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The Use of Chitosan Nanoparticles for Delivery of CpG ODN in Treatment of Allergic Balb/C Mice

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Abstract

Background: This study aims to prepare high stability chitosan nanoparticles (CNP) and examine the ability of CNP in CpG-ODN delivery when treating allergic mice model.

Methods: Preparation and characterization of CNP were performed by ionic gelation, dynamic light scattering, and zeta sizer. The CNP cytotoxicity and activation ability of CpG ODN delivered with CNP were tested using a cell counting kit-8 and Quanti blue method. Allergic mice were injected intraperitoneal with 10 ug ovalbumin on day 0 and 7, and then treated with intranasal CpG ODN/CpG ODN, delivered with CNP/CNP, on the third week three times per week for three weeks. The ELISA method measured cytokine and IgE profiles in the allergic mice's plasma and spleen.

Results: CNP results have sizes 27.73 nm±3.67 dan 188.23 nm±53.47, spherical in shape and non-toxic, and did not alter the NF-κB activation of CpG ODN in RAW-blue cells. The application of CpG ODN delivered by chitosan nanoparticles shows no statistical difference between groups of IFN-γ, IL-10, and IL-13 in Balb/c mice's plasma and spleen, in contrast with IgE level.

Conclusions: The results showed that using chitosan nanoparticles as a delivery system for CpG ODN has the potency to safely CpG ODN efficacy.

Keywords: Allergy, Chitosan nanoparticle, CpG ODN, Immunotherapy, Mice spleen.

Introduction

The prevalence of allergic diseases increases every year. Nowadays, 300 million people worldwide have asthma. It is estimated that asthma will affect an additional 100 million people by 2025 (1, 2). The common manifestations of allergies are allergic rhinitis, asthma, food allergy, dermatitis, and anaphylaxis (3). To treat these allergies, approaches include avoidance of the allergen, pharmacologic treatments, and/or immunotherapy. The concept behind immunotherapy is that the immune system can be desensitized to specific allergens, thereby eliminating allergic symptoms. Immunotherapy is proven to be effective; it is a form of therapy that aims to stimulate or restore the body's ability to fight infection and disease (4).

CpG ODN is a TLR9 agonist, a synthetic form of CpG deoxynucleic acid (CpG DNA). The unmethylated CpG DNA sequence is expressed in bacteria and recognized by TLR9. TLRs are a family of pattern recognition receptors (PRRs) expressed on multiple cell types that recognize various microbial products. There are nine different functional TLRs in humans, namely TLR1 through TLR9. TLR is a type I integral

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membrane glycoprotein, containing leucine-rich repeats flanked by characteristic cysteine-rich motifs in its extracellular region. TLR9 plays a role in allergic diseases; it is expressed mainly in the intracellular compartment of plasmacytoid dendritic cells (pDC) and B lymphocytes. Prior to stimulation, TLR9 is in the endoplasmic reticulum (ER) and translocated to the Golgi complex and lysosomes after stimulation by ODN (CpG or non-CpG). The binding of CpG-ODN with TLR9 causes an immunity shift from the T-helper (Th) 2 phenotype to Th1, along with a maturation of regulatory T cells (Tregs) (5, 6).

Due to the negatively charged plasma membrane, targeting the intracellular compartment with negatively charged CpG ODN is complicated. This condition necessitates the vital role of a drug delivery system (7). Therefore, nanoparticles are used to deliver immunotherapy drugs to target cells. One of the most widely used nanoparticles is the chitosan nanoparticle (CNP). Chitosan is a chitin derivative that is biodegradable and has high bioavailability. Chitosan is positively charged and is mucoadhesive. The positive charge of chitosan may cause it to easily attach to the negatively charged cell membrane. Additionally, chitosan continuously releases active substances in small doses (8, 9).

Our prior study prepared CNP by ionic gelation, which showed that synthetized chitosan nanoparticles have two dispersion peaks, with positive zeta potential and no cytotoxicity to RAW264 cells. However, the chitosan nanoparticles showed poor stability due to less than 10 mV zeta potential (10). Our in vitro experiment using peripheral blood mononuclear cells from allergic rhinitis patients and healthy persons as a control shows that the administration of CpG ODN delivered by chitosan nanoparticles did not significantly increase IFN-γ and IL-10, while decreasing IL-13 and IgE (unpublished data). This study aims to prepare high stability CNP and examine the ability of CNP in CpG-ODN delivery when treating allergic mice model.

Materials and Methods

Chitosan Nanoparticles preparation

Chitosan nanoparticles were prepared using the ionic gelation method, following Mardliyati (11), with a slight modification. A 0.2% chitosan powder (Polyscience. Inc) was dissolved in 0.3% acetic acid (Merck), and the solution was stirred overnight with a magnetic stirrer and then filtrated using a 0.45 µm syringe filter (Millipore). A 0.1% tripolyphosphate (TPP) (Chameleon reagent), was dissolved in distilled water and then filtered using a 0.22 µm syringe filter (Millipore), and the TPP solution was then added to the chitosan solution, with the combined solution being stirred for one hour on a magnetic stirrer at room temperature. The solution was dialyzed using dialysis membrane (Spectra, MWCO:3500) in phosphate buffer saline pH 7.4 overnight at temperature of 4°C.

Nanoparticle characterization

Nanoparticles were characterized using dynamic light scattering (DLS) (DLS-8000) with a He-Ne laser for size determination. Zeta potential was analyzed with a zeta sizer (ELS-Z version 3500/2.13. Otsuka Electronic. co, LTD). CNP were mixed to 0, 0.9 uM, 1.8 uM, 4.5 uM and 9 uM final concentration of CpG ODN then incubated for 15 min before zeta potential measurement. The shape of the nanoparticles was examined under a transmission electron microscope (TEM) (Microscope tecnai 200kV D2360).

Viability of RAW-blue cells stimulated with chitosan nanoparticles

RAW-blue cells (Invivogen) were added by nanoparticles in a serial dilution to evaluate the viability of RAW-blue cells under chitosan nanoparticles stimulation. The cell viability was analyzed using the cell counting kit-8 method (Sigma-Aldrich). A 100 µL of 5 x 10^4 cells/µL RAW-blue cell was pipetted into a 96-well plate and incubated overnight, while a 10 µL serial dilution of chitosan nanoparticles was added into the plate and incubated overnight as well. After adding 10 µL CCK-8 and incubating plate for four hours, the

absorbance was measured using a 450 nm spectrophotometer.

