ソバ甘皮粉加水分解物は歯周病菌由来LPS誘導性NF-κBシグナルの抑制を介し炎症を減弱させる

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Buckwheat Bran Flour Hydrolysate Attenuates *Porphyromonas gingivalis*-LPS-Induced Inflammation in Human Gingival Fibroblasts *Via* Suppressing NF-κB Signaling

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Periodontal disease is a major public health problem that affects over half of the adult population worldwide. Lipopolysaccharides (LPS), produced by the periodontopathic bacterium *Porphyromonas gingivalis*, induces the expression of inflammatory cytokines that promote inflammatory bone destruction. Buckwheat bran (BB), a traditional pseudocereal crop, belongs to a group of grains having high nutritional value and has been reported to be an effective agent. However, the anti-inflammatory effects of BB on *Porphyromonas gingivalis*-lipopolysaccharide (*P.g.*-LPS)-stimulated human gingival fibroblasts (HGFs) have not been completely elucidated yet. This study determined that the buckwheat bran flour hydrolysate powder (BBFHP) significantly inhibited the *P.g.*-LPS-induced production of inflammation-associated genes, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α) and Toll-like receptor 4 (TLR4). Moreover, BBFHP suppressed *P.g.*-LPS-induced NF-κB signaling. The results suggest that the BBFHP attenuates the production of inflammation-associated molecules and suppresses the activation of NF-κB in *P.g.*-LPS treated HGFs.

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Key words : *buckwheat bran flour hydrolysate, human gingival fibroblasts, Porphyromonas gingivalis, inflammation, NF-κB*
ソバ甘皮粉加水分解物，歯周病，*Porphyromonas gingivalis*，炎症，NF-κB

Human periodontal disease causes an inflammatory condition in the oral cavity and is a major public health problem affecting more than half of the adult population worldwide[1]. *Porphyromonas gingivalis* (*P. gingivalis*) is an anaerobic Gram-negative rod-shaped bacterium in dental biofilms, which can damage the periodontal ligament and alveolar bone through the progression of periodontal inflammation[2]. Lipopolysaccharide (LPS) from *P. gingivalis* (*P.g.*) is a potent stimulator of inflammatory cytokines that induces bone resorption[3]. More importantly, *P. gingivalis* derived-LPS (*P.g.*-LPS) and inflammatory cytokines enhance osteoclast-mediated bone resorption by promoting the expression of receptor activator of nuclear factor kappa B (NF-κB) ligand[4]. Previous studies have demonstrated that natural products such as quercetin and cynaropicrin possess anti-inflammatory properties against oral pathogen-induced cytokine release in model systems of inflammation using human gingival fibroblasts (HGFs)[5),6]. Natural products with anti-inflammatory properties, therefore, may suppress inflammation in periodontal tissues.

Buckwheat, a traditional pseudocereal crop, belongs to a group of grains with high nutritional values[7]. Grown in several countries (Europe, USA, Canada, Brazil, South Africa, Australia, Korea, central and northern parts of China, and Japan), it is used in processed foods such as noodles, pizza, and a type of dish like polenta called soba-gaki in Japan[8]. It is a rich source of carbohydrates, proteins, lipids, dietary fiber, vitamins, minerals or phenolic

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compounds. Rutin and phenolic acids, in particular, are known as compounds with high antioxidant activity[9)-11)] and are concentrated in the outer layers of buckwheat grain (buckwheat bran ; BB)[12)]. Although 10 to 24 % of BB flour is produced during the process of buckwheat seed processing into flour[13)], BB flour (BBF) is not commonly used as food products. Functional peptides produced from food hydrolysates have recently received increasing attention. For example, the peptide produced by enzymatic hydrolysis from salmon milt inhibits dipeptidyl peptidase Ⅳ (DPP-Ⅳ), which inactivates hormones to participate in the secretion of insulin such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulin tropic polypeptide (GIP)[14)]. Furthermore, the hydrolysates of various foods (broccoli, rape seed, spinach, and greater weaver) exhibit inhibitory activity against angiotensin I-converting enzyme (ACE), a Zn-metalloprotease that affects the renin-angiotensin system and is one of the principal regulators of blood pressure[15)-18)]. Although BB inhibits DPP-Ⅳ and ACE activity in hydrolysates[19),20)], little has been reported on the BBF hydrolysate (BBFH) peptide. However, to the best of our knowledge, no studies have been conducted on the precise molecular mechanisms of the inhibition of *P.g.*-LPS-induced inflammation by BBF hydrolysate powder (BBFHP) in HGF. The findings, therefore, suggest that BBFHP, as a potential agent, may be able to protect against *P.g.*-LPS induced inflammation.

