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Effects of chitooligosaccharides on rabbit neutrophils in vitro

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Abstract

The effects of chitooligosaccharides(COS) on resting and PMA-activated neutrophils were estimated. MTT assays, NO estimation and superoxide detection revealed that chitooligosaccharides at concentrations of 25, 50, 75, and 100 μg/ml increased the viability, ability to produce reactive oxygen intermediates and nitrogen intermediates of resting neutrophils. Superoxide detection, degranulation assay and adhesion assay suggested that chitooligosaccharides at concentrations from 50 to 150 μg/ml reduced PMA-induced activation of neutrophils.

Keywords: Neurophil; Chitooligosaccharide; PMA-activated

1. Introduction

Chitosan has been widely used in pharmaceutics, foods and cosmetics because of its renewable, nontoxic, biocompatible, and biodegradable properties (Hiroshi, Takashi, & Toru, 2001). Chitooligosaccharides can be obtained by chemical and enzymatic hydrolysis of chitosan. In addition, chitooligosaccharides have been demonstrated to have antimicrobial, antibacterial, antitumour and antiangiogenesis properties (Chung, Kuo, & Chen, 2005; Harish Prashanth & Tharanathan, 2005; Pranoto, Rakshit, & Salokhe, 2005; Yukita, Asano, Okamoto, Mizutoni, & Suzuki, 2000). Moreover, it affects the mitogenic response and the chemotactic activities of animal cells.

Neutrophils are active phagocytic cells which not only facilitate the production of oxygen intermediates and nitrogen intermediates but also contain primary and secondary granules in response to microbial infection. In general, they are the primary cell type to bind to inflamed endothelium and extravasate to the tissues (Babior, 2000). PMA irritates the neutrophils by associating and activating PKC directly (Liao, Lou, Ma, & Wu, 2005). In the acute phase response (APR), neutrophils are excessively activated and a large amount of cytokines are released from these cells which then lead to critical damages to tissues and organs (Middleton, Kandaswami, & Theoharides, 2000). Inhibition of excessive activation of neutrophils is of great benefit for protecting the body from the damage during the APR.

It has been reported that lipopolysaccharides (LPS) can change β_2 integrin activation of human neutrophils. Also, LPS could make CD62L (L-selectin) shedding and stimulate intracellular production of oxidant species (Ruchaud-Sparagano, Ruivenkamp, Riches, Poxton, & Dransfield, 1998). Increase in NOS activity, L-arginine transport and its utilization, free radical generation and anti-oxidant enzyme have been proved in the PMNs after LPS administration (Sethi, Sharma, & Dikshit, 2001). Furthermore, TLR2 has been well documented to play a key role in inhibiting LPS-induced decrease of neutrophil apoptosis (Goshima, Kotani, Lowry, & Calvano, 2004). Previous studies suggested that *N*-acetyl-D-glucosamine (GlcNAC) oligomer could induce chemotactic migration while

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D-glucosamine (GlcN)-induced chemotactic and chemokinetic migration of canine neutrophils. The GlcN oligomer-induced slight enhancement of chemiluminescence of canine neutrophils to zymosan. Neither oligomers displayed cytotoxicity for canine neutrophils (Yasuyuki, Yoshiharu, Takahiro, Yoshihiro, & Saharo, 1998). Phosphated-chitin has been reported to exert protective effects by restoring deformability of neutrophils when the cells were made to loose migrating ability to mimic the in vivo condition as it occurs during acute lung injury (Khanal et al., 2002).

In the present study, we investigated the effects of chitooligosaccharides on the resting and PMA-activated neutrophils separated from the whole blood of New Zealand white rabbit.

2. Experimental

2.1. Materials

PMA (phorbal myristate acetate), MTT (Thiazolyl blue), NBT (nitroblue tetrozolium), TMB (3, 3, 5, 5-tetromebenaidine), fibronectin (fragment 1977–1991), *p*-Nitroaline, *N*-SUCCINYL-ALA-ALA-ALA, Cyto B, RPMI-1640, and HBSS were purchased from Sigma.