NF-κB activation analysis

A quanti blue assay (Invivogen) was used to determine the NF-κB activation ability of CpG ODN (Fasmac) with or without chitosan NP in RAW-blue cells. A 190 µL of 5 x 10^4/µL RAW-blue cells were pipetted into a 96-well plate. After the addition of 10 µL samples, the plate was incubated overnight in a 0.5% CO2 incubator. After harvesting the cells' product due to the separation of supernatant, 10 µL of the supernatant was pipetted into another 96-well plate, while adding a 90 µL Quanti-blue solution. The absorbance of the solution was measured using a 630 nm microplate reader for several incubation time including one, two and three hours.

Animals

Mice

The in vivo study protocol was approved by PT. Bimana Indomedical Bogor Animal Care and Use Committee (ACUC) No.R.04-17-IR. Balb/c mice, aged eight to ten weeks, were obtained from PT. Indoani Lab; they were acclimated to room temperature and given ad libitum food for two weeks.

Allergy stimulation and treatment

Earlier, a preliminary in vivo study of the allergic mice model was reported (12). Twenty-five Balb/c mice were divided into five groups, including a healthy control group (group I), an allergic control group (group II), a CpG ODN and CNP treatment group (group III), a CpG ODN treatment group (group IV), and a CNP treatment group (group V). For allergy stimulation in groups II through V, mice were injected intraperitoneally with 10 µg low endo purified ovalbumin (Worthington) in 2% alhydrogel Vacci Grade™ (Invivogen) (1:1) on day 0, and they received a booster with an equal dose on day 7. Then, the mice were dosed with 10 µL intranasal ovalbumin (5 mg/mL) in 0.9% NaCl (1:1) in each nostril on days 21, 22, 23, 28, 29, 30, 35, 36, and 37. The first group of mice was injected with sterile phosphate buffer saline (PBS) and dosed with 0.9% NaCl using a similar dosing schedule (13).

A CpG ODN and nanoparticle treatment was applied one hour after administering the intranasal ovalbumin, with a preparation 10 µg (45 µM) final concentration of CpG ODN with CNP (1:1). For group III, a 10 uL solution was applied intranasally following each ovalbumin dose in each nostril. Group IV was treated with the previous concentration of CpG ODN without chitosan nanoparticles, and group V was treated with 10 uL 0.2% CNP in each nostril.

After the final treatment, on day 38 the mice were sacrificed, and the blood and spleen were collected and processed for the next measurement. Whole blood was collected in an EDTA anticoagulant tube; it was then centrifuged for 15 minutes at 800g. Supernatant was collected and kept at -20^0C.

ELISA measurement

The mice's anti-ovalbumin IgE Elisa kit (Cayman chemical), IFN gamma, IL-10, and IL-13 Elisa kit (Invitrogen), were used to examine anti-ovalbumin IgE and cytokine level. The captured antibody was diluted and coated onto a 96-well half area microplate (Corning costar) overnight at 4^0C. After washing, the microplate was added with a blocking buffer for one hour. Standard and samples were pipetted into the microplate and incubated for two hours. The remaining solution was discarded, and the microplate was washed with 175 uL wash buffer three times. A 50 uL secondary antibody was then added to the wells, followed by a room-temperature incubation period of one hour. The remaining solution was discarded, and the microplate was washed in the same manner as previously mentioned. An HRP enzyme was added to the microplate and incubated for 30 minutes at room temperature. After the washing procedure, for a total of five times, TMB solution was added to the wells. The microplate was then incubated for about 30 minutes and then added with stop solution.

Finally, the microplate was measured at 450 nm using a microplate reader.

Statistical analysis

For statistical analysis, we used IBM SPSS Statistic version 20 software. A normality test using the Shapiro Wilk test and Levene's homogeneity test was performed, followed by ANOVA, where post hoc LSD to analyze between groups with $P< 0.05$ is considered significant. Data transformation was performed for abnormally distributed data. For abnormally distributed and non-homogeneous data, a non-parametric Kruskal Wallis statistical test was performed, followed by the Mann Whitney test for analysis between groups, where $P< 0.05$ is considered significant. Normally distributed data was presented in mean±SD, while median (min-max) is designated for the abnormal data. The Pearson test was used to correlate normally distributed data, while the Spearman test was used for abnormally distributed data.

Results

Chitosan nanoparticle characteristics

CNP's size is presented in table 1. The data shows that there were double peaks of CNP, 13.3±1.6 nm and 185.5±46.8 nm respectively. Dialyzed CNP show double peaks as well, 38.8±5.7 nm and 207.5±61.1 nm, while dialyzed and filtered CNP's size are 27.7±3.7 and 188.2±53.5 nm respectively. The zeta potential of CNP shows that the chitosan nanoparticles have a positive charge (Fig. 1). While it was bound with negatively charged CpG ODN, it maintained a stable positive charge until 1.8 µM final concentration. The shape of the nanoparticles was observed under a transmission electron microscope and the spherical shape of the chitosan nanoparticles was found (Fig. 2).

Table 1. Nanoparticle's size measured by dynamic light scattering

Nanoparticles	First peak (nm)	Second peak (nm)
Chitosan nanoparticles without dialysis	13.3±1.6	185.5±46.8
Dialyzed chitosan nanoparticles	38.8±5.7	207.5±61.1
Dialyzed and filtered chitosan nanoparticles	27.7±3.7	188.2±53.5

Triple measurement was performed for each sample.

