Original Article
บทวิทยาการ

Effect of lipopolysaccharide from *Porphyromonas gingivalis* on expression of heparanase in human gingival epithelial cell line, Ca9-22

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Abstract

Objective The progression of chronic periodontitis depends partly on a direct invasion ability of *Porphyromonas gingivalis (P. gingivalis)* against host cell supporting tissue and/or degradation of host extracellular matrix by the inflammatory response elicited by the bacteria. The aim of this study was to investigate the stimulation of host cells by lipopolysaccharides from *P. gingivalis* (Pg-LPS) using a human gingival epithelial cell line, Ca9-22, as a model system for the latter mechanism.

Materials and methods In this study, we treated Ca9-22 cells with various concentrations (0, 0.1, 1 and 10 μg/mL) of Pg-LPS for 24h and extracted the mRNA. We investigated mRNA expression of heparanase, a heparan sulfate degrading enzyme, as a marker of host cell inflammatory response, *Toll-like receptor (TLR) 2* and *TLR4* expression at the mRNA level using reverse transcriptase polymerase chain reaction (RT-PCR) analysis.

Results Ca9-22 cells grew as well as control even in the highest concentration of Pg-LPS. The detection of heparanase, *TLR2* and *TLR4* mRNA transcripts showed no statistically significant difference at all concentrations of Pg-LPS.

Conclusion There was no difference in expression levels of heparanase, *TLR2* and *TLR4* transcripts at all concentrations of Pg-LPS tested. Protein level of heparanase should be investigated in further experiment.

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Key words: *heparanase; lipopolysacharide; Porphyromonas gingivalis; TLR2; TLR4*

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Introduction

Periodontitis is a disorder involving a relationship between primarily Gram-negative anaerobic bacteria and host cell response. If bacteria can invade through host immune response, this will lead to the destruction of connective tissue in periodontium, i.e., gingiva, periodontal ligament, cementum and alveolar bone[1]. The interactions between periodontal pathogens and host cells have become the aspect of intensive investigations[2].

P. gingivalis is a Gram-negative, black-pigmented anaerobic bacterium that has been considered as a major pathogen in the induction and progression of periodontal diseases, especially chronic periodontitis[3,4]. *P. gingivalis* has also been identified as a risk factor for coronary heart disease[5] and pulmonary infections[6]. Many studies suggested that *P. gingivalis* produced an array of potential virulence factors[4] such as extracellular proteases (e.g. gingipain which is a cysteine proteinases), adhesins (e.g., fimbriae and haemagglutinins) and lipopolysaccharides (LPS). The outer membrane of Gram-negative bacteria is asymmetric in molecular composition and the outer leaflet of which contains the LPS. The LPS is a large (> 10 kDa) amphipathic molecule, with the hydrophilic polysaccharide exposed to the environment on the exterior surface of the outer membrane, and the core region, buried within the outer leaflet which connects the *O*-antigen to the hydrophobic end of the molecule or lipid A[3]. All virulence factors

from bacteria seem to play a role in invasion of host tissue by damaging against various host cells, including macrophages, neutrophils, fibroblasts, dendritic cells, endothelial cells and, oral epithelial and non-oral epithelial cells[4]. Toll-like receptors (TLRs), the transmembrane receptors, are important signal transducers that mediate inflammatory reactions induced by microbes through pattern recognition of virulent molecules such as LPS and lipoproteins[7]. TLR2 has now been established as an essential component in the recognition of asymmetric LPS from *P. gingivalis* and TLR4 for LPS from other Gram negative bacteria[8].

The major classes of extracellular matrix (ECM) molecules in connective tissues and bone can be classified as fibrous (e.g., collagen and elastin) and non-collagenous molecules (e.g., laminin and proteoglycans). Heparan sulfate proteoglycans (HSPGs) are subclasses of proteoglycans which are composed of heparan sulfate glycosaminoglycan (repeating disaccharide chains of D-glucuronic acid and N-acetyl-D-glucosamine) linked to a protein core[9]. They are synthesized by virtually all cells in invertebrates and vertebrates. They play important roles in cell-cell and cell-matrix interactions, cell proliferation and migration, and are involved in viral and bacterial infection[9]. Heparanase is a β-endoglucuronidase that selectively degrades HSPGs in mammalian tissues. Human heparanase cDNA contains an open reading frame that encodes a latent form of 543 amino acids with

molecular weight of approximately 65 kDa[10]. A number of studies suggested that heparanase was post-translationally processed to be an active form of heterodimer consisting of 50 kDa peptide[11-13]. From many lines of evidence, heparanase is thought to play crucial roles in biological processes especially in tumor cell migration, invasion and involve in tumor metastasis[10,14,15]. Heparanase is also involved in inflammation and tissue destruction[16]. Heparanase activity was remarkably elevated in synovial fluid and synovial tissue from rheumatoid arthritis patients, suggesting an inflammation role of heparanase in promoting joint destruction[17].