2.2. Preparation of chitooligosaccharides (COS)

COS was prepared from enzymatic hydrolysis of chitosan (the degree of *N*-acetylation is below 5%) and separated with membrane (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999). In brief, chitosan (0.5 g) was dissolved in 2% AcOH (10 ml), and then the PH of the solution was adjusted to 5.6. Enzyme mixture (5 mg) in 0.05 mol/L acetate buffer was added and the mixture was incubated for 40 min at 40 °C. The reaction was stopped by boiling for 10 min. The hydrolyzates were fitered on a hollow-fiber membrane. Excess crude COS was added to ethanol and the mixture was stirred forming an supersaturated solution and stored at 4 °C overnight. The insoluble precipitate was filtered under vacuum through Whatman-1# filter paper. The obtained COS solution with a concentration of ~20% (v/v, COS/ethanol) was concentrated to dryness with a rotary evaporator under diminished pressure. An aliquot of COS was dissolved in water and analyzed with a matrix-assisted laser desorption-ionization time-of-flight mass spectrometer.

2.3. Separation of neutrophils

Neutrophils were isolated from heparin anticoagulated whole blood of New Zealand white rabbit. In brief, the isolation method includes density gradient centrifugation (S.G. = 1.085) and gelatin sedimention as described previously (Fairbaim, Page, Lees, & Cunningham, 1993). Above 95% surviving rates and purities were demonstrated by Trypan blue exclusion and Wright–Gimesa stains respectively. Neutrophils were resuspended in RPMI 1640 (for MTT assay and NO assay) or in PBS and in HBSS (for degranulation assay) for the tests below.

2.4. MTT assay

The viability of neutrophils was determined using the previously described MTT assay (Metello et al., 2001). MTT was prepared at 5 mg/ml in 0.1 M PBS and was stored at 4 °C. Ninety microliters of neutrophils suspension (2×10^5 cells/ml) was incubated in 96-well microtiter plates. Ten microliters of COS at final concentrations of 0, 25, 50, 75, and 100 μg/ml were added to each well and incubated at 37 °C for 44 h. Then 20 μl of MTT solution was added to each well and incubated at 37 °C for 4 h. After incubation, 100 μl of DMSO was added to each well to dissolve the formazan product, and pipetted to mix the product. The absorbance of each well was measured at an optical density of 570 nm using 630 nm as reference filter with a microplate reader.

2.5. NO estimation

NO_2^- accumulation was used as an indicator of NO production as previously described (Young et al., 2005). Neutrophils were plated at 2×10^5 cells/ml and incubated with COS at final concentrations of 0, 25, 50, 75, and 100 μg/ml at 37 °C for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 10 min. Nitrite production was determined by measuring the optical density at 550 nm.

2.6. Superoxide anion (O_2^-) detection

O_2^- production was measured by the NBT (nitroblue tetrozolium) reducing test (Zhou, Fa, Jiang, & Lu, 1998). Two groups were set: (1) 90 μl neutrophils (2×10^5 cells/ml) + 10 μl COS (final concentrations of 0, 25, 50, 75, 100, 125, 150 μg/ml); (2) 80 μl neutrophils (2×10^5 cells/ml) + 10 μl COS (final concentrations of 0, 25, 50, 75, 100, 125, and 150 μg/ml) + 10 μl PMA (10^{-7} M, final concentration). One hundred microliters NBT (0.2%) was added to the above 2 groups after incubation for 20 min at 37 °C, cells were collected by centrifugation and DMSO solution was added to dissolve the formazan product in the incubation. Then colorimetric analysis was carried out at 570 nm to measure O_2^- production.

2.7. Degranulation assay

Elastase and myeloperoxidase (MPO) are two enzymes in azurophil granules of neutrophils and secreted to the cell exterior on degranulation. For the two analyses, experimental groups were set as follow: 80 μl neutrophils (2×10^5 cells/ml) + 10 μl COS (final concentrations of 0, 25, 50, 75, 100, 125, and 150 μg/ml) + 10 μl PMA

(10^{-7} M, final concentration). In the measurement of elastase production, neutrophils were co-incubated with substrate *N*-SUCCYNL-ALA-ALA-ALA (2 mM) at 37 °C for 4 h, colorimetric analysis was carried out at 405 nm (Quade & Roth, 1997). In the measurement of MPO secretion, supernatants were collected after 30-min-incubation at 37 °C to determine the activity of MPO with 2.5 mM TMB as substrate. Neutrophils were lysed with cetyltrimethylammonium bromide (0.02% in water) to determine total MPO activity. Colorimetric analysis was carried out at 450 nm (Zhou, Niewiarowski, & Stewart, 1995).

