Formulasi Nasal Spray Anti-Influenza yang Mengandung Nanopartikel Perak

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Artikel Penelitian

Abstract: *Influenza A virus is one of the most common causes of respiratory disease in the world. Even though, vaccines and anti-influenza virus are become the first line for therapy, but the mutation ability of influenza virus is able to cause several outbreaks in the world. Silver nanoparticles (AgNP) have been proven to exhibit antiviral activity, however the use of AgNP in pharmaceutical products is still limited. In this study, we aimed to formulate nasal spray containing AgNP, to evaluate its physicochemical properties, and its antiviral activity toward H5N1 influenza A virus. AgNP were synthesized using chemical reduction method with polyvinyl alcohol as stabilizer, and further prepared into nasal spray product. Physicochemical properties and anti-hemagglutination activity of nasal spray were further evaluated. The nasal spray contained different size of AgNP (less and more than 50 nm) showed physical stability after 28 days storage. However, Anti-influenza evaluation of nasal spray contained AgNP less than 50 nm exhibited better anti-hemagglutination activity against influenza A virus.*

Keywords: *silver nanoparticles, spray, influenza*

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Abstrak: Influenza virus merupakan salah satu penyebab penyakit pernapasan menular dengan tingkat transmisi yang cukup tinggi. Meskipun vaksin dan penggunaan obat antiviral merupakan tata laksana utama dalam mengurangi tingkat keparahan penyakit ini, kemampuan influenza A virus untuk bermutasi tetap mampu menyebabkan kejadian luar biasa dibeberapa negara. Perak nanopartikel (AgNP) merupakan salah satu logam mineral yang memiliki aktivitas antiinfluenza, namun penggunaannya dalam sediaan obat farmasi masih terbatas. Penelitian ini bertujuan untuk memformulasikan AgNP yang dibuat dengan metode reduksi untuk membuat sediaan *nasal spray*, mengevalusi sifat fisikokimia dan aktivitas anti influenza terhadap virus influenza A H5N1 dari sediaan yang dibuat. Pada penelitian ini AgNP dengan ukuran lebih kecil dan lebih besar dari 50 nm telah berhasil dibuat (11.2 nm dan 90 nm). Setelah diformulasikan, *Nasal spray* dengan kedua ukuran AgNP menunjukan stabilitas fisikokimia yang baik. *Nasal spray* dengan ukuran AgNP lebih kecil dari 50 nm menunjukan aktivitas inhibisi hemaglutinin terhadap virus influenza A H5N1 yang lebih baik.

Kata kunci: silver nanoparticles, spray, influenza

Introduction

Influenza is an infectious respiratory disease caused by influenza A and B virus (1). Influenza transmission mainly occurs through droplets produced by infected patients when they talk, sneeze or cough (2). The World Health Organization estimates that annual epidemics of influenza result in 1 billion infections, 3 to 5 million cases of severe illness and 3 to 5 hundred thousand deaths (3). Due to its high transmission rate, handling of influenza is mainly focused on its prevention rather than treatment. Some of the preventive measure includes vaccination, wearing mask, hand washing and disinfection (4). Among those preventive measures, vaccination is the most recommended action to be done annually (5). However, current seasonal influenza vaccines have only sub-optimal effectiveness across all age groups (4). A suggested method for limiting respiratory disease transmission is by the intranasal delivery of antiviral medications or agents (6).

Several studies had been demonstrated the antiviral activity of the silver nanoparticles (AgNP) including against adenovirus, hepatitis B virus, herpes simplex virus, human immunodeficiency virus, influenza A virus, norovirus, poliovirus, respiratory syncytial virus, SARS-CoV-2, chinkungunya virus and white spot syndrome virus (7,8). The different form of AgNP which are singular and combination or composite, has been proofed exhibiting anti influenza virus in vitro (9,10). In addition to its form, recent finding showed the smaller size of AgNP, the better antibacterial activty observed. Therefore, application of intranasal delivery such as nasal spray containing silver nanoparticles with suitable viscosity will be prospective to prevent influenza virus infection.

It should be noted that there is still little study on AgNP formulations for nasal spray. Thus, this study aimed to formulate and evaluate activity of a nasal spray product containing silver nanoparticles with different size. The formulation were further evaluated for its physicochemical properties and its antiviral activity toward H5N1 influenza A virus using the hemagglutination inhibition assay (HAI) method.