The destruction of periodontal tissue in chronic periodontitis might be caused by two pathways, e.g., firstly, by the direct insult by *P. gingivalis* and its secreted molecules, and secondly, by the host inflammatory response elicited by the first mechanism. For the first pathway, *P. gingivalis* can attach to, invade and replicate in epithelial cells[2]. Then, the internalized *P. gingivalis* can inactivate immune response and activate host processes leading to tissue destruction[2]. They also accelerate proliferation of epithelial cells through cell cycle which effects to integrity and gingival turn over *in vivo*[18], results in expansion of periodontal pocket depth[19].

The second pathway, host inflammatory response can mediate the tissue destruction through virulence factor of *P. gingivalis* such as gingipain and LPS. A recent study showed that *P. gingivalis* could promote the host inflammatory response which in turn to tissue destruction in periodontitis[20]. Gingipain, a cysteine proteinase secreted from *P. gingivalis*, can stimulate inflammation and tissue destruction mediating the shedding of epithelial cell membrane-anchored glycoprotein, EMMPRIN, which induced pro-inflammatory cytokine and matrix metalloproteinase (MMP) secretion by gingival fibroblast[20]. Culture supernatant from *P. gingivalis* increased the collagen degradation

in human gingival fibroblast by increasing MMP activity[21]. *P. gingivalis* could produce and secrete heparitinase (a bacterial eliminase, which should not be confused with the above described heparanase) to degrade HSPGs in human gingival tissue[22], suggesting degradation of HSPGs might be involved in the initial invasion of gingival epithelium, permitting the ingress of other microbial virulence factors. These raise an interesting question whether product from bacteria can stimulate host response through heparanase that aids in HSPGs degradation forming a vicious cycle. Unfortunately, there are few, if any, reports about a biological role of heparanase, especially in terms of the relationship between products from pathogenic microorganisms and heparanase expression of host cells in periodontal disease. The aim of this study was to investigate a relationship between lipopolysaccharide from *P. gingivalis* (Pg-LPS) and heparanase production at mRNA level in human gingival epithelial cell line, Ca9-22 as a model of gingival cells. Outcome of the study here would shed light on interactions between periodontopathogens and host cells, and would help improving diagnostic and therapeutic methods for periodontitis.

Materials and methods

Cell culture

The human gingival epithelial cell line, Ca9-22 was obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). The Ca9-22 cells were cultured in 10-cm culture dishes and grown in minimum essential medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 0.6 mg/mL glutamine and 1% penicillin/streptomycin at 37°C in humidified atmosphere of 95% air, 5% CO_2. The medium was replaced every other day during cells growth. After reaching confluence, cells were subcultured at a 1:4 ratio.

Morphology of Ca9-22 cells upon expose to lipopolysaccharide from *P. gingivalis*[23]

Ca9-22 cells were seeded into 24-well plates at a density of 6 x 10^5 cells/well and allowed to attach for 12 h. Cells were cultured overnight (12 h) in serum-free medium before the treatment. Then, cells were treated with various concentrations of Pg-LPS (InvivoGen, USA) at 0, 0.1, 1, 10 and 50 μg/mL in serum-free medium. After 24 h incubation, cell were observed under light microscope.

Stimulation of Ca9-22 cells with lipopolysaccharide from *P. gingivalis*

Ca9-22 cells were seeded in 6-well plates at a density of 2 x 10^6 cells/well and allowed to attach for 6 h. Cells were treated with or without LPS from Pg-LPS; e.g., 0, 0.1, 1 and 10 μg/mL, in serum-free conditions. Cell cultures were done in triplicate for each Pg-LPS concentration. After 24 h, the treated cells were collected and extracted for total RNA with RNeasy Mini Kit (QIAGEN, Germany). The approximate amount of total RNA was calculated by measuring absorbance at wavelength 260 and 280 nm. Extracted RNA was kept at -20°C until use.

