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SCREENING OF IMMUNOSTIMULATORY ACTIVITY FROM INDONESIAN KAMPUNG (*GALLUS DOMESTICUS*) EGG WHITE WATER EXTRACT: *IN VITRO* STUDY

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ABSTRACT

The most popular Indonesian native chicken, known as kampung chicken, is maintained under free-range conditions therefore it is prone to high environmental stress. Indonesian native chicken and its product are always regarded as having health benefits compared to commercial chicken by Indonesian society. But there is still limited report regarding Indonesian native chicken and its product. This study focused on screening immunostimulatory activity from Indonesian native chicken egg white using *in vitro* approaches as a functional food. Indonesian native chicken egg white (NEW) was extracted using distilled water supplemented to human-human hybridoma HB4C5 cells to examine the IgM production stimulating activity using ELISA. The gene expression was also examined using qRT-PCR. The ability of NEW on stimulating immunoglobulin production by mouse splenocytes was analyzed. Commercial egg white water extract (CEW) was used as a comparison. The data were analyzed using One-way ANOVA and continued by using post-hoc analysis using Tukey's multiple comparison test. The results showed that NEW and CEW modulated IgM production by the HB4C5 cells 8.72-fold and 6.75-fold, respectively, compared to control. NEW stimulated immunoglobulin (Ig) production by the mouse splenocytes higher than CEW. To conclude, NEW provides an immunostimulating activity that can potentially act as a health-promoting food.

Keywords: gallus domesticus, HB4C5 cells; immunoglobulin production; immunostimulatory activity; Indonesia native chicken egg white

ABSTRAK

Ayam asli Indonesia yang paling banyak diternakkan, dikenal dengan nama ayam kampung, dipelihara dengan keadaan diumbar sehingga rentan terhadap tekanan lingkungan yang tinggi. Ayam Kampung dan produknya oleh masyarakat Indoneisa selalu dianggap memiliki manfaat kesehatan dibandingkan dengan ayam komersil. Namun penelitian terkait ayam kampung dan produknya masih terbatas. Penelitian ini difokuskan pada skrining aktivitas imunostimulasi dari putih telur ayam kampung dengan menggunakan pendekatan *in vitro* sebagai potensi bahan pangan fungsional. Pertama, putih telur kampung diekstraksi terlebih dahulu menggunakan akuades. Kemudian ekstrak akuades telur kampung (NEW) ditambahkan ke sel HB4C5 untuk dilakukan pemeriksaan aktivitas stimulasi IgM menggunakan ELISA. Selain itu, ekspresi gen menggunakan qRT-PCR juga diperiksa. Tidak hanya itu, kemampuan NEW dalam menstimulasi produksi imunoglobulin pada splenosit tikus juga dianalisis. Ekstrak akuades putih telur komersial (CEW) digunakan sebagai perbandingan. Data dianalisis menggunakan ANOVA satu arah dan dilanjutkan analisis posthoc menggunakan uji perbandingan berganda Tukey. Hasil penelitian menunjukkan bahwa NEW dan CEW menstimulasi produksi IgM oleh sel HB4C5 masing-masing 8,72 kali lipat dan 6,75 kali lipat dibandingkan dengan kontrol. Tidak hanya itu, NEW menstimulasi produksi immunoglobulin (Ig) oleh splenosit tikus lebih tinggi dari CEW. Sebagai kesimpulan, NEW menyediakan aktivitas imunostimulan yang berpotensibertindak sebagai makanan yang dapat meningkatkan kesehatan.

Kata kunci*:* aktivitas immunostimulant; gallus domesticus; produksi immunoglobulin; putih telur ayam kampong; sel HB4C5

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INTRODUCTION

Eggs are widely known as a nutritious food source containing essential nutrients such as amino acids, unsaturated fatty acids, vitamins, minerals, and folate. Egg protein has always been regarded as an excellent source of bioactive peptides distributed in the egg white and egg yolk amounting to 50% and 40%, respectively (Kovacs-Nolan et al., 2005; Mine, 2007). Besides their nutritional properties, some bioactivities such as antibacterial, anti-inflammatory, antihypertensive activities were also observed and contributed to the health-promoting functions (Lee et al., 2018; Tagashira et al., 2018).