Materials and Methods

Materials

Materials used in this research were silver nitrate (Merck, Germany), fully hydrolyzed polyvinyl alcohol (viscosity of 4% aqueous solution at 20°C is 15.0 cps, Mw of 89,000 to 98,000) (Sigma-Aldrich, Singapore), sodium borohydride (Merck, Germany), Sodium carboxymethylcellulose (viscosity of 2% aqueous solution at 25°C is 561 cps) (Sigma-Aldrich, Singapore), Sodium chloride (Merck, Germany), Citric acid monohydrate (Golden Sinar Sakti, Indonesia), demineralized water (Brataco, Indonesia), H5N1 virus clade 2.1.3 (Veterinary faculty, Institute Pertanian Bogor, Indonesia), red blood cell (Veterinary Faculty, Institute Pertanian Bogor University, Indonesia), phosphate-buffered saline pH 7,4 composed of sodium phosphate, sodium chloride and potassium phosphate (Sigma-Aldrich, Singapore), avian influenza serum antibody (Veterinary faculty, Institute Pertanian Bogor, Indonesia), Spectrophotometer UV-Vis (Shimadzu UV 1800-PC, Japan), Transmission electron microscope (Tecnai G2 20S-Twin, America), Particle size analyzer (Malvern Mastersizer, England), Atomic absorption spectrophotometer (Shimadzu AA 6300, Japan), pH meter (Oakton, United States), Viscometer (Cole-Palmer 98965-40, Germany).

H5N1 Influenza A virus was kindly obtained from Veterinary faculty, Institute Pertanian Bogor, Indonesia. Red blood cells were collected from chicken which breed at veterinary faculty, Institute Pertanian Bogor, Indonesia, and kept at 4°C before use.

Method

Synthesis of silver nanoparticles

AgNP were synthesized using chemical reduction method with polyvinyl alcohol as stabilizer. Variation in concentration of $AgNO_3$ and synthesis condition is described in **Table 1**. Briefly, 10 mL of PVA 2% was added into 20 mL $AgNO_3$, and then stirred. The mixture was further added with 0,6 mL $NaBH_4$ 0.2 M, and stirred for 3 hours. Formation of AgNP was confirmed as yellow suspension visually observed.

Table 1. Preparation of silver nanoparticle

Silver Nanoparticle*	Concentration of AgNO3 (mM)	Temperature of synthesis (°C)
AgNP F1	0.5	30
AgNP F2	1	60

*AgNP F1 and F2 refer to Silver nanoparticles with expected size less than 50 nm and more than 50 nm.

To optimizing reduction process of $AgNO_3$, the silver AgNP were stored for a day after the synthesis (11). The AgNP were further stored in airtight glass bottle (12).

Characterization of silver nanoparticles

AgNP morphology were analyzed using the Transmission Electron Microscope. Particle size distribution, polydispersity index and zeta potential of silver nanoparticle was measured using Particle Size Analyzer (Malvern Mastersizer, England) after dilution with demineralized water.

Formation of AgNP was confirmed using UV-Vis spectrophotometer (Shimadzu UV 1800-PC, Japan) after 5 times dilution of samples with demineralized water. Demineralized water was used as the baseline and UV-Vis spectra of the samples was measured in the wavelength of 200 – 600 nm. Concentration of AgNP in the sample was then measured using atomic absorption spectrophotometry (Shimadzu AA 6300, Japan). AgNP with Particle size distribution less than 50 nm and between 100 nm to 50 nm were further marked as AgNP F1 and AgNP F2, respectively.

Formulation of silver nanoparticles nasal spray

Formulation of nasal spray of silver nanoparticle (AgNP NS) is described as depicted in **Table 2**, respectively. In brief, 184.8 mL demineralize water was heated to 30°C, and then added into 90 mL of silver nanoparticles. The mixture was then added with 15 mL of NaCl, 7.8 mL of 2M citric acid and 2.4 mL of sodium carboxyl-methyl cellulose (Na-CMC) 1%. The mixture was stirred for 1 hour, and then stored in airtight and lightproof container at room temperature.

Evaluation of silver nanoparticles nasal spray

AgNP nasal spray was evaluated for its pH, viscosity, AgNP content, and spray pattern. pH was measured with pH meter (Oakton, United States), while its viscosity was measured using Cole-Palmer viscometer (Cole-Palmer, Germany), using spindle 1 at 50 rpm (13). AgNP content in nasal spray was measured using atomic absorption spectrophotometer. Spray pattern was performed by measuring diameter of the spraying pattern from a specific range.

