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Fucoidan Increases Porcine Neutrophil Extracellular Trap Formation through TNF-α from Peripheral Blood Mononuclear Cells

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Abstract Fucoidan extracted from brown seaweed has a variety of biological activities. Neutrophil extracellular traps (NETs) formation is an immune response for the invasion of pathogens. Neutrophils release granule protein and chromatin that form extracellular fibers that bind microbes. These NETs degrade virulence factors and kill bacteria. The aim of this study was to investigate the effect of fucoidan on NET formation of porcine peripheral blood polymorphonuclear cells (PMNs). The NET formation was determined by fluorescence emission of propidium iodide (PI) in PMNs by a fluorescence microplate reader. The production of tumor necrosis factor (TNF)-α from peripheral blood mononuclear cells (PBMCs) was measured by ELISA method. Fucoidan itself did not show any direct effect on NET formation. However, NET formation of PMNs was increased by the culture supernatant from PBMCs treated with fucoidan. The NET formation of PMNs were also enhanced by treatment with recombinant porcine (rp) TNF-α. The ability of culture supernatant from PBMCs treated with fucoidan to increase the NET formation of PMNs was inhibited by addition of goat anti-rp TNF-α polyclonal antibody (pAb) (IgG) prior to the culture. The increase of NET formation by rp TNF-α was also inhibited by goat anti-rp TNF-α pAb (IgG). The level of TNF-α in culture supernatant from PBMCs was increased by treatment with fucoidan. These results suggest that fucoidan increases porcine NET formation, which is mediated by TNF-α produced from PBMCs.

Key words neutrophil extracellular trap (NET) formation, fucoidan, peripheral blood mononuclear cells, tumor necrosis factor-α, porcine.

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Introduction

Neutrophils are immunocytes for defense against invading pathogens and they immediately begin antibacterial functions such as phagocytosis, and production of antimicrobial products and proinflammatory cytokines (17,34). When neutrophils encounter pathogen, they release net-like structures capable of capturing and destroying microbes (1). These structures, known as neutrophil extracellular traps (NETs), are consisted of granule proteins and nuclear constituents that bind and kill bacteria extracellularly (5). This process of NET formation is called NETosis, a different type of cell death, both necrosis and apoptosis (12). NET formation is stimulated by pathogens, cytokines and chemical compounds (e.g., phorbol-12-myristate-13-acetate) (40). After stimulation of neutrophils, reactive oxygen species (ROS) are generated and chromatin decondensations are promoted by neutrophil elastase (NE) and myeloperoxidase (MPO), which are enzymes stored in the azurophilic granules (33).

Fucoidan is sulfated polysaccharides found in the extracellular matrix of various brown algae (3). In the last decades, fucoidan has been extensively studied due to their numerous biological activities: anticoagulant and antithrombotic, antiinflammatory, antitumour, and antiviral (8,28,42). Fucoidan has been shown to modulate inflammatory reaction (8). Also fucoidan enhances the secretion of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in human T and B cells (14). TNF-α is one of NET triggering agents (20) and encouraging neutrophil phagocytosis (26).

It has been known that fucoidan upregulates chemotactic activity of PMNs (21). It increases phagocytic capacity and oxidative burst activity (OBA) of PMNs (26). Fucoidan has been reported to induce tumor necrosis factor (TNF)-α secretion from monocytes, which is triggerng NET formation (16). These observations suggest that fucoidan has considerable potential as an immunomodulatory molecule for NET formation of PMNs. Therefore, the aim of the study is to investigate the effect of fucoidan on porcine NET formation of peripheral blood polymorphonuclear cells (PMNs).

Materials and Methods

Chemicals and reagents

Fucoidan purified from Focus vesiculosus, was purchased commercially (Sigma-Aldrich, St. Louis, MO, USA). The stock solution of fucoidan was prepared to 10 mg/mL at final concentration in phosphate buffered saline (PBS). Phorbol 12-myristate 13-acetate (PMA), propidium iodide (PI) solution, RPMI 1,640 medium, and rabbit anti-recombinant mouse (rm) IL-6 polyclonal antibody (pAb) (IgG) were purchased commercially (Sigma-Aldrich). Recombinant porcine (rp) TNF-α and goat anti-rpTNF-α pAb (IgG) (R&D Systems Inc., Minneapolis, MN, USA) were also purchased commercially.