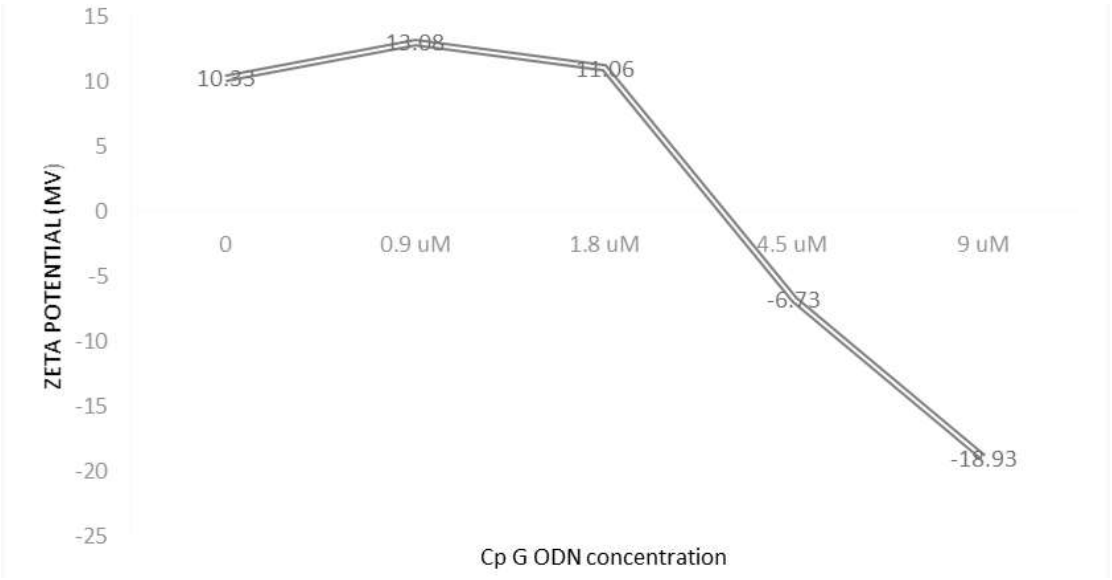

Fig. 1. Zeta potential of CpG ODN bound CNP measured by Zeta sizer. CNP were bound to several concentrations of CpG ODN and incubated for 15 min before zeta potential measurement.

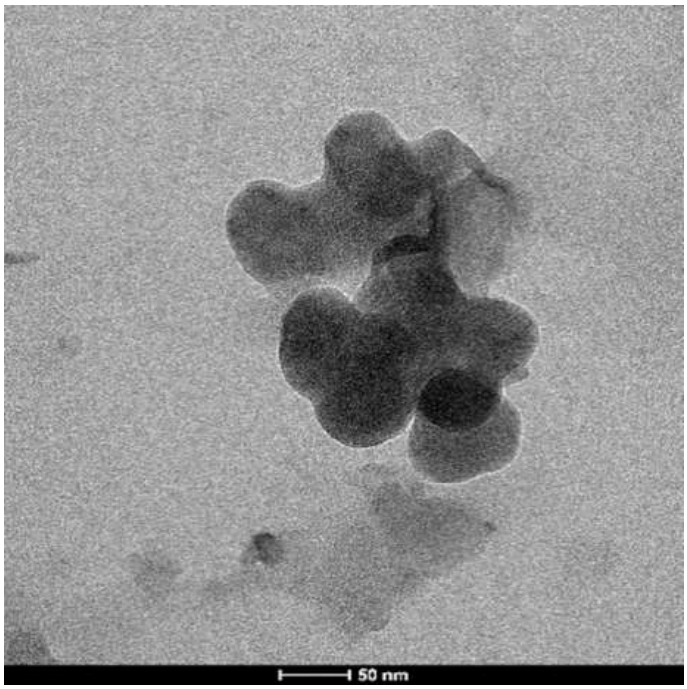

Fig. 2. Shape of chitosan nanoparticles observed under transmission electron microscope

Viability of RAW-blue cells stimulated with chitosan nanoparticles

RAW-blue cells showed more than 90% viability while stimulated with several dilution of CNP including 100%, 25%, 12.5% and 6.25% (Fig. 3).

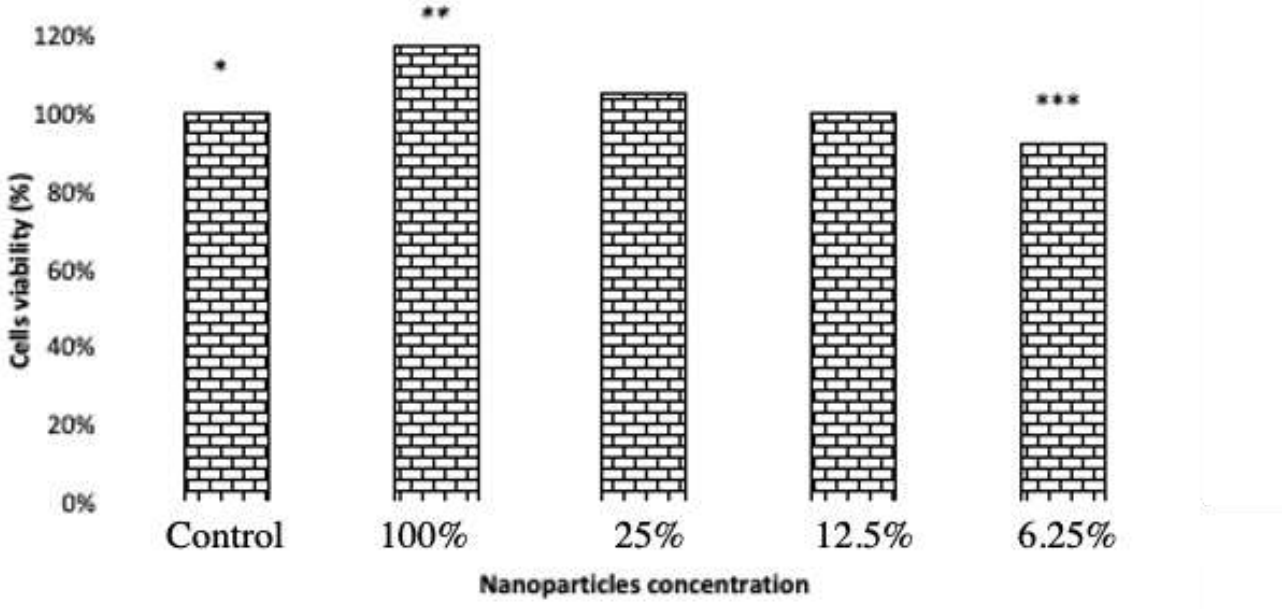

Fig. 3. Viability of RAW-blue cells stimulated with chitosan nanoparticles for 4 hours (7 repetition).
*There was a significant difference with groups 100% and 25% (P< 0.05, Kruskall Wallis, post hoc Mann Whitney).
**There was a significant difference with groups 25%, 12.5%, and 6.25% (P< 0.05, Kruskall Wallis, post hoc Mann Whitney).
***There was a significant difference with groups 25% and 12.5% (P< 0.05, Kruskall Wallis, post hoc Mann Whitney).