Recently, there is a high demand for natural products, especially food-source, as health-promoting agents. This is due to the increasing evidence of immunological disease (Nieman and Wentz, 2019). Immunostimulatory agents are essential to regulate immune response by enhancing the optimal defensive capacity of human immunity (Veldhoen and Brucklacher-Waldert, 2012). Thus, fresh and innovative idea approaches are needed to develop.

Researchers found that certain daily nutritional interventions are advantageous compared to chemical compounds as immunostimulants in terms of side effects and price (Veldhoen and Brucklacher-Waldert, 2012). Some bioactive protein fractions obtained from food products were reported to have immunostimulatory effects. Besides, animal protein is considered to be more nutritious and effective in improving innate and adaptive immunity, resulting in the enhancement of the human immune system from infection and disease (Chalamaiah et al., 2017).

Previous studies added that egg white water-soluble protein fractions could modulate the immune system through the innate and adaptive immune system. Based on *in vitro* study, ovotransferrin, ovomucin, and lysozyme were found to modulate the immune system through the innate immune mechanism (Tanizaki et al., 1997; Lee et al., 2018; Tagashira et al., 2018). Meanwhile, lysozyme was found to be a strong candidate as an immunomodulator due to its ability on stimulating adaptive immunity through immunoglobulin M (IgM) production by HB4C5 cells (Sugahara et al., 2000).

There are few factors that influence the composition and concentration of egg white, such as the wide variety of chicken breeds, physiological status, and the age of the laying hens (Robert, 2004; González Ariza et al., 2021). A previous study reported that the traditional breed had a different egg white concentration. This might be due to immunological adaptation, reflecting their original need to fight pathogens under environmental stress of free-rearing of traditional chicken (Bílková et al., 2018). Moreover, Javůrková et al. (2019) found out that there was a close correlation between eggshell pigmentation in traditional egg and the egg concentration, especially lysozyme and ovotransferrin. They explained that tinted eggshell on the traditional egg was more likely to have a higher lysozyme component compared to other traditional eggs. Another research on characterizing the protein component in traditional egg also reported lysozyme and other egg white components vary widely in traditional egg (Bílková et al., 2018).

Kampung chicken (*Gallus domesticus*) is well known as an Indonesian native chicken. Indonesian native chicken is maintained under free-range conditions; therefore, they are prone to environmental stress (Muladno, 2008). In addition to that, the differences in strain and feed of native chickens and commercial layer chickens cause differences in the eggs' exterior and interior quality (Robert, 2004). So far, the study conducted by Wulandari et al. (2015) has reported that the lysozyme isolated from Indonesian native chicken egg worked as an antibacterial agent towards *Staphylococcus aureus* and *Escherichia coli*. Ragland and Criss (2017) found that the ability of lysozyme to work as antibacterial was correlated with its ability to modulate immune system because components released from bacteria in a lysozyme-dependent manner can alter innate immune function. However, the reports on the effect of the Indonesian native chicken egg white on immunological aspects are not available.

Considering all the previous reports, it is reasonable to evaluate the immunostimulatory

activity of Indonesian native chicken egg white using *in vitro* approaches. Thus, this finding could be beneficial for the search for the new and effective natural immunostimulatory agent as well as potential functional food agent.

MATERIALS AND METHOD

Materials

A total of 90 eggs from commercially available Indonesian native chicken egg (*n*=45) and commercial chicken egg (*n*=45) was purchased from Yogyakarta, Indonesia. The eggs were kept in the refrigerator no more than three days before further analysis. ERDF medium was purchased from Kyokuto Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS), insulin, transferrin, ethanolamine, and sodium selenite were obtained from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-human IgM antibody, horseradish peroxidase (HRP)-conjugated anti-human IgM antibody, goat anti-mouse IgM, goat anti-mouse IgG, rabbit anti-mouse IgA, HRP-goat anti-mouse IgM, HRP-goat anti-mouse IgG, and HRP-goat anti-mouse IgA were obtained from Invitrogen (Carlsbad, CA, USA). Oxalic acid, ABTS (2.2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), trypan blue solution, and Sepasol were obtained from Nacalai Tesque (Kyoto, Japan). MMLV reverse transcriptase was from Promega (Madison, WI, USA), Thunderbird SYBR qPCR Mix was obtained from Toyobo (Osaka, Japan).