Reverse-transcription polymerase chain reaction (RT-PCR)

One μg or maximum amount (in cases when the amounts of total RNA were less than 1 μg) of each RNA sample was converted to cDNA by reverse transcription using reverse transcriptase and oligo(dT)s (Superscript™III First-Strand Synthesis System for RT-PCR, Invitrogen, USA) according to manufacturer's instructions. Subsequent to the reverse transcription, polymerase chain reaction (PCR) was performed for detection of heparanase, TLR2 and TLR4 cDNA. The primers specific to heparanase, TLR2[24], TLR4[24] and

glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were prepared. The oligonucleotide sequences of primers were: **Heparanase** forward 5′-AGA ACA GCA CCT ACT CAA GAA GC-3′, reverse 5′-ATT CCC ATT CGG GCT GAC AGG-3′; **TLR2** forward 5′-GCC AAA GTC TTG ATT GAT TGG-3′, reverse 5′-TTG AAG TTC TCC AGC TCC TG-3′; **TLR4** forward 5′-TGG ATA CGT TTC CTT ATA AG-3′, reverse 5′-GAA ATG GAG GCA CCC CTT C-3′; **GAPDH** forward 5′-TGC CTC CTG CAC CAC CAA CTG C-3′, reverse 5′-AAT GCC AGC CCC AGC GTC AAA G-3′.

The PCR reaction was performed using Taq polymerase (Takara Taq™, Takara, Japan) with a reaction volume of 25 μL. The reaction mixtures contained 10 μM each of sense and antisense primers and 1 μL of RT reaction products. The PCR conditions were as follows: for heparanase and GAPDH, denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and elongation at 72°C for 45 sec; for *TLR2* and *TLR4*, denaturation at 94°C for 30 sec, annealing at 58°C for 1 min, elongation at 72°C for 1 min. All amplifications were pre-heated at 94°C for 3 min and underwent the final extension at 72°C for 10 min on DNA thermal cycler (MyCycler, Bio-Rad, USA). The number of amplification cycles used were 27, 32, 35 and 17 for detecting heparanase, *TLR2, TLR4* and *GAPDH* cDNAs, respectively. The amplified DNA was then electrophoresed on a 2% agarose gel in TBE buffer and visualized by ethidium bromide staining. The PCR products of heparanase, *TLR2, TLR4* and *GAPDH* cDNA were 649 bp, 347 bp, 504 bp and 457 bp, respectively. Bands on agarose gel were photographed and the densities were analyzed using ImageJ software. The PCR reaction of each experiment was done in triplicate.
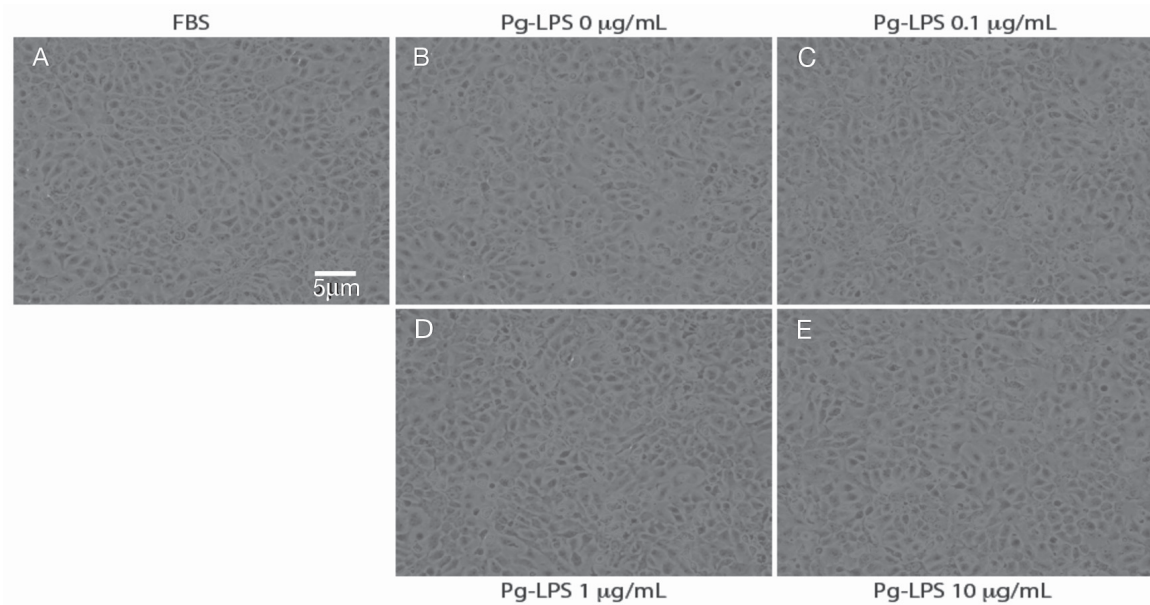
Fig. 1 Morphology of Ca9-22 cells treated with LPS from *P. gingivalis.* Ca9-22 cells were treated with or without LPS from *P. gingivalis* (Pg-LPS) at 0 (B), 0.1 (C), 1 (D) and 10 μg/mL (E) in serum-free condition or in 10% FBS condition (A), and incubated for 24 h at 37°C.

