), each primer (300 nM) and master mix for SYBR green I (10 µl) (Eurogentec, Belgium), making a total volume of 20 µl. Amplification was performed in the MX3000P Multiplex quantitative PCR system with initial denaturation for 10 min at 95°C, following this denaturation was carried out at 95°C for 15 seconds followed by annealing at 60°C for 1 min and finally extension at 72ºC for 30 seconds. Amplification of the internal control (β-actin) was performed simultaneously in separate tubes. All reactions were performed in triplicate along with no template control (NTC) and results were analysed using MX3000P Multiplex quantitative PCR system software (Stratagene). To ensure the amplification of every product dissociation curve analysis was performed after each reaction. The relative comparative CT method was used to calculate the amount of mRNAs(Figure-1).

Figure 1: Instruments and materials used for study

a- Culture of 3T3 cells in DMEM, b- Beta Sitosterol, c- Fibroblast cells under microscope, d- vortexing the extracted RNA before PCR

Results

The negative control group neither had LPS nor Beta Sitosterol (BS). The mRNA expression of TGF-β and Insulin Growth Factor (IGF-1) significantly decreased in LPS induced 3T3 cell line ($p<0.05$) compared to the control. With Beta Sitosterol treatment, TGF-β mRNA increased at 25 and 50 µM ($P=0.0047$) of BS compared to LPS induced cell line while IGF-1mRNA showed an increase at 25 µM of beta sitosterol ($P=0.0354$). The highest expression of TGF-β mRNA was seen at 25 µM of BS and the highest expression IGF-1 mRNA was observed at 50 µM of BS. There was no significant difference in the mRNA level of TGF-β and IGF-1 at 10, 25 and 50 µM concentration of beta sitosterol. The level of TNF-α mRNA significantly increased in LPS induced groups ($p<0.05$) compared to the control. There was a significant decrease in TNF-α mRNA levels at 10, 25 and 50 µM of beta sitosterol concentration ($P=0.0003$). There was a significant difference in level TNF-α mRNA between 10 µM and 25 µM and between 10 µM and 50 µM and no significant difference

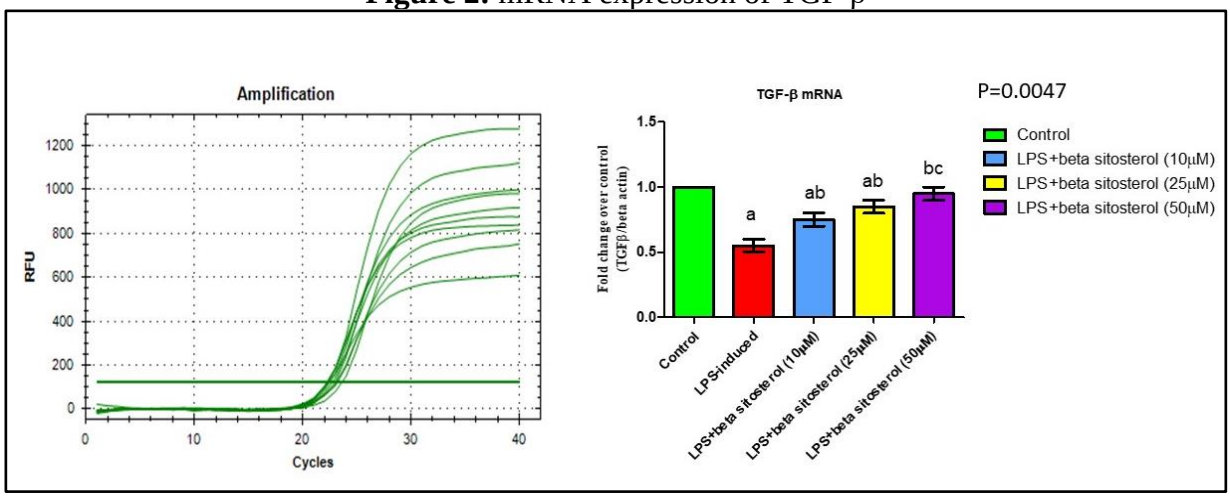
between 25 and 50µM. The maximum reduction in TNF-α mRNA level was at 50 µM of BS treatment (Table 1, Figure 2-4).