Stability study of AgNP nasal spray was performed towards product which stored for 28 days in airtight and lightproof container at room temperature. Samples was taken from AgNP nasal spray at day 0, 7, 14, 21 and 28. Furthermore, its pH, viscosity and antiviral activity was analyzed at those time points.

Antiviral activity of silver nanoparticles nasal spray

Antiviral activity of AgNP nasal spray toward H5N1 influenza A virus was performed in two steps.

Table 2. Formulation of AgNP nasal spray

Ingredient	Concentration (% v/v)
Silver nanoparticles	30
NaCl	5
Citric acid 2 M	2,6
NaCMC 1%	0,8
Demineralized water	61,6

First, hemagglutination assay (HA) was performed to determine the 4 Hemagglutination unit (HAU). Second, hemagglutination inhibition assay (HAI) was performed on AgNP nasal spray formulas to evaluate its activity in inhibiting hemagglutination at red blood cells caused by 4 HAU of H5N1 influenza A virus.

HA was performed with modified Kaufmann *et al* method (14). The steps are as follow: 25 µL of phosphate buffered saline (PBS) was added to the 96 well plate, from line A1 to A12 and B1 to B12, followed by addition 25 µL of H5N1 influenza A virus to well A1 and B1. The mixture was diluted by a factor of two from each column to the next column. After each column was diluted in accordance with their concentration, 25 µL of 1% red blood cell was added from well A1 to A12 and B1 to B12. The 96-well plate was further incubated for 30 minutes at room temperature. Red coloured solution indicates the hemagglutination activity, red coloured precipitate formed at the bottom of the well indicate no hemagglutination activity.

Hemagglutination Inhibition Assay (HAI) was performed with modified Kaufmann *et al* method (14). The steps are as follow: 25 µL of 1 × PBS was added to the 96 well plate, from line A1 to A12, B1 to B12, C1 to C12, D1 to D12, E1 to E12 and F1 to F12. Nasal spray product containing silver nanoparticles less than 50 nm (NS F1) was added to A1 and B1, then the product containing silver nanoparticles more than 50 nm (NS F2) was added to C1 and D1. Meanwhile E1 and F1 was added with avian influenza serum antibody, as a control. Each column in line A to D was then diluted by a factor of 2 from each column to the next column. After the dilution was done, 25 µL of H5N1 influenza A virus was added to all tested wells. The plate was incubated for 30 minutes at room temperature, followed by addition 25 µL of 1% red blood cells. The plate was incubated for another 30 minutes at room temperature. Inhibition of- or no hemagglutination activity was shown by red colored precipitate formed at the bottom of the well. Hemagglutination activity was shown by red-colored solution.

Statistical analysis

Statistical analysis of particle size distribution, polydispersity index and zeta potential of silver nanoparticle were calculated directly by software of Particle Size Analyzer (Malvern Mastersizer, England), the data were written as mean. The pH was measured two times, the data were written as Mean ± Standard deviation.

Results and Discussion

Synthesis and characterization of silver nanoparticles

AgNP were synthesized using chemical reduction method with 2% polyvinyl alcohol as stabilizer. The reduction procedure of $AgNO_3$ was proceed by $NaBH_4$. Ag^+ received electron from $NaBH_4$ and shift from Ag^+ to AgO (15). Silver (Ag) is an unstable substance and tends to agglomerate, therefore stabilizer was included in the formula of AgNP to prevent and reduce the agglomeration. In this study, polyvinyl alcohol (PVA) was selected as the stabilizer as it well-known for its compatibility and ability to shape-control of AgNP (16).

Formation of AgNP was confirmed by observing the formation of yellow suspension in the solution, and analyzing UV-Vis spectra of the solution as shown in **Figure 1**, respectively. AgNP morphology was also observed which showed its spherical shaped, as depicted in **Figure 2**. AgNP have a unique-optical properties called surface plasmon resonance (SPR) which occurs due to the coherent interaction between electron and light electromagnetic field which cause oscillation at metal surface. SPR properties cause an intense interaction of silver nanoparticles at a certain wavelength in the range of 380 - 450 nm (17, 18). As shown in **Figure 1**, the peak wavelength of silver nanoparticles occurred at 399.20 and 384.70 nm for AgNP F1 and AgNP F2, respectively. Thus, this result confirmed that both formulas successfully produce silver nanoparticles.