Isolation of porcine peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs)

Healthy pigs aged 6 months in a slaughterhouse (Donga food Co. Ltd., Cheongju, Chungbuk, Republic of Korea) as blood donors were used. Heparinized peripheral blood from anterior vena cava was diluted with a same volume of PBS, and overlaid 1:1 on a Percoll® solution (Sigma-Aldrich; 1.080 gravity). After centrifugation at 400 g for 50 min at room temperature, the PBMCs between plasma and Percoll® solution were harvested and treated with erythrocyte lysis buffer (iNtRON biotechnology, Seongnam, Gyeonggi, Republic of Korea) for 3 min to lyse erythrocytes. After removing the PBMCs layer, the PMNs were obtained from the upper layer of precipitated erythrocytes. To purify PMNs, the erythrocytes were precipitated with 1.5% dextran (molecular weight, 200,000; Wako Ltd., Osaka, Japan) in PBS for 60 min. Floating cells were gently collected and pelleted by centrifugation at 900 g for 5 min. Residual erythrocytes were lysed by erythrocyte lysis buffer. The obtained PBMCs and PMNs were washed three times with PBS. The PBMCs population consisted of approximately 90% lymphocytes and 10% monocytes (27). The purity of neutrophils in the final PMNs suspension was greater than 95% (38). The viability of PBMCs and PMNs determined by a method of trypan blue dye exclusion always exceeded 98%. All cells were resuspended at 37°C in RPMI 1,640 medium containing 5% heat-inactivated FBS (Gibco Company, Grand Island, NY, USA), 1% 100 U/mL penicillin and 100 μg/mL of streptomycin (Gibco Company) in a 5% CO_2-humidified atmosphere.

PBMCs culture supernatants

The PBMCs seeded at a density of 2×10^6 cells/mL in a twenty-four-multi well plate (Nunc company, Naperville, IL, USA) were incubated with fucoidan (0 to 200 μg/mL) for 24 h at 37°C in a 5% CO_2-humidified atmosphere. Control cells were treated with an equal amount of PBS. All culture supernatants were obtained after centrifugation at 900 g for 15 min and stored at –70°C until used.

Quantification of NET formation

NET formation was measured by the enhanced fluorescence emission of propidium iodide (PI) after its interaction with extracellular DNA. PI which does not penetrate the

neutrophils membrane binds to extracellular DNA. Therefore, fluorescence emission of PI means only NET formation. The PMNs (8×10^5 cells/mL/well) at each well of a 24-well plate were incubated for 4 h with PMA (10 nM) as positive control, fucoidan (0-250 μg/mL), culture supernatants from fucoidan (0-200 μg/mL) treated PBMCs and rpTNF-α. The cultured cells were gently harvested, and centrifuged at 900 g for 15 min. Cells were gently washed with 37°C Dulbecco's phosphate-buffered saline (DPBS) (containing magnesium and calcium) (Gibco Company). Then, cells were mixed with 400 μL of 2.5 μM PI diluted with DPBS. Cells were placed in black 96-well plate (SPL life science, Pocheon, Gyeonggi, Republic of Korea) and incubated for 30 min in the dark at room temperature. The resulting fluorescence was measured at an excitation wavelength of 535 nm and emission wavelength of 617 nm using a SpectraMax M2e microplate reader (Molecular devices, Oreleans Drive Sunnyvale, CA, USA).

Neutralization test

PMNs (8×10^5 cells/mL) were incubated for 4 h either fucoidan (200 μg/mL) treated PBMCs culture supernatant or rpTNF-α (20 ng/mL) in the presence or absence of various concentrations of anti-rpTNF-α (IgG) pAb at 37°C in a 5% CO_2-humidified atmosphere. Rabbit anti-rmIL-6 pAb as a control isotype IgG was used.

Measurement of TNF-α in the culture supernatant from PBMCs treated with fucoidan

The culture supernatants of PBMCs treated with fucoidan (200 μg/mL) were collected after 24 h incubation. The amount of TNF-α in the culture supernatants was measured by direct ELISA using Quantikine® porcine TNF-α immunoassay kit (R&D Systems Inc.). All samples, standard, and controls were analyzed in triplicate. The optical density was measured with an automate microplate reader (Elx808, Bio-Tek Instruments Inc., Winooski, Vermont, USA) at 450 nm. TNF-α levels in the samples were quantified in a standard curves created with purified porcine TNF-α tested at eight titration points.

Statistical analyses

All statistical analyses were assessed by the program of GraphPad prism 6 software (GraphPad software, San Diego, CA, USA). Comparisons of two groups were performed using the Student's t-test. One-way analysis of variance (ANOVA) was used to examine differences between control and treatment groups followed by Dunnett's post hoc test. p value of less than 0.05 was considered statistically significant. Data are expressed as means ± standard deviations (SD).