Table1: Effect of Beta Sitosterol on TGF-β, TNF-α and IGF-1 mRNA in the fibroblast cell line

GENE	Negative Control	LPS induced	LPS+10 BS	LPS+25 BS	LPS+50 BS	P value
TGF-β	1.00±0.00	0.55± 0.07^{a}	0.75±0.07^{ab}	0.85± 0.07^{ab}	0.95±0.07^{bc}	0.0047
IGF-1	1.00±0.00	0.65±0.07^{a}	0.85±0.07^{ab}	1.00±0.14^{ab}	0.95±0.07^{b}	0.0354
TNF-α	1.00± 0.00	1.65±0.07^{a}	1.35±0.07^{ab}	1.05± 0.07^{bc}	0.85±0.07^{bcd}	0.0003

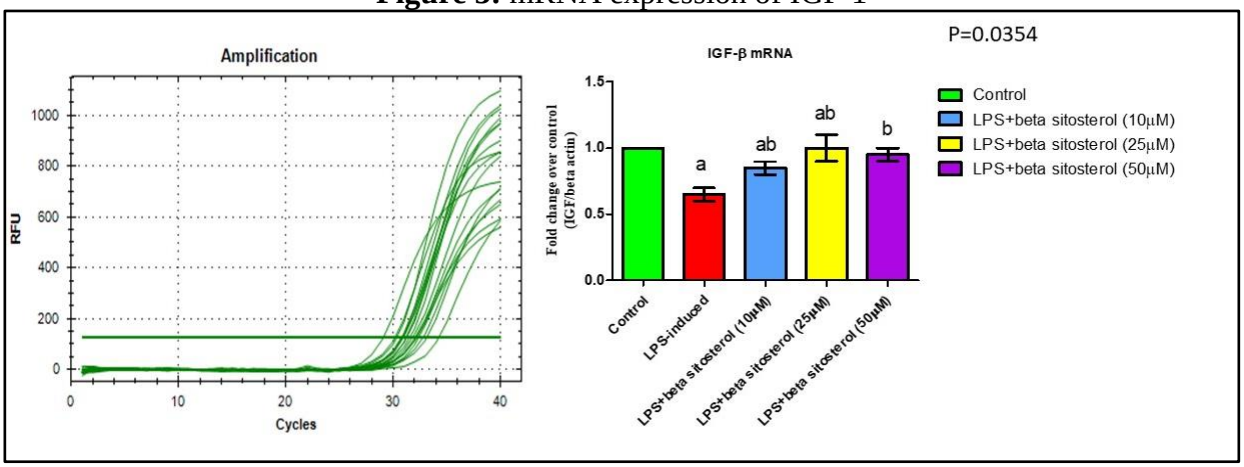
The Mean±SD (Fold change) of the gene expression effect of BS on TGF-β, TNF-α and IGF-1 mRNA in the fibroblast cell line. The mRNA expressions were assessed by Real Time-PCR. Significance at P <0.05, 'a' Significantly different from control,'b' Significantly different from LPS-induced, 'c'- compared with 10µM treated cells, 'd'- compared with 25µM treated cells. BS- Beta Sitosterol