Concentration of $AgNO_3$ and reaction temperature were varied to obtain nanoparticles with various size, as described in **Table 1**. The result showed that the higher concentration of $AgNO_3$, the bigger particle size of AgNP, as shown at **Table 3**, respectively.

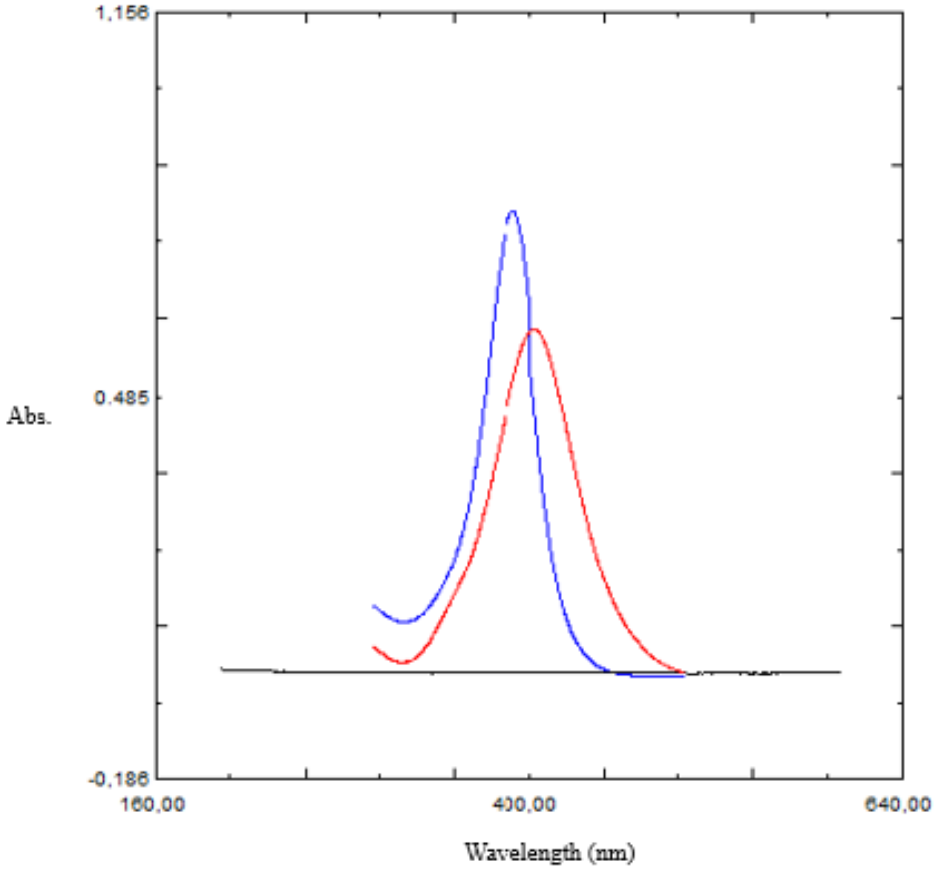

Figure 1. The UV-Vis spectrum of AgNP F1 (Dv90 11.2 nm) indicated by red line; AgNP F2 (Dv90 92.2 nm) indicated by blue line