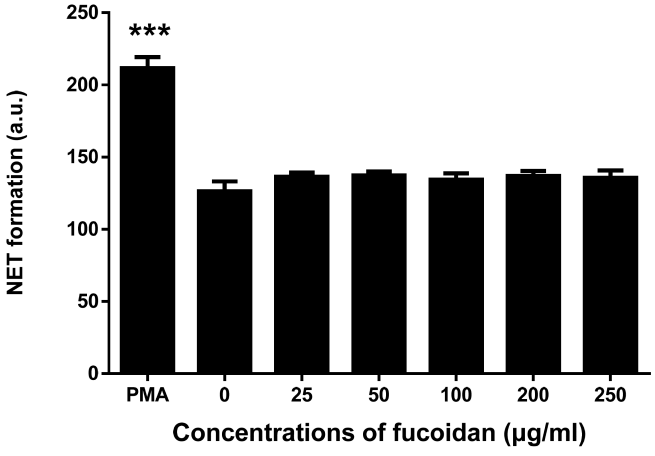

Fig. 1. Direct effect of fucoidan on NET formation. PMNs (8×10^5 cells/well/mL) were treated with 10 nM PMA (positive control) or fucoidan (0-250 μg/mL) for 4 h. Control cells were incubated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye, propidium iodide (PI). Data represent means ± SD (n = 3). One-way ANOVA was used for statistical analyses, followed by a Dunnett's post hoc test. Comparison of two groups was performed by Student's t-test. a.u., arbitrary units. ***p < 0.001 vs. control (0 μg/mL).

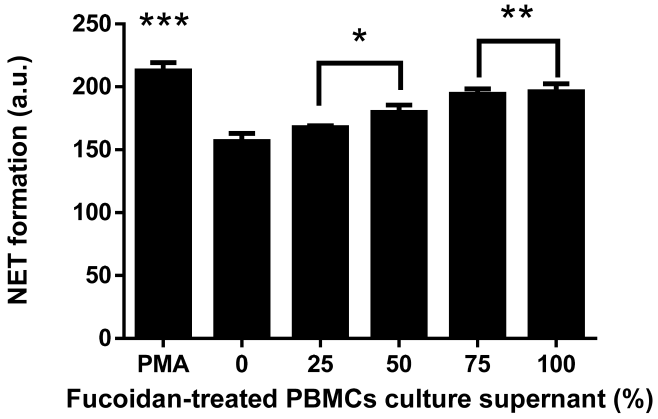

Fig. 2. The effect of fucoidan (200 μg/mL)-treated PBMCs culture supernatant on NET formation. PMNs (8×10^5 cells/well/mL) were treated for 4 h with 10 nM PMA (positive control) or culture supernatants (0-100%) from PBMCs (2×10^6 cells/mL) treated with fucoidan (200 μg/mL) for 24 h. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye, PI. Data represent means ± SD (n = 3). One-way ANOVA was used for statistical analyses, followed by a Dunnett's post hoc test. Comparison of two groups was made by Student's t-test. a.u., arbitrary units. *p < 0.05 vs. control. **p < 0.01 vs. control. ***p < 0.001 vs. control (0%).

Results

Fucoidan itself does not increase the NET formation

To examine the direct effect of fucoidan on the NET formation of PMNs, freshly isolated PMNs were incubated with fucoidan 0 to 250 μg/mL for 4 h. Treatment of PMA, as a positive control, significantly ($p < 0.001$) increased the NET formation of PMNs compared to control cells treated without fucoidan. But the direct treatment of fucoidan did not increase any effect on NET formation as compared with control cells (Fig. 1).

Culture supernatant from PBMCs treated with fucoidan increases the NET formation

Isolated PMNs were incubated with culture supernatants (0-100%) from fucoidan (200 μg/mL)-treated PBMCs for 4 h. The treatment of PMA significantly ($p < 0.001$) increased the NET formation of PMNs compared to control. The NET formation of PMNs was significantly (25 and 50%; $p < 0.05$, 75 and 100%; $p < 0.01$) increased by culture supernatant from PBMCs treated with fucoidan as compared with control (0%) (Fig. 2).