NF-κB activation

The study the ability of CpG ODN with or without chitosan nanoparticles to activate NF-κB showedthat there was no significant difference between 1 hour, 2 hours and 3 hours incubation period in the ability of NF-kB activation (Fig. 4).

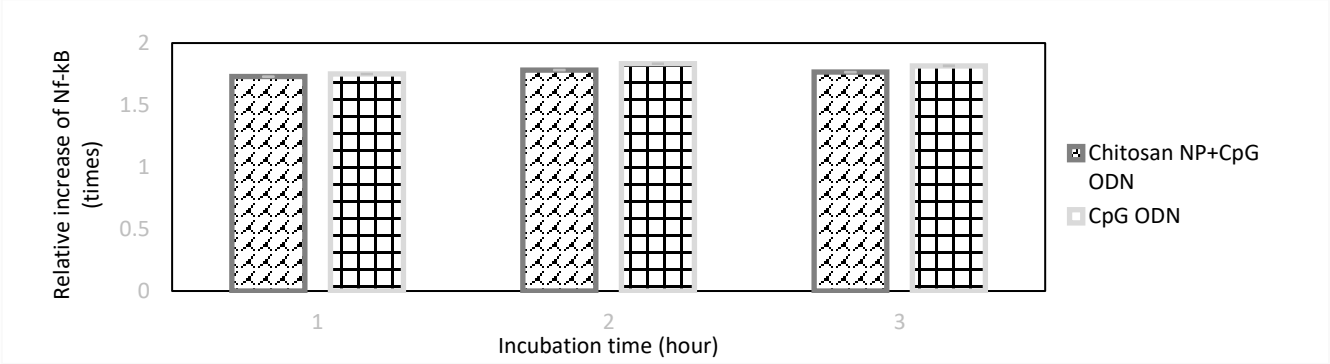

Fig. 4. The relative increase of NF-kB activation on RAW-blue cells after stimulation with CNP+CpG ODN or CpG ODN compared to PBS control (5 repetition). No significant difference between groups of 1-hour, 2-hour, and 3-hour incubation periods (independent t test, P> 0.05)

Cytokine levels of allergic mice

The comparison of cytokine levels of the mice's plasma did not show statistical difference between the groups in plasma concentration in IFNγ, IL-10, and IL-13 (Fig. 5). However, nanoparticles tend to enhance the ability of CpG ODN to stimulate IFN production. On the other hand, CpG ODN also stimulates IL-10 production. The CpG ODN showed no apparent suppression of IL-13 synthesis on Balb/C mice.

The IFNγ concentration in the group stimulated by CpG ODN+chitosan, 4.16 pg/mL, (0.8-5.6), the group stimulated by CpG ODN (3.12 pg/mL (1.5-42), and the chitosan-stimulated group 2.5 pg/mL (0.3-9.2) was higher when compared to the allergy control (2.2 pg/mL (0.2-17.5) and non-allergic control (2.15 pg/mL (0.8-4.1). In the non-parametric test, Kruskall Wallis did not show a significant difference between the treatment groups of mice (P> 0.05).

The highest IL-10 concentration was in the group stimulated by CpG ODN (190 pg/mL±124.9). When compared, the concentration of IL-10 in the group stimulated with CpG ODN+chitosan (167.0 pg/mL±35.9) and the group stimulated with chitosan (150 pg/mL±79.5) was higher than the allergy control (118.3pg/m L±32.6). Meanwhile, the lowest IL-10 concentration was in the non-allergic control (105 pg/mL±8.7). In the parametric statistical test after transformation, there was no significant difference between the groups (P> 0.05, ANOVA).

The IL-13 concentration in the CpG ODN group (24 pg/mL±2.7), chitosan group (1.8 pg/mL±1.4), and CpG ODN+chitosan group (1.4 pg/mL±1.2) was higher than the concentration of IL-13 in the allergic (1.3 pg/mL±0.8) and nonallergic controls (1.0 pg/mL±1.0). There was no significant difference between the groups (P> 0.05, ANOVA) in the plasma cytokine concentrations of Balb/c mice is presented (Fig. 5).

The IFN-γ concentration of the chitosan stimulated group of the mice's spleen (1.181 pg/mg±0.403) and the CpG ODN nanoparticle chitosan group (1.078 pg/mg±0.155) was higher than the control group (0.763 pg/mg±0.583) and the allergy group (1.011 pg/mg±0.877) (Fig. 6). The group stimulated by CpG ODN (0.686 pg/mg±0.588) had the lowest IFN-γ concentration of the four groups of mice. Based on the ANOVA statistical test results, there was no significant difference between treatment groups (P> 0.05).