Characterization of albumen from Indonesian native egg and commercial egg

The shell cleanliness of prepared egg was checked through direct observation. First, the eggshells were cracked open. The presence of meat spot and bloodspot inside the egg was checked through direct observation. The albumen and yolk were separated. The height of the thick albumen was measured within a tripod micrometer at three different points. The height of albumen was measured to describe the quality of the egg. The egg white was then pooled, homogenized, and further lyophilized. The moisture and protein content of albumen from each egg was measured using the method from Association of Official Analytical Chemist (AOAC) method (AOAC, 2005) with three replications.

Water extraction of egg white samples

Lyophilized Indonesian native egg and commercial egg white was dissolved in distilled water and stirred for 24 h at 10°C. After that, they were centrifuged at 25,000 × g at 4°C for 20 minutes to remove insoluble substances. Then, supernatant was adjusted to pH 7.4 and sterilized using 0.22 µm filter. The sterilized samples were kept in the refrigerator and used as egg white water extracts for further experiments.

HB4C5 cell culture and assay condition

This experiment utilized human hybridoma cell line HB4C5 cells. This cell line was used to assess the IgM production stimulation activity of egg white samples. HB4C5 cells were cultured in ERDF medium supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2.

The ability of NEW and CEW on stimulating IgM production was examined by measuring the concentration of IgM secreted by HB4C5 cells into the culture medium.—HB4C5 cells were sub-cultured in ERDF medium supplemented with 10 µg/mL of insulin, 20 µg/mL of transferrin, 20 µM ethanolamine, and 25 nM sodium selenite (ITES-ERDF medium). HB4C5 cells were inoculated in 96-well culture plate at cell density of 3×10^5 cells/well in ITES-ERDF medium supplemented with various concentrations of NEW or CEW (0.04 mg/mL, 0.16 mg/mL, 0.625 mg/mL, 2.5 mg/mL, and 10 mg/mL). Distilled water was added to ITES-ERF medium instead of sample as control. After six (6) hours incubation period, the supernatant was collected, and the concentration of IgM secreted into the culture medium was measured by the enzyme-linked immunosorbent assay (ELISA).

First, 100 µL of the primary antibody using goat anti-human IgM antibody diluted 1000 times with 50 mM carbonate buffer as coating solution was applied to the 96-well plate and incubated at 4°C overnight. Phosphate-buffered saline with Tween

20 (PBS-T) was used three times as a washing solution in between the reaction. After that, each well was blocked with PBS containing 5% skim milk for two hours at 37°C. Next, each well was treated with 50 µL of culture supernatant and distilled water as a control for one hour at 37°C. Next, each well was treated with washing solution. After that, 100 µL of secondary antibody consisting of HRP-conjugated anti-human IgM antibody diluted 2,000 times with PBS containing 5% skim milk was added to each well and incubated for one hour at 37°C. Then, 0.6 mg/mL of ABTS dissolved in 50 mM citrate buffer (pH 4.0) containing 0.03% H_2O_2 was applied to the well at 100 µL/well. After that, 1.5% oxalic acid was added to the well to stop the reaction. The absorbance was measured at 415 nm with 655 nm as the reference wavelength (Sugahara et al., 2000).

Determination of cell viability

Trypan blue dye exclusion test was conducted to evaluate the cytotoxicity of samples to HB4C5 cells. HB4C5 cells were inoculated at concentration 3×10^5 cells/well with various concentration of NEW and CEW (0.04 mg/mL, 0.16 mg/mL, 0.625 mg/mL, 2.5 mg/mL, and 10 mg/mL) into a 24-well culture plate. In addition to that, distilled water was added as a control instead of sample. After inoculation, HB4C5 cells were cultured for 6 h in CO_2 incubator at 37°C and 5% CO_2. After that, the cell suspension was collected from each well into microtubes and centrifuged 3,000 rpm at 4°C for 10 min. The supernatant was removed, and the cell pellet was resuspended with 10 µL medium. Next, 10 µL trypan blue solution was added to each cell suspension in dark room. After 5 min, 10 µL of cell suspension was put in the haemocytometer. The number of living cells and dead cells were counted. Cell viability (%) was calculated by dividing the number of viable cells with total number of cells times 100.