2.8. Adhesion assay

We chose 30 min as the time interval for measuring the adhesion to fibronectin of PMA-activated neutrophils as previously described (Liao et al., 2005). Ninety-six-well microtitre plates were coated with 10 μg/ml fibronectin dissolved in PBS and blocked with 2% BSA. Plates were then stored at 4 °C, immediately prior to the assay, the plates were pretreated at 37 °C for 10 min. Experimental groups were set up as follows: 80 μl neutrophils (2×10^5 cells/ml) + 10 μl COS (final concentrations of 0, 25, 50, 75, 100, 125, and 150 μg/ml) + 10 μl PMA (10^{-7} M, final concentration). Nonadherent neutrophils were removed by PBS washing for three times, adherent neutrophils were stained with crystal violet (0.25%). Inner cell dye was dissolved with glacial acetic acid (10%). Then colorimetric analysis were carried out at 570 nm using Microplate Reader (Foster, McCabe, Sanjar, & Cunningham, 1997).

2.9. Statistical analysis

Statistical analysis was performed using Student's *t*-test. MTT and NO assays were carried out in quintuplicate and repeated twice, other assays were carried out in triplicate and repeated three times.

3. Results and discussion

1. Fig. 1 showed the TOF-MS of COS sample.
2. The effects of COS on resting neutrophils.
3. The MTT assay of rabbit neutrophils incubated with COS show that four concentrations of COS increased the viability of neutrophils in vitro. There is a peak at the concentration of 75 μg/ml as shown in Fig. 2.
4. The effects of COS on production of NO are shown in Fig. 3. When COS were at the concentration of 25 μg/ml, the production of NO was slightly reduced, but at the following concentrations the production of NO was significantly heightened.
5. The effects of COS on O_2^- production of resting neutrophils are shown in Fig. 4. COS increased the reactive oxygen intermediates-O_2^- at the concentration from 50 to 150 μg/ml.

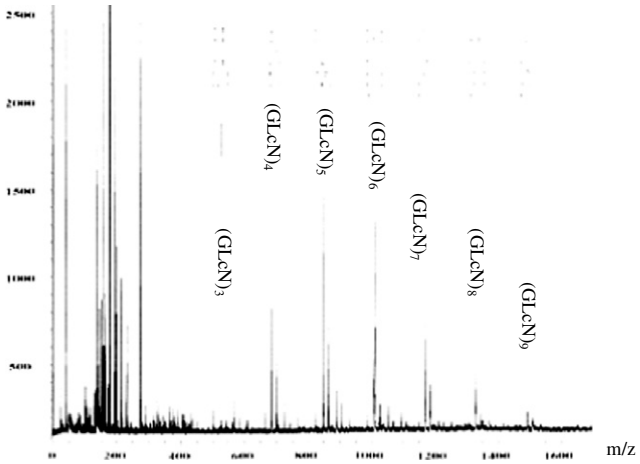

Fig. 1. TOF-MS of chitooligosaccharide sample.

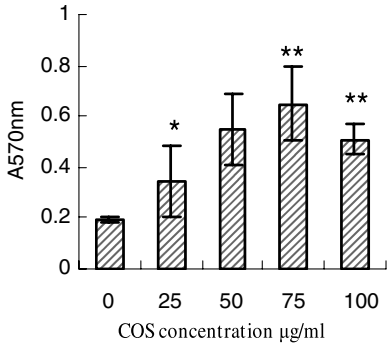

Fig. 2. The effects of chitooligosaccharides on viability of neutrophils. Data were displayed as mean ± S.D. for five groups and two respective experiments. *$p < 0.05$, **$p < 0.001$ vs control.

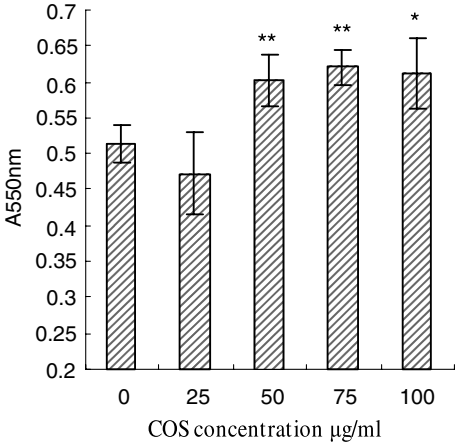

Fig. 3. The effects of chitooligosaccharides on production of NO. Data were displayed as mean ± SD for five groups and two respective experiments. *$p < 0.05$, **$p < 0.001$ vs control.