The present study investigated the anti-inflammatory effects of BBFHP extract on *P.g.*-LPS-induced expression of inflammatory signals, including NF-κB signaling, in HGF cells, which are the major cells in periodontal connective tissues.

Materials and methods

1. Preparation of BBF hydrolysate powder

Alcalase 2.4 L FG and Flavourzyme were procured from Novozymes Co. (Bagsvaerd, Denmark). Methanol was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Then, 0.1 M phosphate buffer (pH8.0) was prepared by mixing 0.1 M sodium dihydrogen phosphate$_{aq.}$ and 0.1 M disodium phosphate$_{aq.}$ to adjust the pH to 8.0. The BBFHP was procured from a local store in Tokyo, Japan. The sample was then stored at − 20℃ until required.

A 10 g sample was dispersed in 150 mℓ of 0.1 M phosphate buffer (pH 8.0), to which 500 μℓ of Alcalase and 10 mg of Flavourzyme were then added. The mixture was continuously stirred using a magnetic bar at 450 rpm for 17 h at 70℃ with a magnetic stirrer (REXIM RSH-4DN; AS ONE Co., Osaka, Japan). The hydrolysate was then centrifuged at 4,000 g for 10 min at 4℃ after the enzymatic reaction, and the supernatants were filtered through a filter paper (No. 5 A ; ADVANTEC, Tokyo, Japan) using suction filtration. The filtrate, the enzyme was terminated at 450 rpm for 1 h at 115℃ and was then centrifuged at 4,000 g for 10 min at 4℃. The supernatants were filtered through filter paper using suction filtration. The obtained filtrate was filled up to 200 mℓ with 0.1 M phosphate buffer (Fig. 1-A).

Next, 200 mℓ of BBFH was applied to reverse phase columns (ϕ 2.5 × 4 cm, Cosmosil 140C_{18}-OPN; Nacalai Tesque, Inc., Kyoto, Japan) and washed with 100 mℓ of water and 20% methanol at a flow rate of 3.5 mℓ/min. The maintained component was eluted with 100 mℓ of 40% methanol after washing and then concentrated using a rotary evaporator (N-1300 ; Eyela ; Tokyo Rikakikai Co., Tokyo, Japan). BBFH powder was then obtained (BBFHP), which was dissolved in water and dried using a freeze dryer (FDU-1200 ; Eyela, Tokyo Rikakikai Co., Tokyo, Japan) (Fig. 1-B). This was followed by determining the comparative effects of BBFHP on *P.g.*-LPS-induced inflammatory HGF cells.

2. Cell culture

HGFs were cultured in Dulbecco's modified Eagle's medium (DMEM) (phenol red-free) (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), 100 U/mℓ penicillin, and 100 mg/mℓ streptomycin (Gibco BRL/Invitrogen) at 37 ℃ in 5% CO_2.

3. Measurement of cell viability

The HGF cells (1.0×10^3 cells/well) were cultured in 96-well plates. The cells were then treated with various concentrations of BBFHP for 24 h. This was followed by assessing the cell viability using a Cell Counting Kit-8 (CCK-8 ; DOJINDO, Kumamoto, Japan). The effect of BBFHP on cell viability was expressed as a percentage of cell viability, with that of cells treated with a phosphate-buffered saline (PBS) (−) vehicles set at 100%. The HGF cells were incubated in a 6-well plate at 1.0×10^5 cells/well for 24 h. The experiment was then divided into untreated, 1 μg/mℓ *P.g.*-LPS (InvivoGen, San