Determination of gene expression using reverse transcription-polymerase chain reaction (qRT-PCR)

The effect on IgM gene expression was measured using quantitative real-time RT-PCR (qRT-PCR). The method for determining gene expression was conducted under previous experiment from Nishi et al. (2011) with few modifications. HB4C5 cells were cultured in ITES-ERDF medium at 5×10^5 cells/mL containing egg white water extract at 37°C for 6 h. The harvested cells were washed twice with PBS and stored at −80 °C. Sepasol RNA I Super G Total RNA and chloroform (Nacalai Tesque, Kyoto, Japan) were used for isolating RNA according to the manufacturer's instructions, and the isolated RNA was spectrophotometrically quantified using a Nanodrop (Biospec-nano, Shimadzu, Kyoto, Japan). The first strand cDNAs were synthesized from total RNAs using an oligo-dT primer, MMLV reverse transcriptase, and dNTP. The qRT-PCR mixture consisted of 2.5 µg of a cDNA sample, Thunderbird SYBR qPCR Mix, 10 µM forward primer, and 10 µM reverse primer were spined for 5 minutes before applying to StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative gene expression was calculated by the comparative CT method using StepOne Software v2.1 (Applied Biosystems). Data are shown as the number-fold differences in IgM expression normalized to the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), housekeeping gene, as an endogenous reference. The PCR primer sequences used in this experiment for amplification were as follows: human GAPDH 5'-GCACCGTCAAGGCTGAGAAC-3' (sense) and 5'-TGGTGAAGACGCCAGTGGA-3' (antisense); and human IgM 5'-CTCCCAAAGTGAGCGTCTTC-3' (sense) and 5'-CAGCCAGGACACCTGAATCT-3' (antisense) (Nishi et al., 2011).

Determination of Ig-production stimulating activity of egg white water extract on mouse splenocytes *in vitro*

Animal experiment was carried out under an approved protocol by Ehime University Animal Care and Use Committee and was performed under applicable guidelines and regulations. Three 6-week-old female BALB/c mice were bought from Japan CLEA (Tokyo, Japan). The mice were housed with a pelleted basal diet and water *ad libitum* in the animal room under controlled environment: 12 h light/dark cycle at a temperature of 24 ± 1°C.

The mouse splenocytes were prepared under a previous experiment by Kumalasari et al. (2012). The mice were sacrificed and the spleens were taken out from mice. The spleen was minced through 40 μm pore size mesh into the culture dish. The splenocytes suspension was centrifuged at 190 × g at 4°C for 5 min and hemolyzed 2 times using hemolysis buffer. After that, the splenocytes were washed with PBS and centrifuged at 190 × g for 5 min. Following the centrifugation, the cells were suspended in 5% FBS-RPMI 1640 medium and inoculated into each well of a 96-well culture plate at 1×10^6 cells/well. After that, the NEW or CEW was the added into the 96 well-plate. As a comparison, distilled water was also added as a control. The mixture of mouse splenocytes and the samples were incubated in a CO_2 incubator at 37°C for 48 h. The availability of cells was checked using WST-8 test. Thereafter, the IgA, IgG, and IgM secreted into culture medium were determined by ELISA as previously described by Nishi et al. (2011).

First, the 100 μL coating solution was added into a 96-well plate for 2 h at 37°C. The coating solution consisted of goat anti-mouse IgA, goat anti-mouse IgG, or rabbit anti-mouse IgM diluted 1,000-folds with 50 mM carbonate buffer. After that, the 96-well plate was washed using PBS-T. Following the washing step, each well was blocked with 250 μL of 5% skim milk-PBS solution for 2 h at 37°C to prevent non-specific binding which suppresses accurate measurement. After washing, each well was treated with 50 μL of culture supernatant for 1 h at 37°C. Distilled water was added to blank well. After incubation, the plate was washed and treated with 100 μL secondary antibody consisting of HRP-goat anti-mouse IgA, HRP-goat anti-mouse IgG, or HRP-goat anti-mouse IgM diluted 1,000 times with 5% skim milk-PBS for 1 h at 37°C. After washing the wells, 0.6 mg/mL of ABTS dissolved in 50 mM citrate buffer (pH 4.0) containing 0.03% H_2O_2 was added to the wells at 100 μL/well. The absorbance was measured using a microplate reader at 415 nm with 655 nm as the reference wavelength (to eliminate non-specific absorbance) after the addition of 1.5% oxalic acid to terminate the coloring reaction at 100 μL. The assays were triplicated.