TNF-α also increases the NET formation

The NET formation of PMNs by rpTNF-α significantly (1 and 2.5 ng/mL; $p < 0.05$, 5, 10 and 20 ng/mL; $p < 0.01$) increased in a dose-dependent fashion as compared with control (Fig. 3). The NET formation of PMNs was climaxed at 20 ng/mL rpTNF-α.

Anti-rpTNF-α pAb neutralizes the increasing effect of NET formation by fucoidan

To examine whether the enhanced NET formation of PMNs by culture supernatant from fucoidan-treated PBMCs is due to TNF-α, the neutralization test using the anti-rpTNF-α pAb was performed. The NET formation of PMNs in response to culture supernatant from PBMCs treated with fucoidan was significantly (10, 25 and 50 μg/mL; $p < 0.05$, 100 μg/mL; $p < 0.001$) inhibited in a dose-dependent fashion by the addition of anti-rpTNF-α pAb as compared with culture supernatant from PBMCs treated with fucoidan (200 μg/mL) alone (Fig. 4). Similarly, the increased NET formation of PMNs by rpTNF-α at 20 ng/mL was also significantly (0.1 and 1 μg/mL; $p < 0.01$, 10 μg/mL; $p < 0.001$) reduced by the addition of anti-rpTNF-α pAb in a dose-dependent fashion (Fig. 5). However, in the examination of the possibility of nonspecific inhibition for

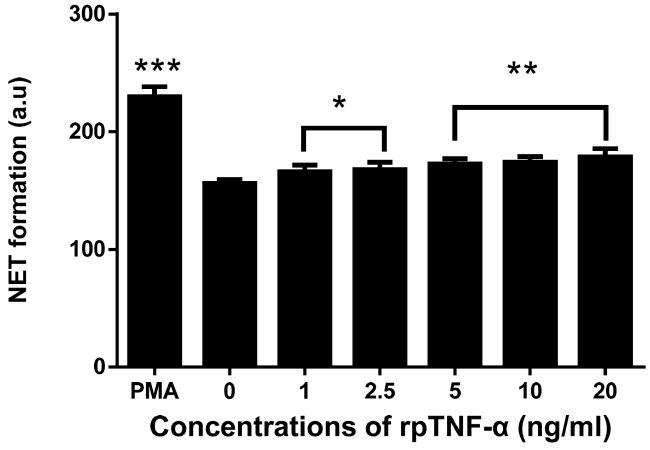

Fig. 3. The effect of rpTNF-α on NET formation. PMNs (8×10^5 cells/well/mL) were treated with 10 nM PMA (positive control) or rpTNF-α (0-20 ng/mL) for 4 h. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye, PI. Data represent means ± SD (n = 3). One-way ANOVA was used for statistical analyses, followed by a Dunnett's post hoc test. Comparison of two groups was performed by Student's t-test. a.u., arbitrary units. *$p < 0.05$ vs. control. **$p < 0.01$ vs. control. ***$p < 0.001$ vs. control (0 ng/mL).

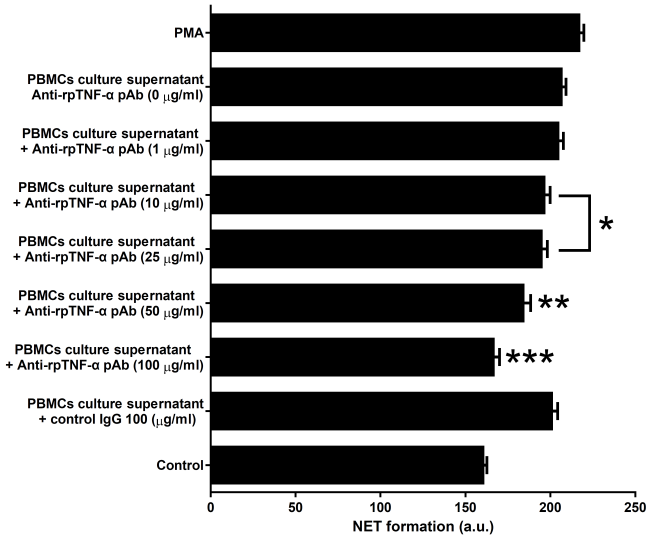

Fig. 4. The effect of anti-rpTNF-α pAb on NET formation by culture supernatant from PBMCs treated with fucoidan. Anti-rpTNF-α pAb (0, 1, 10, 25, 50 and 100 μg/mL) and anti-rmIL-6 pAb (100 μg/mL) were mixed with the fucoidan (200 μg/mL)-treated PBMCs culture supernatant for 30 min at room temperature. PMNs (8×10^5 cells/well/mL) were incubated for 4 h with these mixtures. Control cells were treated with the same amount of PBS. Fucoidan (200 μg/mL)-treated PBMCs culture supernatants were incubated for 24 h. The NET formation was quantified using the membrane impermeable fluorescent DNA dye, PI. Data represent means ± SD (n = 3). One-way ANOVA was used for statistical analyses, followed by a Dunnett's post hoc test. Comparison of two groups was assessed by Student's t-test. a.u., arbitrary units. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with culture supernatant (200 μg/mL) alone.