Figure 2: mRNA expression of TGF-β



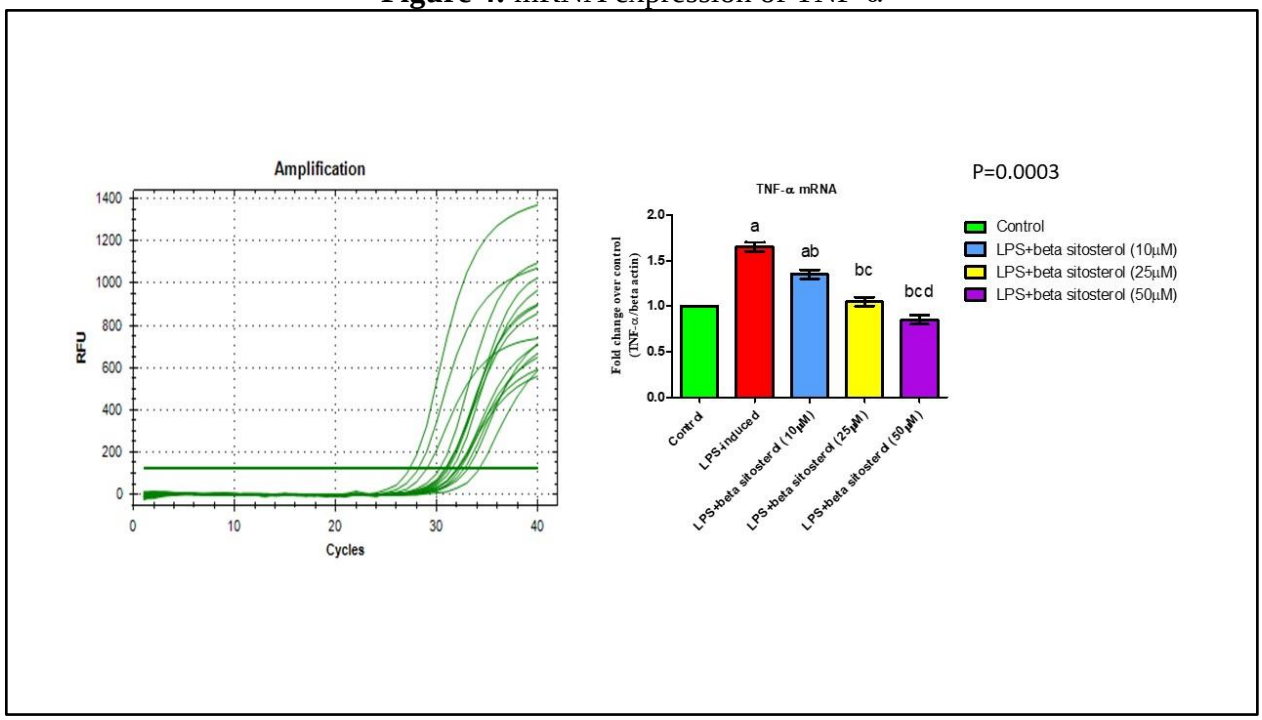
Amplification plot and mRNA expression of TGF-β in the fibroblast cell line. The mRNA expressions were assessed by Real Time-PCR. Significance at P <0.05, 'a' Significantly different from control,'b' Significantly different from LPS-induced, 'c'-compared with 10µM treated cells, 'd'- compared with 25µM treated cells. LPS- Lipopolysaccharide, BS- Beta Sitosterol

Figure 3: mRNA expression of IGF-1



Amplification plot and mRNA expression of IGF-1 in the fibroblast cell line. The mRNA expressions were assessed by Real Time-PCR. Significance at P <0.05, ‘a’ Significantly different from control,‘b’ Significantly different from LPS-induced, ‘c’-compared with 10µM treated cells, ‘d’- compared with 25µM treated cells. LPS- Lipopolysaccharide, BS- Beta Sitosterol

Figure 4: mRNA expression of TNF-α



Amplification plot and mRNA expression of TGF-β in the fibroblast cell line. The mRNA expressions were assessed by Real Time-PCR. Significance at P <0.05, ‘a’ Significantly different from control,‘b’ Significantly different from LPS-induced, ‘c’-compared with 10µM treated cells, ‘d’- compared with 25µM treated cells. LPS- Lipopolysaccharide, BS- Beta Sitosterol