We found that COS upregulated the functions of resting rabbit neutrophils. COS increased the following ability of resting neutrophils: (1) viability in vitro; (2) the production of reactive nitrogen intermediates-NO; (3) the production of reactive oxygen intermediates-O_2^-. Neutrophils have a

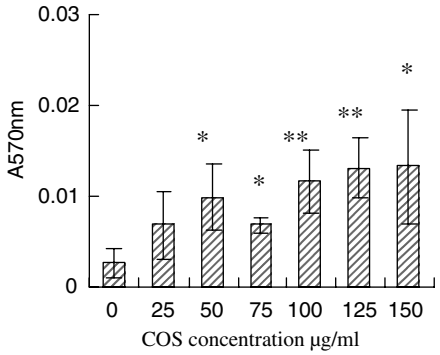

Fig. 4. The effects of COS on production of O_2^- of resting neutrophils. Data were displayed as mean ± SD in triplicate and three respective experiments. *$p < 0.05$, **$p < 0.001$ vs control.

life span of only a few days in vivo; the life time is much shorter in vitro (Whyte, Meagher, MacDermott, & Haslett, 1993). When incubated with COS, the viability of neutrophils was distinctly enhanced. Activated neutrophils produced a number of reactive oxygen intermediates and reactive nitrogen intermediates that had potent antimicrobial activity (Monfardini, Burvenich, Massart-leen, Smits, & Paape, 1999). O_2^- was extremely toxic to ingest microorganisms and also generated other powerful oxidizing agents, including hydroxyl radicals and hydrogen peroxide. NO also could combine with the superoxide anion to yield even more potent antimicrobial substances. Co-incubation with COS made resting neutrophils activated maybe by enhancing the viability and the production of O_2^- and NO.

3.1. The effects of COS on PMA-activated neutrophils

1. COS decreased O_2^- production of PMA-activated neutrophils. Fig. 5. showed that COS decreased the O_2^- production of PMA-activated neutrophils and prominently decreased at concentrations of 25, 50, and 75 μg/ml.
2. Figs. 6 and 7, represented the degranulation assay of PMA-activated neutrophils incubated with COS. COS at the concentration of 50 μg/ml markedly decreased the secretion of myeloperoxidase. Fig. 6. showed that COS at the concentrations of 25, 50, 75, and 100 μg/ml decreased the secretion of elastase, and there is a prominent decrease at the concentration of 100 μg/ml.
3. Fig. 8, showed that COS decreased the adhesion to fibronectin of PMA-activated neutrophils, there were prominent decrease at the concentrations of 100, 125, and 150 μg/ml.

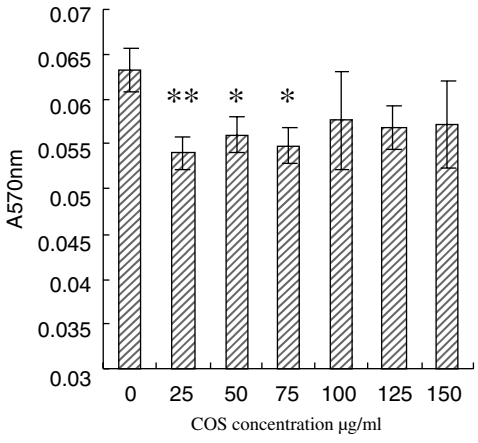

Fig. 5. The effects of COS on the O_2^- production of PMA-activated neutrophils. Data were shown as mean ± SD for three experiments. *$p < 0.05$, **$p < 0.001$ vs control vs control.

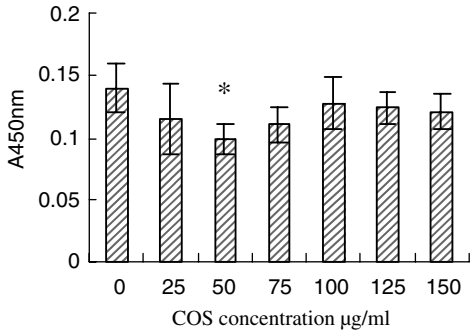

Fig. 6. The effects of COS on the secretion of myeloperoxidase of PMA-activated neutrophils. Data were shown as mean ± SD in triplicate for three experiments. *$p < 0.05$ vs control.