Statistical analysis

All the data are expressed as a mean ± standard deviation (SD). The data of egg quality was analyzed using t-test. Meanwhile, all the data of immunostimulatory activity were analyzed using one-way variance (ANOVA) using SPSS (ver. 22). Tukey HSD was used as a post-hoc analysis. Values of $p<0.05$ were regarded as statistically significant.

RESULTS AND DISCUSSION

One of the utmost strategies on promoting and maintaining a human health is through human diet containing natural bioactive compound especially the one that has low toxicity to normal cells (Faria et al., 2013; Teodoro, 2019). The finding of the more effective natural compounds is a very important goal. Therefore, this research was succeeded to identify the immunostimulatory potential agent from native chicken egg white through stimulating IgM production by HB4C5 cells and antibody production by mouse splenocytes.

Characterization of Indonesian native chicken egg white

First, the characterization of the egg was conducted to understand the difference between native egg and commercial egg (**Table 1**). The interior quality of the egg was described by looking at the height of the egg white. While the chemical characteristic of egg white was described by examining the moisture and protein content. This study described that native egg has a lower egg white height than commercial egg ($p<0.05$). In addition, native egg has 86.91% moisture content, which was lower than ($p<0.05$) that of commercial egg (87.72%). In addition, the protein content of native egg showed a higher percentage ($p<0.05$) than that of the commercial egg white, which was 10.89% and 10.26%, respectively.

A good quality egg has higher thick albumen describing the freshness of the egg. As the temperature and storage time increase, the height of albumen will decrease periodically (Samli et al., 2005). Not only that, but the height of thick

albumen is an important indicator of calculating Haugh Unit (HU) as well. Haugh Unit (HU) describes the internal quality of egg based on the height of its albumen and egg weight. The height of thick albumen is directly correlated with HU. It is generally accepted that a good quality egg has higher HU (Robert, 2004). However, previous research reported that the egg's weight could be a biased factor in calculating HU and might influence the height of thick albumen(Silversides and Villeneuve, 1994; Shi et al., 2009). It is safe to say that the lower height of thick albumen in native egg was caused by the lower egg weight in native egg. The albumen height of native egg in this experiment still in the range of thick albumen height of native egg according to the previous experiment, which was 3.3-7.22 mm (Wulandari et al., 2015).

Protein content in raw native chicken egg white was slightly higher than that in commercial chicken egg white (Table 1). This result was supported by previous result (Wei et al., 2019). Previous study has reported that the water-soluble protein content in egg white was known to have immunostimulatory activity, such as ovalbumin, ovomucoid, ovotransferin, and lysozyme (Huang et al., 2012; Rupa et al., 2015; Sugahara et al., 2000). We assume that NEW contained mostly water-soluble fractions, such as ovotransferrin, ovomucin, lysozyme, unknown peptides, and amino acids. It is reasonable to say that water-soluble fraction that has immunostimulating activity in NEW is higher than in CEW.

Table 1. Comparative physical and chemical properties of fresh indonesian native chicken egg and commercial chicken egg using proximate analysis (mean ± SD, *n*=3).

Parameters	Native egg	Commercial egg	*p* value
Egg white height (mm)	4.87±1.16	8.11±1.18	<0.05
Moisture (%)	86.91±0.01	87.72±0.01	<0.05
Protein content (%)	10.89 ±0.07	10.26 ±0.01	<0.05

The effect of Indonesian native chicken egg white on IgM production and cell viability of HB4C5 cells

In order to demonstrate the immunostimulatory activity of Indonesian native chicken egg white, it was extracted using distilled water, thereafter activity on IgM-producing stimulation was evaluated using HB4C5 cells. The cells used in this experiment was the human-human hybridomas producing a monoclonal IgM. HB4C5 cells are commonly used in the screening of antibody production stimulation activity in foodstuffs.