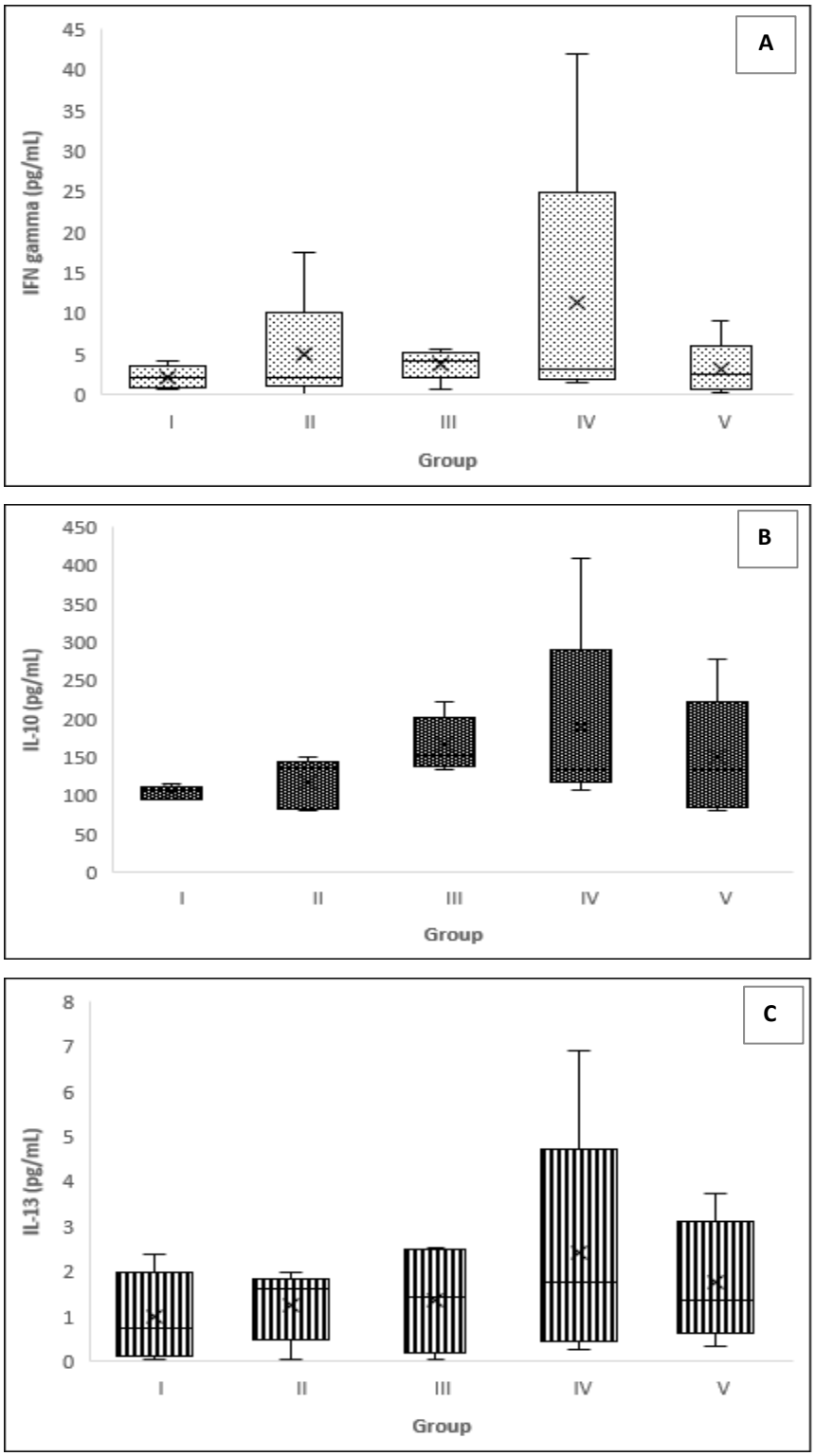

Fig. 5. Comparison of cytokine levels in mice's plasma. (A) IFN-gamma, (n = 5/group, P> 0.05, Kruskal-Wallis test), (B) IL-10, (n = 5/group, P> 0.05. ANOVA test), (C) IL-13(n = 5/group, P> 0.05. ANOVA test). Group I: non-allergic control; group II: allergic control; group III: CpG ODN bound nanoparticle chitosan treatment; group IV: CpG ODN treatment; and group V: nanoparticles treatment. X: mean value.