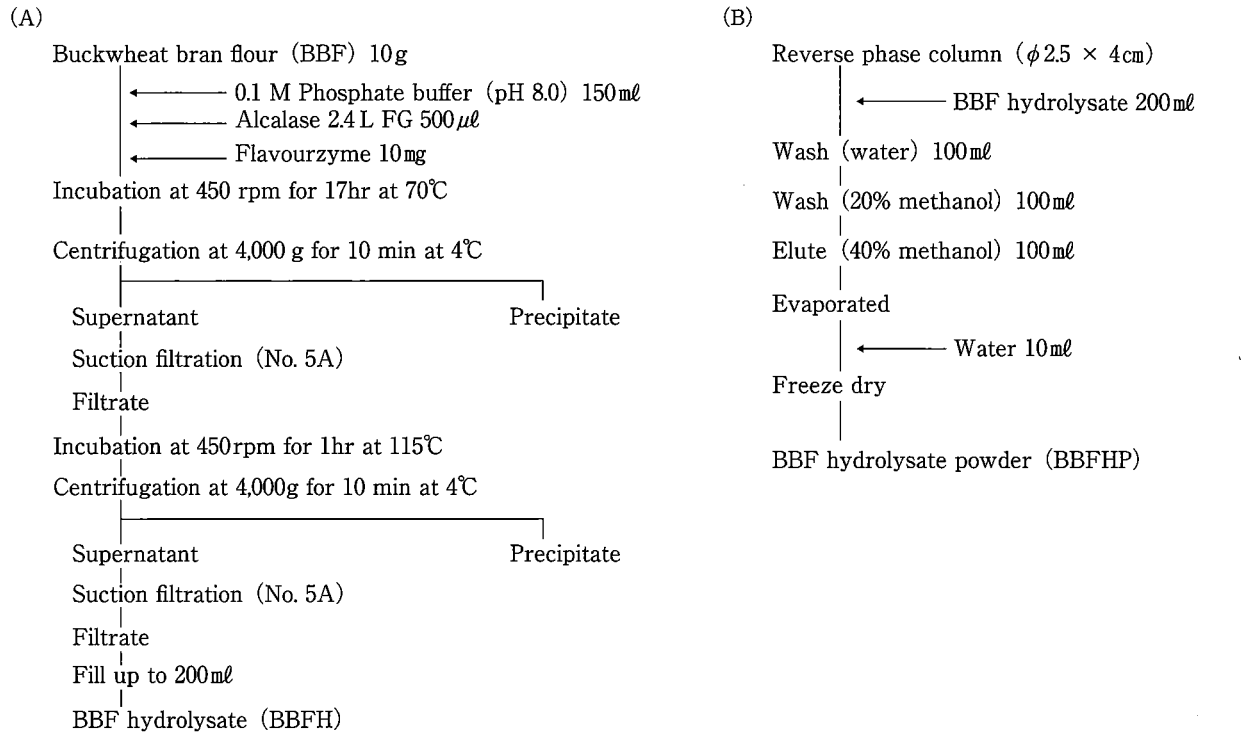

Fig. 1 Preparation of BBF hydrolysate powder

(A) Scheme of BBF hydrolysis with alcalase and flavourzyme. (B) Fractionation of BBF hydrolysate with reverse phase column chromatography.

Diego, CA, USA), and 1 μg/mℓ *P.g.*-LPS +10 or 30 μg/mℓ BBFHP groups.

4. mRNA preparation and real-time polymerase chain reaction (PCR) analysis

The total RNA was isolated from the HGF cells using Sepasol-RNA I Super G (Nacalai Tesque). cDNA was synthesized from the total RNA using a PrimeScript RT reagent kit (Takara Biotechnology, Shiga, Japan). Real-time PCR was performed using an ABI StepOne Plus System (Applied Biosystems, Foster City, CA, USA) with THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan). The primer sequences are presented in Table 1. All the reactions were normalized to the housekeeping gene GAPDH.

Table 1 The primer sequences for real time PCR analyses

Gene		sequences 5′ to 3′
human IL-1β	*sense*	TAGGGCTGGCAGAAAG
	antisense	GTGGGAGCGAATGACA
human IL-6	*sense*	TTCGGTCCAGTTGCCTTCT
	antisense	GAGGTGAGTGGCTGTCTGTG
human IL-8	*sense*	CTTGTCATTGCCAGCTGTGT
	antisense	TGACTGTGGAGTTTGGCTG
human TLR-4	*sense*	CACAGACTTGCGGGTT
	antisense	GGACTTCTAAACCAGC
human TNF-α	*sense*	AACATCCAACCTTCCC
	antisense	TGGTCTCCAGATTCCA
human GAPDH	*sense*	TGCACCACCAACTGCTTAGC
	antisense	GGCATGGACTGTGGTCATGAG