Previous studies screened the IgM production stimulating activity from various foodstuffs using human hybridoma HB4C5 cells (Sugahara et al., 2005; Daifuku et al., 2012; Nishi et al., 2011). In current investigation, as shown in Figure 1, egg white water extract stimulated the production of IgM by HB4C5 cells in a dose-dependent manner. This result suggested that both egg white samples from Indonesian native chicken egg and commercial egg significantly enhance IgM production by HB4C5 cells. However, NEW more effectively activated IgM production compared to CEW, where NEW significantly enhanced 8.72-fold, while CEW enhanced 6.75-fold compared to the control at 20 mg/mL sample concentration.

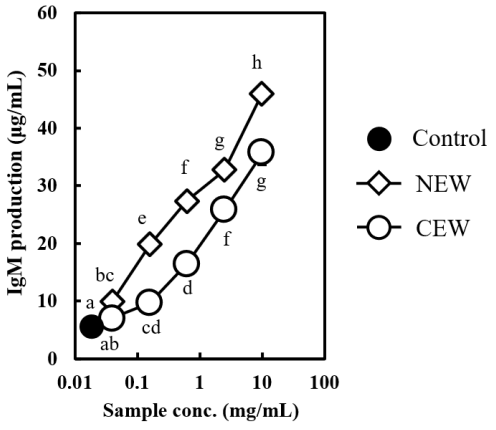

Figure 1. The effect of egg white water extract on IgM production by HB4C5 cells. means denoted by a different letter indicate significant difference between treatments (Tukey's HSD, $p<0.05 n=3$).

Interestingly, at the lowest concentration, CEW did not show any IgM production-stimulating activity (p>0.05) on HB4C5 cells. In the same concentration, NEW stimulated IgM production slightly higher than control (p<0.05). According to the relative cell viability result (Figure 2), both samples did not show cell toxicity at any concentration.

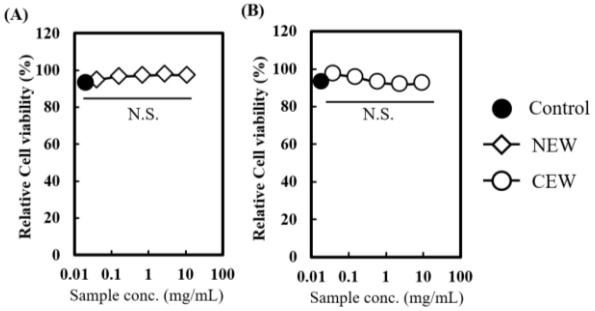

Figure 2. Cell viability of HB4C5 cells treated with egg white water extracts and control. (A) relative cells viability of HB4C5 cells treated with NEW, (B) relative cell viability of HB4C5 cells treated with CEW (Tukey's HSD, p<0.05, n=3).

The effect of NEW on IgM gene expression in HB4C5 cells

To further analyze the IgM production enhancing ability of NEW, the gene expression level of IgM in HB4C5 cells was investigated using quantitative RT-PCR. HB4C5 cells were harvested after cultivation with 150 µg/mL for 6 h. As shown in Figure 3, NEW upregulated IgM gene expression, meanwhile CEW did not sgnificantly different with control. This result showed that NEW more effectively upregulates IgM gene expression level in HB4C5 cells than CEW at the same sample concentration as NEW (p<0.05).

The immunostimulatory activity of Indonesian native chicken egg white has not been explored before. This study added new information regarding the potential immunostimulatory agent from native egg. This study revealed that NEW activates IgM production by HB4C5 cells through elevation of mRNA level. The potential active substance is water-soluble protein from egg white. This is supported by previous experiment conducted by Sugahara et al. (2000) who examine the mode of action of water-soluble fraction, lysozyme, from egg white. The study showed that lysozyme performed immunostimulatory activity through enhancing IgM production by HB4C5 cells through accelerating the translation process. Further experiments are needed to examine the active substance and the mode of action of Indonesian native chicken egg white water extract.