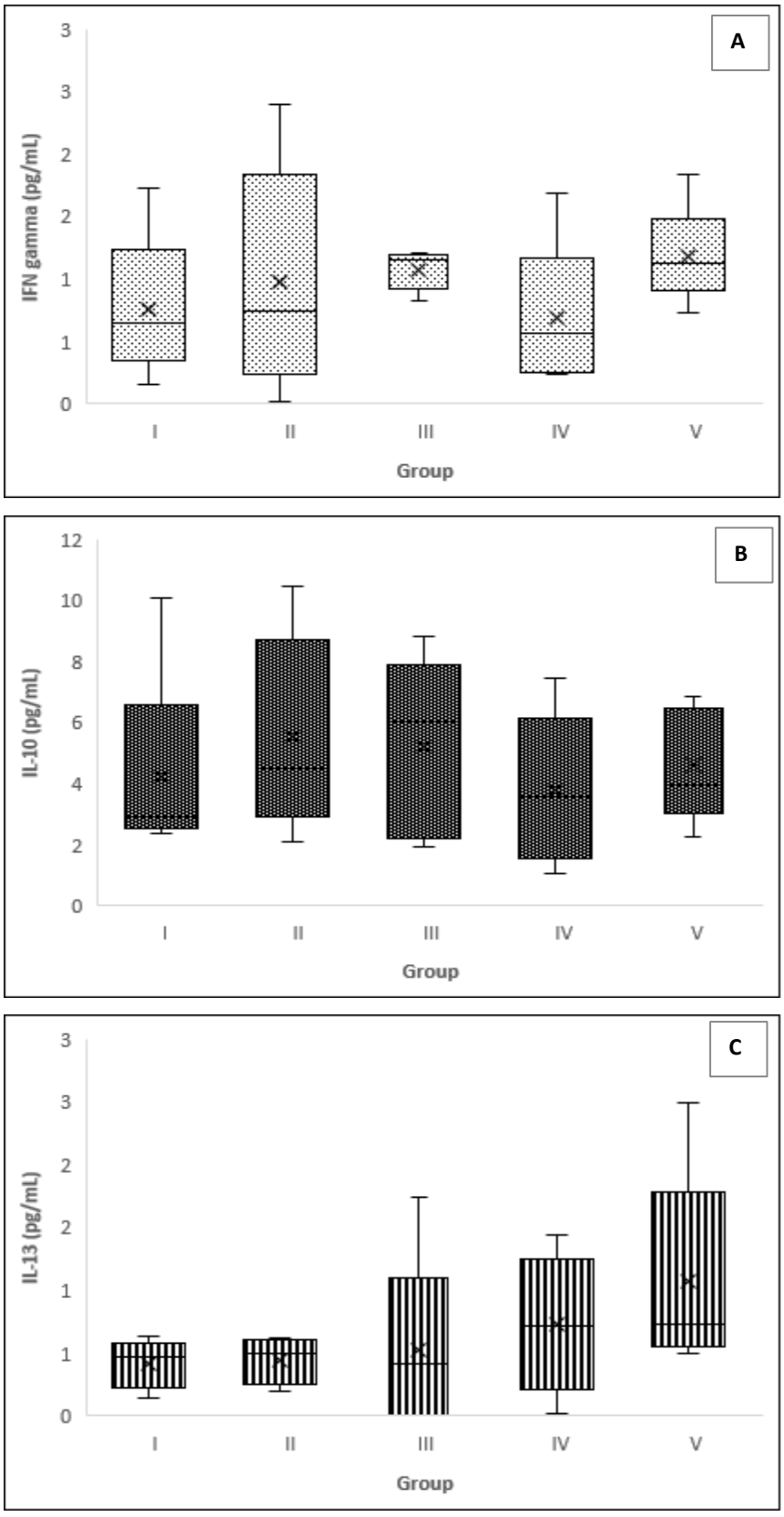

Fig. 6. Comparison of cytokine levels in mice's spleen. A. IFN-γ concentrations in spleens of Balb/c mice (n = 5/group, P> 0.05. ANOVA test). B. IL-10 concentration in spleens of Balb/c mice (n = 5/group, P> 0.05, Kruskal-Wallis test). C. IL-13 concentration in spleens of Balb/c mice (n = 5/group, P> 0.05, Kruskal-Wallis test). Group I: non-allergic control; group II: allergic control; group III: CpG ODN bound nanoparticle chitosan treatment; group IV: CpG ODN treatment; and group V: nanoparticles treatment. X: mean value.

The highest IL-10 concentration was found in the group stimulated by CpG ODN chitosan nanoparticles (6.048 pg/mg (1,932-8,856), followed by the allergy group with a concentration of IL-10 (4.520 pg/mg (2.121-10.450). Furthermore, the group treated with chitosan had an IL-10 concentration of 3.961 pg/mg (2.275-6.858). The CpG ODN group (3.575 pg/mg (1.041-7.474) was lower than the chitosan group, and the lowest IL-10 concentration was in the control group (2.933 pg/mg (2.374-10.010), which can be seen in Figure 1. Additionally, the IL-10 levels were not normally distributed, so a non-parametric Kruskal-Wallis test was performed—the statistical test did not show a significant difference between treatment groups (P> 0.05).

The results obtained in this study showed that the IL-13 concentration in the group stimulated by CpG ODN + chitosan (0.465 pg/mg (0.000-1.739) was lower than in the control group (0.472 pg/mg (0.138-0.633) and the allergic group 0.502 pg/mg (0.202- 0.627). Meanwhile, the group that was treated with CpG ODN stimulation (0.722 pg/mg (0.026-1.441) and chitosan (0.738 pg/mg (0.505-2.492) alone had higher concentrations than the control and allergy groups. This is presented in the form of a box-plot graph in Figure 6. The non-parametric Kruskal-Wallis test was performed because the data was not normally distributed. The test did not show a significant difference between the treatment groups (P> 0.05).

Immunoglobulin E levels

The comparison of IgE levels in the mice's plasma and spleen is presented in Figure 7, in which it shows that the highest concentration of anti-ovalbumin specific IgE was in the allergy control group 1951 ng/uL (971.2-3120.2), while the lowest concentration was in the non-allergic control group 9.6 ng/uL (4.8-43.3). The concentration of specific IgE anti ovalbumin in the group given CpG ODN+chitosan was 1439.9 ng/uL (1072.1-4927.9), the group given CpG ODN was 1555.3 ng/uL (312.5-2269.2), and the group given chitosan was 1617.8 ng/uL (836.5-3067.3). The three treatment groups were lower than the allergy control group. After transformation, the parametric statistical test showed significant differences between the non-allergic control group and the allergic group, the CpG ODN+chitosan, CpG ODN, and chitosan groups (P< 0.05 ANOVA, post hoc LSD).

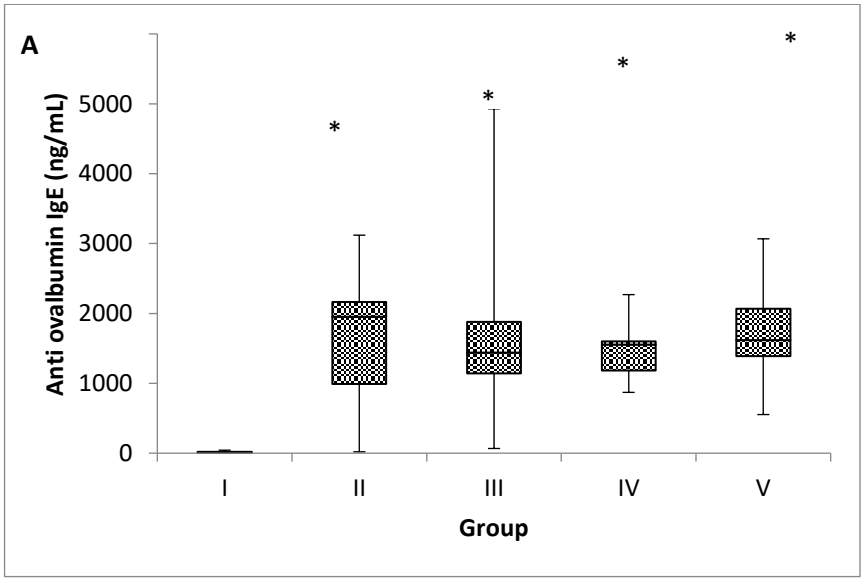

Fig. 7. Comparison of IgE levels in mice's plasma (A) and spleen (B). Group I: non-allergic control; group II: allergic control; group III: CpG ODN bound nanoparticle chitosan treatment; group IV: CpG ODN treatment; and group V: nanoparticles treatment. *There was a significant difference with the non-allergic control group (P< 0.05, ANOVA, post hoc LSD). #There was a significant difference with the non-allergic control group (P< 0.05, Mann Whitney). X: mean value.