5. Western blotting

The HGF cells were washed twice with PBS (−) and lysed in RIPA buffer (Nacalai Tesque, Tokyo, Japan). The proteins in the cell lysates were separated using SDS-PAGE, transferred onto PVDF membranes (Millipore, Bedford, MA), and incubated with specific primary antibodies. All the primary antibodies were diluted in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST; dilution, 1 : 1,000). Immunoblotting was performed using HRP-conjugated anti-rabbit or anti-mouse IgG antibodies (dilution, 1 : 3,000) as secondary antibodies and detected using an ECL system (GE Healthcare, Piscataway, NJ, USA). Antibodies against phospho-p 65, Iκβα, and β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

6. Statistical analysis

The results are presented as the mean ± S.E. All the data represent the means of three independent experiments. Multiple comparisons were performed using Tukey's test after a one-way

analysis of variance (ANOVA). Different letters indicate significant differences at $P < 0.05$, as determined using a one-way ANOVA with Tukey's test.

Results and discussion

1. Effects of BBFHP on the expressions of inflammation-associated gene by *P. g.* -LPS-stimulated HGFs

HGF cells are closely related to the remodeling of periodontal soft tissues and can recognize LPS and mediate the host immune response in periodontal lesions[3]. More importantly, several inflammatory mediators-interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α) and Toll-like receptor 4 (TLR4)-within gingival tissue are positively increased[5),6)]. Suppressing these inflammatory mediators or blocking the involved signaling pathway, therefore, may aid in restricting the initiation and progression of periodontal disease. The HGF cells were treated with various concentrations of BBFHP and evaluated using a cell counting kit (CCK)-8 assay. As presented in Fig. 2-A, several concentrations of BBFHP (3, 10, and 30 μg/mℓ) were found to have no cytotoxic effect on the HGF cells. The comparative effects of BBFHP were then determined on *P. g.* -LPS induced inflammatory cytokines.

Next, to examine the effect of the extract on the expression of inflammatory cytokines in HGFs stimulated by *P.g.*-LPS, they were analyzed using real-time PCR. We observed that *P.g.*-LPS treatment drastically increased the inflammation-associated gene expression of IL-1β, IL-6, IL-8, TNF-α and TLR4, whereas all the inflammation-associated gene expression levels were found to significantly decrease in BBFHP-pretreated HGF cells (Fig. 2-B-F). Several studies have reported increased levels of IL-8 and IL-6 mRNA through the activation of the nuclear factor-κB (NF-κB)[5),6)]. Takashiba *et al.* demonstrated that IL-8 is associated with the pathogenesis of periodontal diseases[21]. Furthermore, levels of IL-6 in the periodontal pockets of patients with periodontitis are higher than those of IL-1 and