Results

Morphology of Ca9-22 cells upon exposure to lipopolysaccharide from *P. gingivalis*

The treated Ca9-22 cells appeared healthy and grew well even at the highest concentration (50 μg/mL) of Pg-LPS tested. The cell morphology was observed under light microscope. The cells were rather round shape. The shape and cell density in all Pg-LPS concentrations were similar to control (Fig 1).

Effect of lipopolysaccharide from *P. gingivalis* on heparanase, TLR2 and TLR4 expression

All primers were tested for specificity of interested genes (data not shown). The PCR conditions for heparanase, *TLR2, TLR4* and *GAPDH* were optimized to detect the differences of cDNA amount (data not shown). The results showed no significant differences in all concentrations of Pg-LPS to

stimulate heparanase, *TLR2* and *TLR4* expression (Fig 2).

Discussion

The periodontal diseases involve destruction of tooth supporting tissue that is caused by Gram-negative anaerobic bacteria such as *Aggregatibacter actinomy-cetemcomitans* (a major cause of acute periodontitis) and *P. gingivalis* (a major etiologic agent of chronic periodontitis). In worse cases, patients with severe periodontitis can loose their teeth. The severity of disease depends on the balance between bacteria and host response. LPS is one of virulence factors of Gram-negative bacteria which is recognized by a family of host transmembrane proteins, i.e., TLRs, and then eliciting immune response in host cells[25,26]. LPS is recognized by TLR4, which activates downstream pathways of host response to infection in many cell types[27]. A recent study showed that LPS derived from *Escherichia coli* could regulate gene expression of
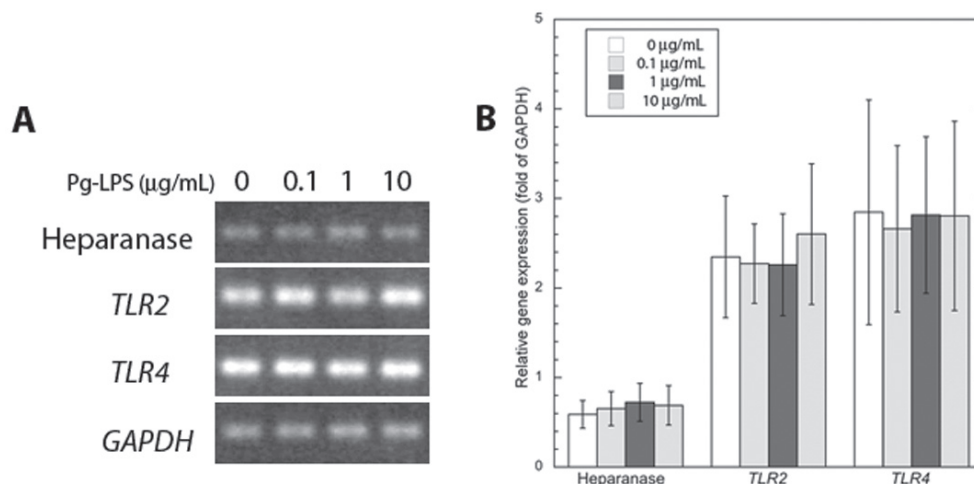
Fig. 2 Effect of Pg-LPS on expression of heparanase, *TLR2* and *TLR4* genes. Ca9-22 cells were treated with or without Pg-LPS (0, 0.1, 1 and 10 µg/mL). The mRNA level was detected with RT-PCR (A). The band density was measured and normalized against *GAPDH* expression (B).

proteoglycans called decorin via TLR4 in odontoblasts[27]. This suggested that proteoglycans are involved in bacteria-host interactions via TLR. Furthermore, the concentrations of chondroitin-4 sulfate and hyaluronan at periodontal site that contain *P. gingivalis* were higher than those in a site with less amount of *P. ginginvalis*[28]. These results demonstrated the relationship between *P. gingivalis* and elevated concentration of proteoglycans[28]. The *P. gingivalis* could induce many biological processes such as bone resorption and inhibited bone formation and fibroblast proliferation through its lipopolysaccharide[29]. The heparanase has been known for a physiologic degradation of heparan sulfate proteoglycans and also regulated their function[30].