The IgE Specific Ovalbumin concentration in the mice's spleen in the control group was 0.050 ng/mg (0.025-0.232), the allergic group was 5.829 ng/mg (1.003-8.520), the group stimulated with CpG ODN + chitosan was 5.797 (0.913-18.752), the CpG ODN stimulated group was 1.619 (0.807-11.367), and the chitosan stimulated group was 0.910 (0.069-5.932). Based on this data, it is clear that the allergy group has the highest IgE levels, with the control group having the lowest levels. The other three groups were somewhere between the control and allergy groups, with the highest in the CpG ODN chitosan nanoparticles group, the second was in the CpG ODN group, and the third was the chitosan treated group. After the Kruskal-Wallis non-parametric statistical test was performed, there was a significant difference ($P< 0.05$), which led to the Mann-Whitney test following the statistical test. Significant differences were found between control group to the other groups ($P< 0.05$). There were no significant differences between the CpG ODN CNP treated group, the CpG ODN treated group, and the group that underwent chitosan stimulation ($P> 0.05$).

Discussion

Chitosan is a heteropolysaccharide, deacetylated product of chitin, which consists of linear β-1,4 linked units (14, 15). Chitosan has emerged in biomedical applications due to its mucoadhesive properties, lower toxicity, biodegradability, and biocompatibility (16). Chitosan is soluble in an acidic environment due to protonation of the amine group. Its positive charge allows for the preparation of nanoparticles by ion gelation using multivalent anions, including tripolyphosphate (TPP). Under acidic conditions, chitosan may crosslink with TPP to form nanoparticles (17, 18).

This study prepared chitosan nanoparticles by using the ionic gelation method. Ionic gelation was reported as a common method for preparing reproducible, monodisperse, and nanoscale chitosan particles (19). This method was chosen to ease preparation, control, and decrease the necessity of organic solvents (20). The other study reported the preparation of chitosan/TPP nanoparticles ranging in sizes of 100 to 615 nm (18, 21, 22). Chitosan nanoparticles in this study were prepared with 0.2% chitosan, 0.3% acetic acid, and 0.1% TPP (chitosan:TPP ratio=5:1). The chitosan and TPP volume ratio affects the distribution pattern (number of peaks formed), particle size, and stability (11). Our study resulted in dialyzed and filtered nanoparticles with 27.73 nm±3.67 and 188.23 nm±53.47. This nanoparticle shows spherical morphology when conducting TEM observation. Factors influencing the nanoparticles' characteristic are chitosan-TPP concentration and ratio, chitosan molecular weight, pH and ionic strength of preparation medium (17), acetic acid concentration, and temperature during the cross-reaction (20).

Moving to a new area, the Zeta potential is related to colloid stability. This study resulted in relatively stable positively charged nanoparticles (+10.33 mV) compared to our previous study, which resulted in +3.mV nanoparticles (10). The Zeta potential of the plasma membrane of human cells varies, ranging from -19.4 mV on HeLa cells to -31.8 mV on erythrocytes (23). For this reason, the use of positively charged nanoparticles is essential in bridging the attachment of negatively charged synthetic oligonucleotide, such as CpG ODN, with the plasma membrane. In this study, our nanoparticles still showed a positive charge after interacting with 1.8 uM CpG ODN. Our nanoparticles showed to be non-toxic, confirmed by the viability of RAW blue cells being more than 90% after incubation. In addition, it is shown that chitosan nanoparticles did not alter the ability of CpG ODN to activate the NF-kB.

Nanoparticles have spherical, triangular, cubic, hexagonal, oval, prism, rod, and helical shapes. In our study, the chitosan nanoparticles had spherical shape. Spherical shaped nanoparticles are less toxic than other shapes due to their ease of being endocytosed. Besides shapes, other factors can increase the toxicity of nanoparticles: size, surface area, surface charge, composition, crystalline structure, aggregation, concentration, surface coating, and surface

roughness. We tested our chitosan nanoparticles' toxicity with RAW-blue cells. The RAW-blue cells had more than 90% viability which means our chitosan nanoparticles are non-toxic. We examined the activation of NF-κB using a Quanti-Blue test after incubating RAW-blue cells with CpG-ODN chitosan nanoparticles. As a result, NF-κB activity increased after incubation (24).

It is also important to understand that the allergic symptoms varied, from a mild urticaria to severe anaphylaxis (3, 4). However, the treatments only relieved the symptoms, not the disease (25). Therefore, it is for this reason why allergy treatment is an interesting topic to discuss. This study used Balb/c mice due to their easy reproduction, low cost, and sensitivity to Ovalbumin (26, 27). There were 25 mice in total, divided into five groups with five mice in each. The first group was the control group; second group was the allergy group; the third group was treated with CpG ODN CNP; the fourth group was treated with CpG ODN; and the fifth group was treated with chitosan nanoparticles (12). We chose plasma and the spleen for our sample because of their roles in the immune system, especially the interaction between APCs and lymphocytes in the spleen (28). APCs express TLR9 that recognizes CpG DNA in bacteria. In humans, TLR9 can recognize CpG ODN, a synthetic form of CpG DNA (29). CpG-ODN was reported to improve the imbalance of Th1/Th2 cytokines, which manifested in allergic reactions (30). CpG ODN needs a delivery system (i.e., nanoparticles) because it can be efficiently degraded (31). In this study, we examined IFN-γ as a Th1 type cytokine, IL-10 as a T-regulator cytokine, and IL-13 as a Th2 type cytokine. IgE was examined in this study to observe the improvement of allergic symptoms.