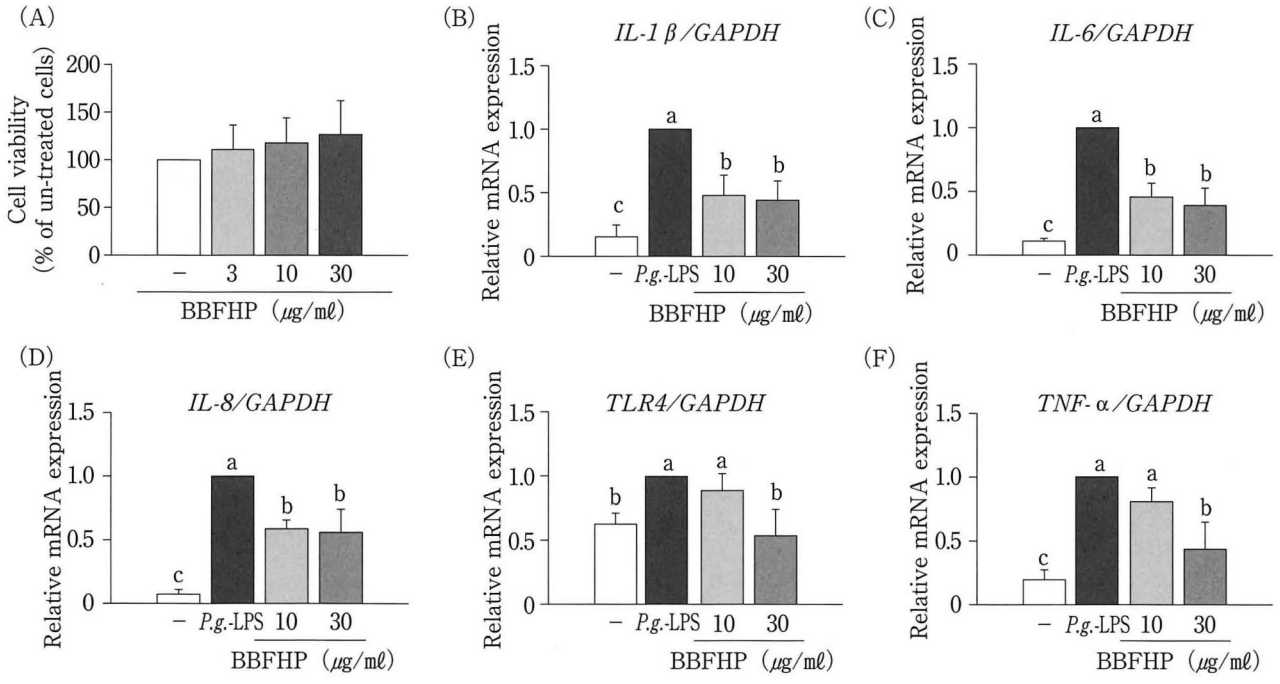

Fig. 2 Effect of BBFHP on the inflammation-associated gene expressions in *P.g.*-LPS stimulated HGF cells

(A) HGF cells were cultured in a 96-well plate and then treated with various concentrations of BBFHP for 24 hours. Cell viability was assessed using the CCK-8 assay. Cell viability is expressed as a percentage of the values obtained for BBFHP-untreated cells. Next, HGF cells were pretreated with BBFHP at a different final concentration of 10 and 30 μg/mℓ for six hours, respectively, followed by *P.g.*-LPS stimulation (final concentration at 1 μg/mℓ) for three hours. Gene expression levels of inflammatory cytokines, such as (B) IL-1β, (C) IL-6, (D) IL-8, (E) TLR4, and (F) TNF-α were then determined using real-time-PCR. GAPDH was used as a control for mRNA loading. Each column and error bar represents the mean and S.E., respectively, based on three independent experiments. Different letters indicate significant differences at $P<0.05$, as determined by a one-way ANOVA with Tukey's test.

play an important role in the progression of periodontal disease[22]. The data, therefore, suggest that BBFHP attenuate *P.g.*-LPS induced inflammation by suppressing the transcriptional levels of IL-6 and IL-8 mRNA in HGF cells.

2. BBFHP inhibits *P.g.*-LPS induced NF-κB activity in HGFs

Periodontal inflammation is, in general, relieved via the inhibition of NF-κB activation[23]. Additionally, NF-κB is usually stored in the cytoplasm as an inactive complex with inhibitor IκBα. However, inflammation stimulation such as LPS treatment induces ubiquitination of IκBα, leading to degradation of IκBα by the 26S proteasome[24]. The study investigated the effects of BBFHP on NF-κB activation to determine its anti-inflammatory mechanism. As presented in Fig. 3, *P. g.*-LPS treatment significantly increased the phosphorylation of the NF-kB subunit p65 at Ser536 and caused IκBα degradation, whereas BBFHP pre-treatment attenuated this effect (Fig. 3-A-C). More importantly, phosphorylation of the NF-κB p65 subunit at Ser 536 is essential for maximal transcriptional activity[24]. Additionally, we observed that BBFHP pretreatment decreased the levels of TLR4 mRNA in the *P.g.*-LPS treated cells (Fig. 2-E). TLR4 mediates the activation of NF-κB in LPS-stimulated HGF cells and subsequently modulates the expression of inflammatory cytokines[3),4)]. BBFHP may, thus, inhibit *P. g.* -LPS-upregulation of inflammation-associated molecules in HGFs via the regulation of the NF-κB signaling pathway.

Conclusion

In conclusion, this study demonstrated that BBFHP is a potent suppressor of *P.g.*-LPS induced inflammatory responses. The findings revealed that BBFHP significantly downregulated the expression levels of inflammation associated genes in *P.g.*-LPS treated HGFs. This anti-inflammatory mechanism suppressed the activation of NF-κB induced by *P.g.*-LPS. These findings are the first to report that BBFHP attenuates *P.g.*-LPS mediated inflammatory responses in HGFs. *In-vivo* studies are further required to evaluate the anti-inflammatory properties of BBFHP. The present work indicates a promising therapeutic potential of BBFHP in the treatment of periodontal disease.