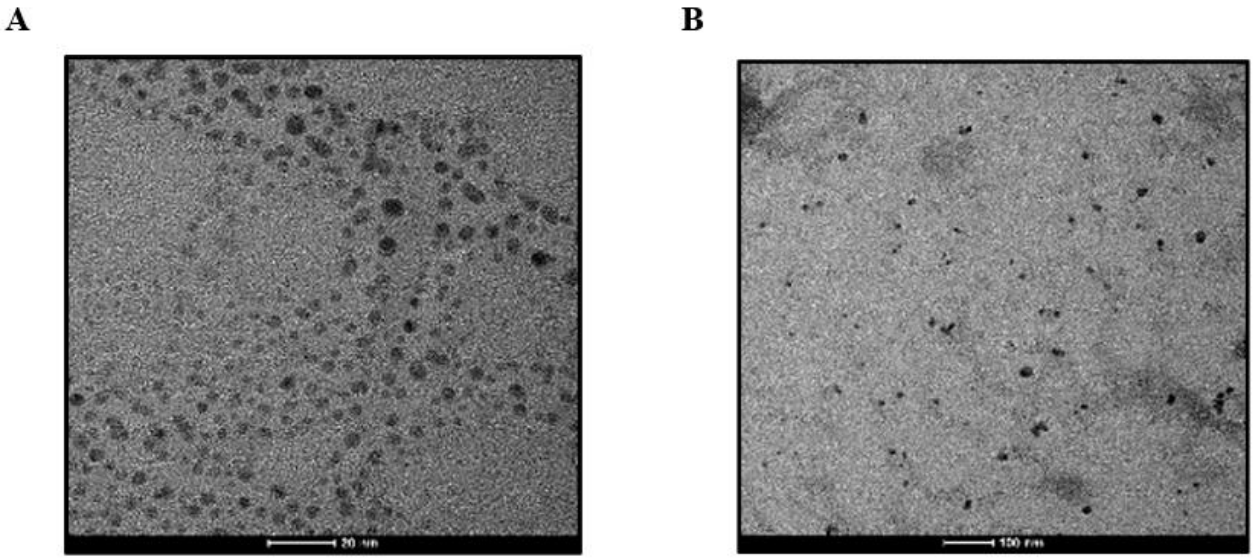

Figure 2. Silver nanoparticles morphology of F1 (A) and F2 (B)

Table 3. Characterization of silver nanoparticles (AgNP)

Parameters	AgNP F1	AgNP F2
Particle size (nm)	11.2	92.2
Polidispersity index	0.467	0.450
Zeta potential (mV)	-19.8	-14.3
Ag content (µg/mL)	38.64	38.17

Note: AgNP F1 mean AgNP with particle size less than 50 nm; AgNP F2 mean AgNP with particle size more than 50 nm

This result was in accordance to the previous study which combination of high concentration and high temperature produced AgNP with bigger particle size (19,20).

Polydispersity index (PDI) which indicates the size heterogeneity of nanoparticles was also measured, as shown at **Table 3**, respectively. The PDI values < 0.05 are more to mono disperse, meanwhile values > 0.7 are common to heterogeneity distribution of particles size (21). It showed that PDI value of both samples were less than 0.5, which indicates a slightly mono dispersed particle distribution. Zeta potential was also measured to predict the stability of particles in liquid product, since it is related to the interactions between particles in a colloidal system. Nanoparticulate system with high zeta potential values (less than -30 mV or more than +30 mV) are considered as stable. On the other hand, low zeta potential value indicates that the particles tend to attract to each other and aggregate (22).

In addition, at **Table 3**, zeta potential value of AgNP were -19.8 mV and -14.3 mV for the AgNP F1 and F2, respectively. This indicated that both AgNP need to be optimized more to get AgNP with higher stability. However, as expected, the smaller nanoparticles had higher zeta potential value as they are more stable and less likely to aggregate compared to the larger nanoparticles. Further concentration of AgNP in the solution was also measured. No significant difference of AgNP concentration in F1 and F2 was obtained. Therefore, concentration of $AgNO_3$ and reaction temperature were only affected physical properties of particles, but not the concentration of AgNP obtained.

Formulation and characterization of silver nanoparticles nasal spray (AgNP NS)

Silver nanoparticle nasal spray (AgNP NS) was prepared by adding AgNP solution into solution containing NaCl, citric acid and Na-CMC. The obtained nasal spray product was then evaluated, and the result is described in **Table 4**, respectively.

Citric acid was added into AgNP nasal spray formula as pH adjuster, to ensure a suitable pH of nasal product. It is suggested that the nasal product has pH 3.5 – 7.5 to prevent disruption of the cilia function in acidic or alkaline environment (23).

Table 4. Characterization of AgNP nasal spray

Parameters	NS F1	NS F2
pH	5.35 ± 0.01	5.29 ± 0.01
Viscosity (cp)	117.1	118.3
Gradient average	1.2305	1.095
Ag content (µg/mL)	5.8	7.28

Note: NS F1 mean nasal spray contained AgNP with particle size less than 50 nm; NS F2 mean nasal spray contained AgNP with particle size more than 50 nm

Table 5. Stability of AgNP nasal spray

Parameters	Storage time (days)	NS F1	NS F2
pH	0	5.35 ± 0.01	5.29 ± 0.01
	7	5.40 ± 0.01	5.39 ± 0.01
	14	5.50 ± 0.01	5.35 ± 0.01
	21	5.55 ± 0.02	5.52 ± 0.01
	28	5.58 ± 0.01	5.60 ± 0.01
Viscosity (cp)	0	117.1	118.3
	7	113.8	119.6
	14	114.5	119.1
	21	114.2	117.2
	28	115.6	117.6
HAI test (µg/mL)	0	0.18	0.23
	28	2.9	not detected