immunoglobulin isotype, IgG, any NET formation of PMNs to either culture supernatant from fucoidan (200 μg/mL)-treated PBMCs or rpTNF-α (20 ng/mL) was not inhibited by the addition of high concentration (100 μg/mL) of anti-rmIL-6 pAb, control IgG (Figs. 4, 5).

Fucoidan increases the production of TNF-α from PBMCs

The amount of TNF-α in culture supernatant from fucoidan (200 μg/mL)-treated PBMCs for 24 h was measured by ELISA method. The level of TNF-α in culture supernatant from PBMCs treated with fucoidan was significantly ($p < 0.001$) higher than culture supernatant from PBMCs without fucoidan (Fig. 6).

Discussion

It has been reported that PMA on the NET formation of PMNs involves an active cell death mechanism leading to disruption of the nuclear membrane, disintegration of the nucleus and cytoplasmic granules with mixing of chromatin and granular constituents, and local extrusion of DNA, histones, and enzymes (39). Thus, in the present study, PMA-treated PMNs were used as a positive control for NET formation (7).

It has been shown that fucoidan increases the production of TNF-α in the culture supernatant from fucoidan-treated PBMCs (26) and treatment of fucoidan induce the TNF-α production on helper T cells and monocytes (36,37). Activated PBMCs generate in a variety of cytokines, such as IL-1, IL-6 and TNF-α (32). Among of these cytokines, TNF-α induces NET formation in neutrophils (4). TNF-α enhances the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase dependent superoxide anion production by PMNs (11). Also, NADPH oxidase leading to ROS and NO production on PMNs induces NET formation (15). Thus, we hypothesized that TNF-α in the culture supernatant from fucoidan-treated PBMCs may be related with NET formation of PMNs. We tested the effects of culture supernatant from fucoidan-treated PBMCs and rpTNF-α on the NET formation of PMNs. The culture supernatant from fucoidan-treated PBMCs and rpTNF-α increased NET formation of PMNs. There may be more cytokines in the culture supernatant from fucoidan-treated PBMCs to enhance the NET formation. It has been recently reported that IL-1β and IL-8 also increase the NET formation of PMNs which was mediated through the activation of NADPH oxidase and MPO (23,40). Next, we examined whether an anti-rpTNF-α pAb neutralizes the increase of NET formation by culture supernatant from fucoidan-treated PBMCs and rpTNF-α. The anti-rpTNF-α pAb neutralized the ability of both the culture supernatant from fucoidan-treated PBMCs and rpTNF-α to increase the NET formation. We found that the production of TNF-α in the culture supernatant from PBMCs treated with fucoidan was remarkably higher than that of PBMCs without fucoidan. These results suggest that

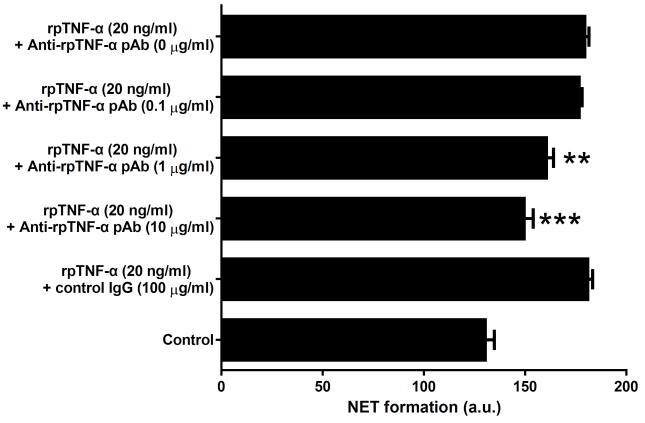

Fig. 5. The effect of anti-rpTNF-α pAb on NET formation by rpTNF-α. Anti-rpTNF-α pAb (0, 0.1, 1, 10 and 100 μg/mL) and anti-rmIL-6 pAb (100 μg/mL) were mixed with the rpTNF-α (20 ng/mL) for 30 min at room temperature. PMNs (8×10^5 cells/well/mL) were incubated for 4 h with these mixtures. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye, PI. Data represent means ± SD ($n = 3$). One-way ANOVA was used for statistical analyses, followed by a Dunnett's post hoc test. Comparison of two groups was assessed by Student's t-test. a.u., arbitrary units. **$p < 0.01$, ***$p < 0.001$ compared with rpTNF-α (20 ng/mL) alone.