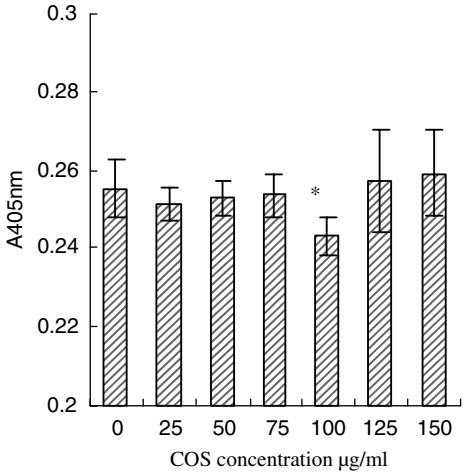

Fig. 7. The effects of COS on the elastase secretion of PMA-activated neutrophils. Data were shown as mean ± SD in triplicate for three experiments. *$p < 0.05$ vs control.

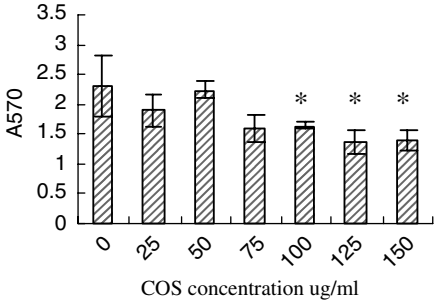

Fig. 8. The effects of COS on the adhesion to fibronectin of PMA-activated neutrophils. Data were shown as mean ± SD in triplicate for three experiments. *$p < 0.05$ vs control.

Though the involvement of neutrophils is the first line against bacterial invasion and is an important component of the inflammatory response, constant activation of neutrophils definitely does harm to the host. Neutrophils are the first line against microbial pathogens invasion, on the other hand they can damage the tissues by degranulation and releasing some cytokines when they are recruited into the inflammatory location (Kanashiro et al., 2004). Previous experiments have shown COS had the effects of antimicrobial and stimulate immune response. The results of our experiments demonstrated that COS downregulate the functions of PMA-activated neutrophils. But the inhibitory effect on PMA-activated neutrophils is not on a dose-dependent mean.

4. Conclusion

By means of MTT assay and nitrite determination and NBT reduction assay, COS were demonstrated that it was a positive regulator of resting neutrophils. The viability and production of NO and O_2^- of resting neutrophils were significantly increased by incubated with different concentrations of COS. At this state the neutrophils had stronger antimicrobial ability. It was proved that COS was a negative regulator of PMA-activated neutrophils by means of NBT reduction and degranulation and adhesion assays. The excessively stimulated state of neutrophils was inhibited by incubation with COS. These observations implied a potential application of COS in treatment with inflammatory response. However, whether or not the structure and degree of polymerization of COS are related to the functions on neutrophils is not clear, further studies should be done to elucidate this problem.

References

Babior, B. (2000). Phagocytes and oxidative stress. *American Journal of Medicine, 109*, 33–44.

Chung, Y. C., Kuo, C. L., & Chen, C. C. (2005). Preparation and important functional properties of water-soluble chitosan produced through Maillard reaction. *Bioresource Technology, 13*, 1473–1482.

Fairbaim, S. M., Page, C. P., Lees, P., & Cunningham, F. M. (1993). Early neutrophil but not eosinophil or platelet recruitment to lungs of allergic horses following antigen exposure. *Clinical and Experimental Allergy, 23*(10), 821–828.

Foster, A. P., McCabe, P. J., Sanjar, S., & Cunningham, F. M. (1997). Agionist-induced adherence of equine eosinophils to fibronectin. *Veterinary Immunology and Immunopathology, 56*, 205–220.

Goshima, M., Kotani, J., Lowry, S. F., & Calvano, S. E. (2004). LPS-induced decrease of neutrophil apoptosis is regulated through TLR2, not through TLR4. *Journal of The American College of Surgeons, 199*(3), 38.

Harish Prashanth, K. V., & Tharanathan, R. N. (2005). Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis. *Biochimica et Biophysica Acta, 1722*, 22–29.