Note: NS F1 mean nasal spray contained AgNP with particle size less than 50 nm; NS F2 mean nasal spray contained AgNP with particle size more than 50 nm

Na-CMC 1% was also added into formula as viscosity increasing agent. Viscosity is one of the factors affecting the spraying pattern and effectivity of a nasal spray product. The greater the viscosity value, the larger the droplet and the spray pattern will be narrower. On the other hand, the lower the viscosity value, the smaller the droplet and the spray pattern will be wider. Moreover, Na-CMC also play as muco-adhesive polymers, to increase residence time of AgNP in nasal cavity and provide adequate time to exhibit antiviral activity (24).

AgNP content in the product at the concentration of 6.25 - 100 µg/mL showed inhibitory effect towards the influenza A virus. However, concentration greater than 25 µg/mL showed cytotoxic effect, which indicates that safety level of usage is under 25 µg/mL (9). Therefore, silver content in the nasal spray was determined ensure that the product is safe to be used without causing any cytotoxic effect. **Table 4**, respectively, showed that Ag concentration in nasal spray were adequate to exhibit inhibitory effect towards the influenza A virus, as well as safe and non-toxic to the cells.

Storage of AgNP nasal spray in room temperature for 28 days showed that pH and viscosity of product were slightly changed, as depicted at **Table 5,** respectively. After 28 days of storage, the pH of AgNP nasal spray F1 and F2 were increased which might indicated the changed of free Ag in nasal spray. On the other hand, the viscosity of AgNP nasal spray were slightly reduced which might reduce residence time of AgNP in nasal cavity. To consider both of parameters, change in pH and viscosity after 28 days of storage might affect the antiviral activity of AgNP nasal spray.

Antiviral activity test

Several reports suggested that the anti influenza activity of AgNP is due to entry inhibition of influenza virus into the cells which caused by binding of AgNP and Influenza glycoproteins (10,25). Hemagglutination inhibition assay is a classical methodology use for the classification of hemagglutinating viruses and predicting compound(s) ability to protect cells against influenza virus entry (26,27).

Hemagglutination Assay (HA) was performed to determine the dilution in which the 4 HAU can be obtained. The result of HA test is shown in **Figure 3**, respectively. According to WHO, 4 HAU was used as standard amount for hemagglutination inhibition assay (HAI) (14). Thus, this result was then applied for further HAI to assess antiviral activity of AgNP nasal spray at day 0 and day 28.

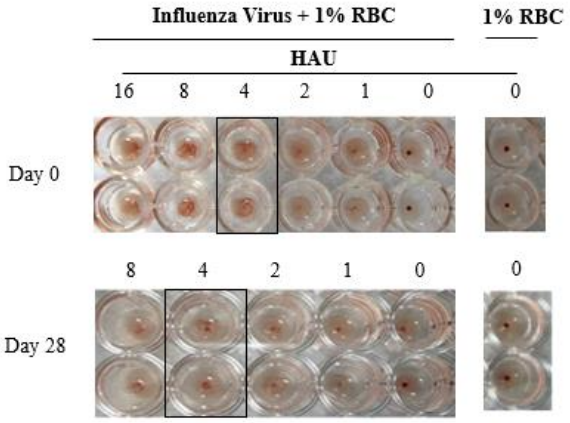

Figure 3. Hemagglutination assay was performed at day 0 and at day 28. A) 4 HAU as mark by black color line at day 0; B) 4 HAU as mark by black-color line at day 28. HAU was referred to hemagglutination unit; RBC was refer to red blood cells.