The Ca9-22 cells showed normal growth rate and morphology when incubated with Pg-LPS up to the highest concentration (50 µg/mL). This result was different from a previous result[31]. Bartold PM and Milar SJ found that LPS from various pathogenic bacteria including *P. gingivalis* inhibited fibroblast proliferation at concentration range of 0.5-50 µg/mL[31]. However, many recent studies showed Pg-LPS at concentration ≤ 10 µg/mL did not inhibit cell proliferation nor toxic

to cells[32,33]. The concentration of Pg-LPS was reported to stimulate TLR2 and TLR4 at concentration ≤ 10 µg/mL in Chinese hamster ovarian (CHO) cell line[34]. In this study the highest concentration of Pg-LPS for gene expression experiment was 10 µg/mL. The production of inflammatory cytokines such as interleukins (ILs) was increased when treated with Pg-LPS in human macrophage cell line, THP-135 and gingival fibroblast[32,33] at highest dose 10 µg/mL. The dose 50 µg/mL of Pg-LPS might induce other inflammatory cytokines which would interfere an heparanase expression.

TLR2 is known to be a receptor for Pg-LPS. The stimulated-TLR2 is the indicator for effective conditions of Pg-LPS such as concentration, incubation time. Kocgozlu L *et al.* tested the incubation time of *TLR2* stimulation from Pg-LPS in endothelial cells and epithelial cells[36]. They incubated 1 µg/mL of Pg-LPS for 0, 0.5, 2 and 6 h to stimulate *TLR2* mRNA expression. The result showed that mRNA level of *TLR2* was significantly different from without Pg-LPS stimulation at 6 h incubation time. Furthermore, preliminary study in each incubation time (6, 12 and 24 h)

was done in triplicates. The results showed no difference in mRNA level of *TLR2*. Thus, the longest incubation time, 24 h, was chosen in this study.

The mRNA expressions of heparanase, *TLR2* and *TLR4* in this study were not different in Pg-LPS treated Ca9-22 cells. The TLR2 and TLR4 were well-known to be a receptor for LPS from bacteria especially from *P. gingivalis*[8]. The heparanase mRNA at all concentrations of Pg-LPS were not distinguishable from that of control. It implied that heparanase might not be induced by Pg-LPS. On the other hand, the effect of LPS from *Aggregatibacter actinomycetemcomitans* (Aa-LPS) inhibited protein and proteoglycan synthesis with dose dependent manner in adult human gingival fibroblast[31]. Ca9-22 cells might adjust themselves to a stressful environment, such as the exposure to Pg-LPS, by decreasing heparan sulfate proteoglycan synthesis instead of increasing the production of heparanase, like adult human gingival fibroblasts responded to Aa-LPS[31]. Heparanase is extensively studied in cancer progression[10,14], tumor metastasis[10,15], angiogenesis[15] and inflammatory diseases[37,38]. The effect of heparanase on inflammatory response in human gingival fibroblast is still unknown. Martin M *et al.*[35] showed that a stimulation of Pg-LPS in THP-1 cells, increased inflammatory cytokine production e.g. IL-1β, IL-6 and TNF-α. Moreover, Pg-LPS could elevate both mRNA and protein level of IL-6, IL-8 and TNF-α in human gingival fibroblasts[32]. These results suggest that the response to Pg-LPS on heparanase and cytokines in gingival fibroblasts might be in different inflammatory pathway.

The lack of responses to LPS in Ca9-22 cells in the present study may be due to two possiblities. First, the effect of Ca9-22 cell to Pg-LPS on mRNA level of heparanase might be independent. The protein level and heparanase enzyme activity should be investigated in the future. Second possibility, Ca9-22 cells are a cell line which some functions might be lost during

their malignant transformation. By using gingival cell system responsive to LPS or other proinflammatory bacterial products, it would become clear whether heparanase may be a biological marker or a therapeutic target for the treatment of periodontitis patients.

Conclusion

There was no significant change in expression levels of heparanase, *TLR2* and *TLR4* transcripts at all concentrations of Pg-LPS tested. Response of Ca9-22 cell to Pg-LPS might be independent to mRNA level. Protein level of heparanase in Ca9-22 cells and other gingival cell systems should be elucidated in a further experiment.