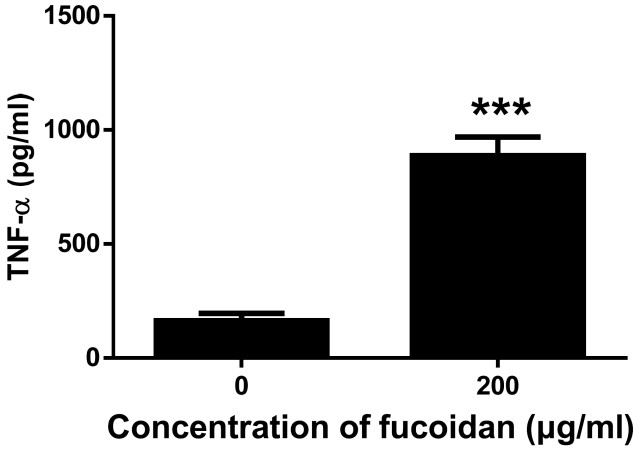

Fig. 6. Amount of TNF-α in the culture supernatant from PBMCs treated with fucoidan. PBMCs (2×10^6 cells/mL) were incubated with fucoidan (200 μg/mL) for 24 h. Control cells were treated with the same amount of PBS. The concentration of TNF-α in the culture supernatant from PBMCs was measured by ELISA. Data represent means ± SD ($n = 3$). Comparison of two groups was made by Student's t-test. ***$p < 0.001$ vs. control (0 μM).

the effect of fucoidan on the NET formation is mediated by TNF-α produced from fucoidan-treated PBMCs.

Neutrophil extracellular traps (NETs) are released by TNF-α induced cell death (35). This progress was recently named NETosis (31). NETosis has been demonstrated following stimulation of neutrophil receptors by triggers such as bacteria, fungi, virues, parasites, and chemical factors such as PMA and TNF-α (29). TNF-α binding to the neutrophil receptors induces the release of endoplasmic reticulum calcium stores and the opening of membrane channels that lead to cytoplasmic calcium increases. And elevated calcium stimulates protein kinase C (PKC) activity and assembly of functional NADPH oxidase, leading to ROS and NO production (22). NADPH-oxidase dependent ROS degrades cytoplasmic granules and neuclear envelop (22). Upon ROS stimulation, NE and MPO are released from the azurophilic granules and translocate to the nucleus (31). In the nucleus, NE cleaves histones and promotes chromatin decondensation, while MPO binds to chromatin promoting further decondensation (30,33). Decondensed chromatin threads are breakdown of nuclear and granule membranes and the mixing of nuclear, granular, and cytoplasmic antimicrobial contents (22). Finally, the cell membrane is disrupted and releasing NETs (6).

In this study, fucoidan itself did not cause any effect on the NET formation. Numerous studies have shown that neutrophil apoptosis can be delayed by proinflammatory cytokines and other inflammatory mediators (18,41). NETosis is a progress of cell death when neutrophuils encounter pathogen or cytokines. Moreover, a study showed that fucoidan delayed neutrophil apoptosis (19). Direct treatment of fucoidan on PMNs may delay neutrophil apoptosis. Therefore, these observations are suggested that fucoidan does not directly affect the NET formation.

NET formation occurs in sites of a proinflammatory response. Since NET immune response activity was measured in virtually all lymph and blood samples (10). Several studies indicate the NET formation in autoimmune diseases such as small vessel vasculitis, lupus nephritis, systemic lupus erythematosus (SLE), and rheumatoid arthritis (13,24,25). NETs were also detected in the cerebrospinal fluid (CSF) of *Streptococcus suis*-infected piglets (9). These NET formation may be useful as indicators of immune-mediated diseases. In addition the effect of fucoidan on PMNs may be applicable for the development of feed additives in swine inflammatory diseases such as mastitis, ulcerative colitis, atrophic rhinitis and arthritis (2). Overall, the results of the present study suggested that fucoidan has an enhancing effect on porcine NET formation.

Conflicts of Interest

The authors have no conflicting interests.

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