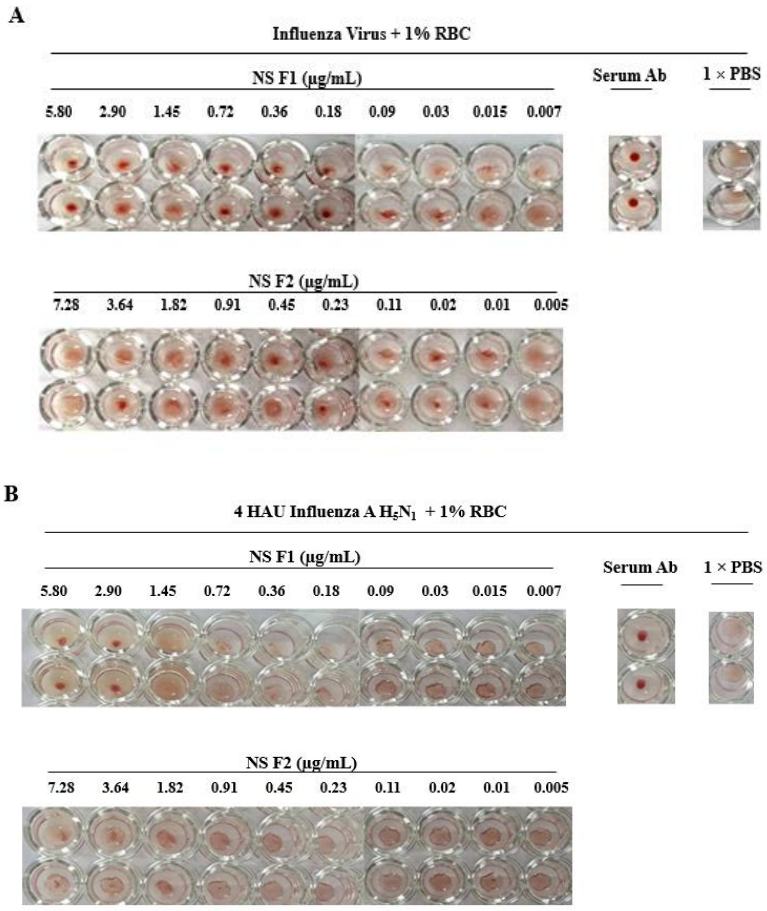

Figure. 4 Inhibition of 4 HAU influenza A H5N1 Hemagglutination activity at red blood cells by nasal spray nanoparticle. A) Inhibition of Hemagglutination activity at red blood cells by NS F1 and NS F2 at day 0 post preparation. Hemagglutination inhibition was observed more consistent at addition of NS F1 compare to NS F2; B) Inhibition of Hemagglutination activity in red blood cells occurred at addition of NS F1 only at day 28 post preparation. NS F1 refer to nasal spray contain Ag nanoparticle with size ~11.2 nm; NS F2 refer to nasal spray contain Ag nano particles with size ~92.5 nm; Serum Ab refer to Serum containing influenza A H5N1 antibody; PBS refer to phosphate buffer saline

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No hemagglutination activity was indicated by the presence of red-colored precipitate, meanwhile hemagglutination will cause the formation of spread red-colored solution. The HAI results showed difference in effectiveness between the AgNP nasal spray F1 and F2 at day 0 and day 28, as shown by **Figure 4A** and **4B**, respectively. AgNP nasal spray F1 which have smaller size, showed consistent and better activity at day 0 and day 28 compared to AgNP nasal spray F2. The particles with smaller size have a larger surface area, resulting in more unsaturated bond which resulting in easiness binding onto the virus protein. AgNP is predicted to bind and cleave the virus's haemagglutinin disulfide bond which then cause the virus to destabilize (8,28). Thus, the destabilization of Hemagglutination glycoprotein will inhibit the initiation of influenza virus infection which is binding onto the human cell's sialic acid and further replication process.

Based on the comparative result of HAI at day 0 and day 28 as shown at **Figure 4B**, the AgNP nasal spray F1 shown reduction in their HA inhibition effectiveness after 28 days. At day-0 post preparation, AgNP NS F1 was effective at 0.18 µg/mL, meanwhile on day-28, it inhibited the HA influenza virus at 2.9 µg/mL. The effectiveness reduction may cause by the aggregation during storage which due to presence of electrolyte or buffer at a high concentration (29). Therefore, further study should be performed to get optimum formula with better stability. In conclusion, this study showed that in term of Ag nanoparticles, the smaller size of Ag nanoparticles, the more effective HAI activity observed.

Conclusion

Silver nanoparticles nasal spray contained AgNP with particle size less than 50 nm was indicated able to inhibit hemagglutination caused by influenza virus. This result may broaden the information regarding the application of AgNP in the Nasal spray form against Influenza virus. Although, studies on the formulation stability needs to be performed in upcoming research.

Acknowledgement

The author would like to acknowledge Universitas Indonesia for funding this research through "Hibah PPM Penugasan Universitas Indonesia 2021" with no ST-178/UN2.F15.D/PPM/00/2021.

Conflicts of interest

There are no conflicts of interest.

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