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ผลของไลโพโพลีแซคคาไรด์จากเชื้อพอร์ไฟโรโมแนสจินจิวัลลิสต่อการแสดงออกของเฮพพาแรนเนสในเซลล์ไลน์เยื่อบุผิวของเนื้อเยื่อเหงือกในมนุษย์ (ซีเอ 9-22)

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บทคัดย่อ

วัตถุประสงค์ การลุกลามของโรคปริทันต์อักเสบเรื้อรัง ขึ้นกับสองกลไก ได้แก่ ความสามารถในการบุกรุกของเชื้อพอร์ไฟโรโมแนสจินจิวัลลิสเข้าสู่เซลล์เจ้าบ้านของเนื้อเยื่อรองรับ และ/หรือ การทำลายสารองค์ประกอบนอกเซลล์ โดยกระบวนการตอบสนองการอักเสบของเซลล์เจ้าบ้านต่อเชื้อแบคทีเรีย วัตถุประสงค์ของการศึกษานี้เพื่อตรวจสอบการกระตุ้นเซลล์เจ้าบ้านด้วยไลโพโพลีแซคคาไรด์จากเชื้อพอร์ไฟโรโมแนสจินจิวัลลิส(พีจี–แอลพีเอส) โดยใช้เซลล์ไลน์เยื่อบุผิวของเนื้อเยื่อเหงือกของมนุษย์ชื่อซีเอ 9-22 ในการทดสอบกลไกที่สอง

วัสดุและวิธีการ ในการศึกษานี้ผู้วิจัยได้ทำการเลี้ยงเซลล์ซีเอ 9-22 ในอาหารเลี้ยงเซลล์ที่มีพีจี–แอลพีเอส หลายความเข้มข้น (0, 0.1, 1 และ 10 ไมโครกรัมต่อมิลลิลิตร) โดยเลี้ยงเป็นเวลานาน 24 ชั่วโมง จากนั้นนำเซลล์มาทำการสกัดเอ็ม อาร์เอ็นเอ แล้ววัดการแสดงออกของยีนเฮพพาแรนเนส (เอนไซม์ที่ย่อยเฮพพาแรนซัลเฟต ซึ่งเป็นตัวบ่งชี้ของ กระบวนการอักเสบของเซลล์เจ้าบ้าน) โทลล์–ไลค์–รีเซฟเตอร์ 2 (ทีแอลอาร์–2) และโทลล์–ไลค์–รีเซฟเตอร์ 4 (ทีแอลอาร์–4) โดยใช้การวิเคราะห์รีเวิร์สทรานสคริปเทส ปฏิกิริยาลูกโซ่โพลิเมอเรส (อาร์ที–พีซีอาร์)

ผลการศึกษา การเจริญเติบโตของเซลล์ซีเอ 9-22 ที่เลี้ยงในอาหารที่มีพีจี–แอลพีเอส ที่มีความเข้มข้นสูงสุดเจริญเติบโตได้ไม่แตกต่างจากเซลล์ซีเอ 9-22 ที่ไม่ได้ใส่พีจี–แอลพีเอส และผลการวิเคราะห์การแสดงออกของยีนเฮพพาแรนเนส ทีแอลอาร์–2 และ ทีแอลอาร์–4 ในระดับเอ็มอาร์เอ็นเอในกลุ่มเซลล์ซีเอ 9-22 ที่เลี้ยงในอาหารที่มีและไม่มีพีจี–แอลพีเอส พบว่าไม่มีความแตกต่างกันทางสถิติ

สรุป การเปลี่ยนแปลงการแสดงออกของยีนเฮพพาแรนเนส ทีแอลอาร์–2 และ ทีแอลอาร์–4 ไม่แตกต่างกันในกลุ่ม เซลล์ซีเอ 9-22 ที่เลี้ยงในอาหารที่มีพีจี–แอลพีเอสในทุกความเข้มข้นที่ทดสอบ ทั้งนี้ในการทดลองต่อไปควรทำการวัดระดับปริมาณโปรตีนของเฮพพาแรนเนส

(ว ทันต จุฬาฯ 2556;36:143–52)

คำสำคัญ: *ทีแอลอาร์–2; ทีแอลอาร์–4; พอร์ไฟโรโมแนสจินจิวัลลิส; ไลโพโพลีแซคคาไรด์; เฮพพาแรนเนส*

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