Cytokine levels

IFN-γ is a glycosylated protein, weighing 25 kDa, produced by lymphocytes type Th1 (32). IFN-γ suppresses IL-4 mediated Th2 polarization. These cytokines promote the differentiation of naïve T cells to Th2 type cells, inhibiting Th1 activation. In a study conducted by Peng et al., the spleens of mice treated with CpG ODN produced more IFN-γ than non-treated mice. The increased IFN-γ and IL-12 production, as well as the decreased IL-4 production, indicate a shift from the Th2 to Th1 type immune response after CpG ODN treatment (33). In this study, when compared with the control and allergy groups, the group treated with CpG ODN showed higher IFN-γ levels in the plasma, but the group also showed lower IFN-γ levels in the spleen. The problem with the application of CpG ODN is that it is easy for CpG ODN to degrade due to DNase (34). The use of chitosan nanoparticles in this study plays a role in the delivery system to increase the effectiveness of CpG ODN action. This importance of the nanoparticles is shown by the concentration of IFN-γ in the group treated with CpG ODN chitosan nanoparticles being higher than the CpG ODN group. The stimulation of Th0 to Th1 maturation was seen from the increase in the concentration of IFN-γ in the CpG ODN chitosan nanoparticles group, compared to the allergy group. However, there was no statistical significance. The group treated with chitosan showed the highest spleen IFN-γ concentration, compared to all groups of mice. Wu et al. found that chitosan can induce macrophage activation and the production of TNF-α, IL-6, IFN-γ, and nitric oxide (35). The control group showed that the lowest IFN-γ concentration might be caused by the absence of stimulation due to PBS administration.

IL-10 is a potent anti-inflammatory cytokine that plays an essential role in preventing inflammatory and autoimmune pathologies. The primary sources of IL-10 include Th cells, monocytes, macrophages, and dendritic cells. Regulatory T cells (Treg) produce IL-10, functioning in allergy suppression and maintaining immune tolerance (30, 36). CpG-ODN is a synthetic oligonucleotide that binds to its intracellular receptors, TLR9. CpG-ODN can trigger a signaling cascade by binding to TLR9 in APC. This cascade results in cross-presentation of antigens and production of Th1 cytokines, such as IL-12, and the regulatory cytokine, IL-10 (37). In a study conducted by

Kim et al., it was shown that IL-10 and Treg cell concentrations did not significantly increase after administering intranasal CpG ODN. Our study is in line with their study, showing that the spleen IL-10 concentration in the CpG ODN-treated group alone was lower than the allergy group. In contrast, Oliveira et al. found an increase in IL-10 concentration (37). In line with Oliveira's study, our study found that the plasma IL-10 concentration in the CpG-ODN group was the highest compared to other groups. The effect of using chitosan nanoparticles optimized the role of CpG ODN in this study, and the CpG OGN chitosan nanoparticles treated group showed higher concentrations than the allergy group. Although there was an increase in IL-10, no statistical significance was found. The control group had the lowest IL-10 concentration among the other groups in the absence of stimulation.

IL-13 is a Th2 cytokine produced by various types of cells, particularly from Th2 cells. The effect of IL-13 on immune cells is similar to IL-4 because these cytokines share the same receptor subunit (α subunit). IL-13 plays an essential role in allergic inflammation by promoting bronchial hyperreactivity and excessive mucous production. IL-13 also promotes isotype switching from B cells to produce IgE. In a mouse model, IL-13 is an essential mediator for allergic asthma (38). Intranasal CpG ODN treatment has been shown to reduce the specific response to allergic asthma in mice, when compared to untreated mice. There was a decrease of airway hyper response, eosinophils in nasal flush fluid, total IgE, specific IgE, IL-13, and IL-5, when compared to non-ODN treated mice (30, 37). Our study found that the CpG ODN chitosan nanoparticles treated group showed a decrease of IL-13 concentrations in the spleen; however, the plasma IL-13 concentration was not affected. It seems that CpG ODN delivery using chitosan nanoparticles in this study tends to increase the action of CpG ODN shifting the Th2 to Th1 response. This study is in accordance with Kim et al., where the IL-13 concentration of both plasma and spleen in the CpG ODN treated group was higher than the allergic group. This finding may be caused by DNase degraded CpG ODN (34). Plasma and spleen IL-13 concentrations in the chitosan nanoparticles treated group were also higher than the allergy group. These results contradict Li et al., which states that low molecular weight oligosaccharide chitosan has an anti-inflammatory effect (39). However, IL-13 levels were not statistically significant.

IgE Level

In allergic individuals, once the allergen has been captured by pDC, the pDC produces cytokines that direct Th0 to Th2 maturation. This secretion of cytokines induces allergen-specific IgE production by plasma cells. CpG ODN will induce the secretion of IL-12 and differentiation of Th0 cells into Th1 cells by stimulating pDC and B cells. IFN-γ secreted from Th1 cells will decrease the Th2 response and inhibit IgE production (29). A study conducted by Peng et al. showed that after administration of CpG ODN, the specific IgE concentration was suppressed up to 90% compared to the allergic control mice (33). Our result is consistent with their study's finding. The chitosan nanoparticle CpG ODN treated group produced lower concentrations of Ovalbumin-specific IgE than the allergic group, similarly with the CpG ODN treated and chitosan treated groups.

Limitation

Due to technical difficulties, this in vivo study solely examined plasma and spleen samples. The result can be more representative if a measurement can be performed on the treatment site, including the nasal cavity and other respiratory tracts. We recommend observing the bronchoalveolar lavage fluid (BALF) to evaluate the mice's immunological response after administration of CpG ODN CNP. In addition, measurement of other cytokines, such as IL-4, IL-5, IL-12, and IL-6, can also be performed due to their role in allergic diseases.

In this study, we prepared chitosan nanoparticles in a spherical shape with a diameter of 27.73 nm±3.67 dan 188.23

nm±53.47 on average; they were also non-toxic and did not alter the activation of NF-κB in CpG ODN in RAW-blue cells. The application of CpG ODN delivered by chitosan nanoparticles shows an increase of IFN-γ and IL-10 levels in plasma and spleen cells. In contrast, IL-13 levels in the spleen and IgE levels in both plasma and the spleen decreased. The application of chitosan nanoparticles as a delivery system for CpG ODN has the potency to enhance safety and CpG ODN efficacy.

Institutional review board statement

The study was conducted according to the guidelines on the Declaration of Helsinki and approved by Animal Care and Use Committee of PT. Bimana Indomedical No. R.04-17-IR and The Health Research Ethics Committee Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital No.: KET-971/UN2.F1/ETIK/PPM.00.02/2020.

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Conflicts of Interest

All the authors stated there was no conflict of interest of this study.

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