Discussion

In case of pulpal necrosis and periapical lesions, gram negative anaerobic bacteria predominate the root canals (12–14). This is due to the decrease in the oxygen tension in the root canals. The change in environment causes the shift from aerobic to facultative or obligate anaerobic bacteria (12,15)(16,17)(12,15). Endotoxins (LPS) are an important component of the outer membrane of these bacteria(15). Humans are very susceptible to the effect of endotoxins(18)(19,20)(18). LPS contains O antigen, core sugars and lipid A that can elicit host immune response(21,22).The LPS from the bacteria act on neutrophils, macrophages and fibroblasts and cause the release of inflammatory cytokines like interleukins, interferons, prostaglandins and tumour necrosis factor(23), (24–27). High concentrations of TNF-α are found in symptomatic and asymptomatic periapical lesions(28,29). They are also well known to induce osteoclastic activity in the bone(28)(30–34)(28).

The Transforming Growth Factor is a significant cytokine and has an eminent role in cell proliferation, differentiation and migration(35)(36,37)(35). Joyce et al (1990) in his study reported that TGF-β increased the human osteoblast proliferation (28,29,38). Apart from this it was also found that TGF-β inhibits osteoclast formation and resorption of bone(39). Several studies have also reported the key role of IGF-1 in the osteoblast differentiation and bone formation(40–42)(43)(40–42). Pro-inflammatory mediators such as IL-1β and LPS suppress the anti-inflammatory action of TGF-β(44)(45,46). Hence suppression of inflammatory osteoclastic activity of TNF-α is important to heal

the periapical lesions along with the increase in osteoblastic activity of TGF-β and IGF-1. Apart from this beta is also present in the soluble and insoluble components of dentin matrix and play an important role in recruitment of the progenitor cells from the pulp to the site of injury in the regenerative dentinogenesis. TGF beta is also mitogenic for the cells present in the subodontoblastic layer.

In the present study, after the addition of LPS there was a significant reduction in TGF-β and IGF-1 and an increase in TNF-α. This is in agreement with the study by Mitchell et al (2014) , where rat microglia treated with LPS decreased in the expression of the TGF-β receptors, TbR1 and TbR2 along with the reduction of Smad2 responsible for the signaling of TGF-β(47)(48–51)(47). Similarly previous animal studies showed that there is a decrease in IGF-1 with the introduction of LPS in rodents and sheeps (52)(53–56)(52)(57,58).

Several studies have reported the anti- inflammatory properties of the plant derived sterols(59–61)(62,63)(59–61). Phytosterols are also known to reduce the expression of inflammatory mediators(64,65). In this study Beta Sitosterol, a phytosterol, was used as a treatment for LPS induced cells(42-52(66–71)). It was found that BS significantly reduced the expression of TNF-α. This is in agreement with the previous studies where BS showed dose dependent reduction in TNF-α and interleukin(72,73)(74,75)(72,73). Choi et al (2012) reported the decrease in TNF-α in LPS induced d RAW 264.7 murine macrophages with BS treatment(76). It was found that BS significantly increased the expression of TGF-β and IGF-1. This is consistent with the previous study by Kassen et al where BS induced the expression of TGF-β between 1.26 to 1.86-fold compared to cholesterol in Human Prostate Stromal Cells (77)(78–81)(77).

Beta Sitosterol exhibited significant anti-inflammatory effects and its potential osteoblastic effect can be utilised in promoting the healing of periapical lesions(57-61). The limitation of this study is that it is an in vitro study using mouse fibroblast cell lines. Further animal trials and human trials can help us determine the clinical efficacy of BS as an intracanal medicament.

Conclusion

In LPS induced fibroblast cell lines there was significant reduction in Beta sitosterol osteo-inductive and anti-inflammatory properties in LPS treated fibroblast cell lines. The effect of BS is dose dependent. Further animal and human trials are required to determine its clinical efficacy in dentistry. Beta sitosterol can be used as an intracanal medicament and as a vital pulp therapy agent owing to its anti-inflammatory and osteo-inductive property, by upregulating TGF-β and IGF- 1 mRNA and by down regulating TNF-α mRNA in fibroblast cells.

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