Hiroshi, U., Takashi, M., & Toru, F. (2001). Topical formulations and wound healing applications of chitosan. *Advanced Drug Delivery Reviews, 52*, 105–115.

Kanashiro, A., Kabeya, L. M., Polizello, A. C., Lopes, N. P., Lopes, J. L., & Lucisano-Valim, Y. M. (2004). Inhibitory activity of flavonoids from Lychnophora sp. on generation of reactive oxygen species by neutrophils upon stimulation by immune complexes. *Phytotherapy Research, 18*, 61–65.

Khanal, D. R., Miyatake, K., Okamoto, Y., Shinobu, T., Morimoto, M., Saimoto, H., et al. (2002). Phosphated chitin (P-chitin) exerts protective effects by restoring the deformability of polymorphonuclear neutrophil (PMN) cells. *Carbohydrate Polymers, 48*, 305–311.

Liao, X. L., Lou, B., Ma, J., & Wu, M. P. (2005). Neutrophils activation can be diminished by apolipoprotein A-I. *Life Sciences, 77*, 325–335.

Metello, I., Adriana, M., Giuseppe, P., Giuseppe, M., Beate, W., & Domenico, D. P. (2001). Involvement of oxygen radicals in cytarabine-induced apoptosis in human polymorphonuclear cells. *Biochemical Pharmacology, 61*, 1033–1040.

Middleton, J. E., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*, 673–751.

Monfardini, E., Burvenich, C., Massart-leen, A. M., Smits, E., & Paape, M. J. (1999). Effect of antibiotic induced bacterial clearance in the udder on L-selectin shedding of blood neutrophils in cows with *Escherichia coli* mastitis. *Immunopathology, 67*, 373–384.

Pranoto, Y., Rakshit, S. K., & Salokhe, V. M. (2005). Enhancing antimicrobial of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT-Food Science and Technology, 38*, 859–865.

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology and Immunopathology, 58*(3–4), 239–248.

Ruchaud-Sparagano, M. H., Ruivenkamp, C. A., Riches, P. L., Poxton, I. R., & Dransfield, I. (1998). Differential effects of bacterial lipopolysaccharides upon neutrophil function. *FEBS Letters, 430*, 363–369.

Sethi, S., Sharma, P., & Dikshit, M. (2001). Nitric oxide and oxygen-derived free radical generation from control and lipopolysaccharide (LPS)-treated rat polymorphonuclear leukocyte. *Nitric Oxide, 5*(5), 482–493.

Whyte, M. K. B., Meagher, L. C., MacDermott, J., & Haslett, C. (1993). Impairment of functional in aging neutrophils is associated with apoptosis. *Journal of Immunology, 150*, 5124–5134.

Yasuyuki, U., Yoshiharu, O., Takahiro, T., Yoshihiro, S., & Saharo, M. (1998). Effects of *N*-acetyl-D-glucosamine and D-glucosamine oligomers on canine polymorphonuclear cells in vitro. *Carbohydrated Polymers, 36*, 137–141.

Young, H. K., Kyung, L. W., Jun, H. L., Shin, K., Tae, J. L., Eun, M. J., et al. (2005). 8-Hydroxyquinoline inhibits iNOS expression and nitric oxide production by down-regulating LPS-induced activity of NF-κB and C/EBPβ in Raw 264.7 cells. *Biochemical and Biophysical Research Communications, 329*(2), 591–597.

Yukita, A., Asano, M., Okamoto, T., Mizutoni, S., & Suzuki, H. (2000). Suppression of ascites formation and re-accumulation associated with human ovarian cancer by anti-VPF monoclonal antibody in vivo. *Anticancer Research, 20*, 155–160.

Zhang, H., Du, Y. G., Yu, X. J., Mitsutomi, M., & Aiba, S. I. (1999). Preparation of chitooligosaccharides from chitosan by a complex enzyme. *Carbohydrate Research, 320*(3–4), 257–260.

Zhou, D., Niewiarowski, S., & Stewart, G. J. (1995). Efficient flexible method for assay of human granulocyte elastase. *Analytical Biochemistry, 224*(1), 436–437.

Zhou, W., Fa, X., Jiang, X., & Lu, S. (1998). Effect of substance P on the functions of human polymorphonuclear neutrophilic leucocytes. *Chinese Journal of Microbiology and Immunology, 18*(2), 124–127.