Conflicts of Interest The authors declare no conflicts of interest regarding the publication of this paper.

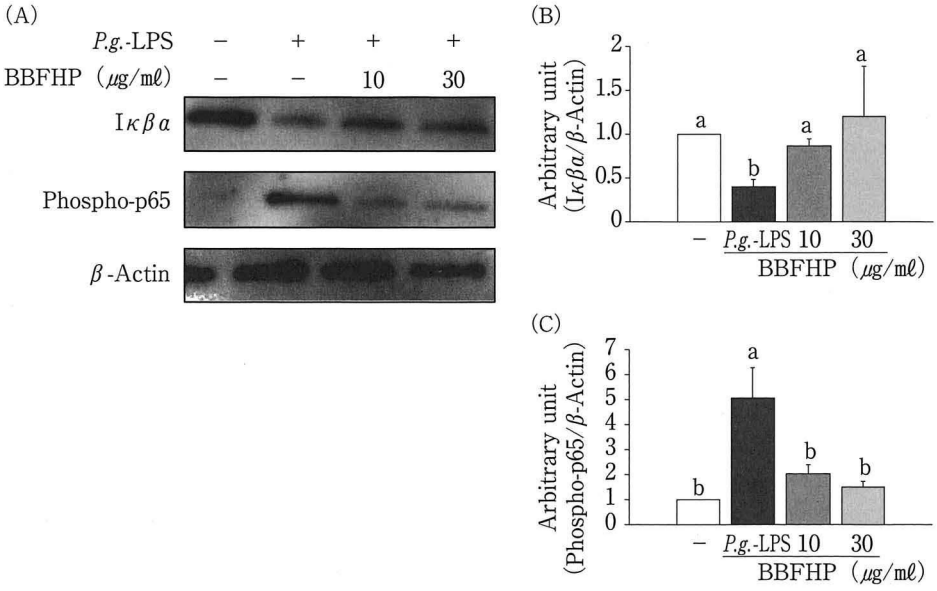

Fig. 3 Effect of BBFHP on NF-κB activation in *P.g.*-LPS stimulated HGF cells

(A) HGF cells were pretreated with BBFHP at a different final concentration of 10 and 30 μg/mℓ for six hours, respectively, followed by *P.g.*-LPS stimulation (final concentration at 1 μg/mℓ) for 30 min. Each column and error bar represents the mean and S.E., respectively, based on three independent experiments. Different letters indicate significant differences at $P<0.05$, as determined by one-way ANOVA with Tukey's test.

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ソバ甘皮粉加水分解物は歯周病菌由来LPS誘導性NF-κBシグナルの抑制を介し炎症を減弱させる

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歯周炎は世界的な健康問題の一つである。これまでに歯周病菌 (*Porphyromonas gingivalis*) 由来のリポポリサッカライド (以下, *P.g.*-LPS) は, 炎症性サイトカインを介し, 最終的に骨吸収能をあげることで歯の損失につながることが明らかとなっている。これまでに我々は未利用資源としてソバ甘皮粉を用い, その加水分解物中ペプチドの同定と機能性に関して研究を進めているが, 歯周炎症抑制効果については検討を行っていない。そこで本研究では, ヒト由来歯肉線維芽 (Human GF) 細胞を用いて, ソバ甘皮粉加水分解物 (以下, BBFHP) が*P.g.*-LPSによる歯肉炎症を抑制するか否かについて明らかにすることを目的とした。まず初めに, HGF細胞に対するBBFHPの細胞毒性評価を行った結果, 本試験で用いた30μg/mℓまで細胞毒性を示さなかった。続いて, *P.g.*-LPS刺激したHGF細胞に対するBBFHPによる炎症性サイトカイン類 (IL-6, IL-8, IL-1β, TLR4およびTNF-α) の遺伝子発現について解析した結果, *P.g.*-LPS刺激による増加した全ての炎症性サイトカイン類の遺伝子発現はBBFHPにより有意に抑制された。同様に, NF-κBシグナルの活性化指標であるIKKαの分解ならびにリン酸化p65の核内移行もBBFHP抽出物処理により有意に抑制された。以上より, ヒト歯肉繊維芽細胞において, BBFHP抽出物は*P.g.*-LPS誘導性炎症性メディエーター群ならびにNF-κBシグナルの不活性化を誘導することで歯肉炎症を抑制することを明らかにした。

(令和5年3月2日受付, 令和5年4月27日受理)