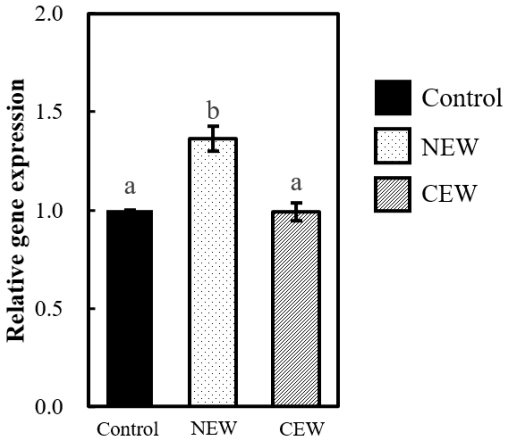

Figure 3. Effect of egg white water extract on IgM gene expression (mean ± SD). means denoted by a different letter indicate significant difference between treatments (Tukey's HSD, p<0.05, n=3).

The effect of NEW on mouse splenocytes *in vitro*

The immunostimulatory activity of NEW and CEW was evaluated by using mouse splenocytes *in vitro*. As indicated in Figure 4, NEW and CEW accelerated the production of IgA and IgM dose-dependently.

NEW and CEW facilitated IgA production 4.4-fold and 1.98-fold, respectively, at the highest concentration compared to control (p<0.05). Unlike NEW, CEW did not significantly enhanced IgA production at the lowest concentration but significantly increased at the highest concentration. IgG concentration was not significantly increased by both NEW and CEW, thus there was no significant difference between both samples (p>0.05). IgM production was enhanced by both

NEW and CEW (p<0.05). NEW effectively enhanced 3.2-fold and CEW enhanced 2.6-fold at the highest concentration compared to control.

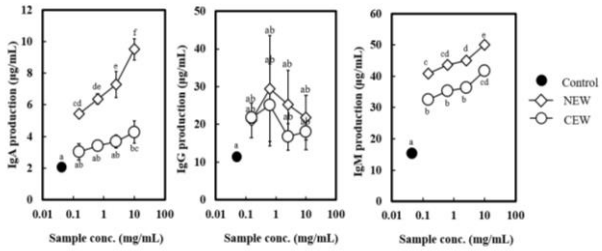

Figure 4. The effect of egg white water extract on mouse splenocytes. means denoted by a different letter indicate significant difference between treatments (Tukey's HSD, p<0.05, n=3)

This study demonstrated that both NEW and CEW were able to stimulate IgA and IgM production by mouse splenocytes. Furthermore, the production of IgG from both samples were not significantly different (p>0.05). In addition, NEW has an ability to enhance IgA and IgM production higher than CEW. In other words, NEW has immunostimulatory activity through stimulating Ig production more effectively compared to CEW by mouse splenocytes. This result is supported by Song et al. (2014) who reported that egg white water extract promoted host defense mechanism through producing immunoglobulin production.

Ig production provides total humoral immunity (short-term and long-term protection) of the body to fight against all sorts of pathogens, cancer cells, and toxic substance (Janeway et al., 2001). The result of this study was supported by the previous findings which pointed out that production of IgA, IgG, and IgM plays an important role to provide humoral immunity in human body (Schroeder and Cavacini, 2010). This finding was considered novel since there was no previous research evaluating the ability of egg white extract on Ig production using mouse splenocytes *in vitro* and this is the first study that evaluated immune stimulating effect of native egg white.

Further detailed *in vitro* studies are needed to determine the exact substance in NEW that modulates IgM production. Besides, *in vivo* humoral and cellular immunological investigations are required to determine its capability on immunostimulating potential and molecular mechanisms of actions.

CONCLUSION

NEW stimulates the immune system through enhancing IgM production by the HB4C5 cells without any cytotoxicity. NEW was also found to more effectively upregulate IgM gene expression level in HB4C5 cells than CEW. This study also demonstrated that NEW has an ability to modulate Ig production by mouse splenocytes *in vitro*. NEW enhanced not only IgA and IgM production but also IgG production only at the highest concentration. the immunostimulating activity of NEW can be a promising source of an immunostimulator agent. Further investigations of active substances and *in vivo* studies are needed to understand the mechanism of immunostimulatory activity.

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