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# The evaluation of anti-NDV hyperimmune sera for serotherapy in chickens infected with Newcastle disease virus field isolate

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**Abstract**
A study was carried out at Faculty of Veterinary Medicine Udayana University from March 2018 to March 2020 to evaluate the possible use of anti-NDV hyperimmune sera (HIS) for immunotherapy in chickens infected with field ND virus. Hyperimmune sera (HIS) against NDV were used in this study with subcutaneous (SC) of HIS administrations. Four groups (I-IV) each consisted of 5 chickens were infected with NDV isolate. All chickens in groups I through III were treated with HIS at a dose of 1 mL per chicken whereas chickens of group IV were HIS-untreated. Chickens in group I was treated twice at 6 and 15 hours post infection (pi). Chickens of groups II were treated once at 48 h pi, whereas those in group III were treated twice at 48 h and 63 h pi. HIS treatments in ND chickens were able to prevent death and clinical symptoms especially when the treatment were given at 6 h and 15 h pi before the onset of clinical symptoms.

**Keywords:** Hyperimmune sera, neuronal sign, pathology, vaccination

## 1. Introduction
Newcastle disease (ND) is one of the most devastating disease among many avian diseases. The causative agent of the disease is avian ortho avulavirus 1 which is also called Newcastle disease virus (NDV) [1]. NDV virus has a very wide genetic diversity and one among those vicious genotypes are genotype VII which is found in Indonesia and several other countries in the world causing high mortality rate in unvaccinated chickens. The mortality rates of NDV genotype VII in susceptible chickens in Indonesia and other countries reported were between 93,3% and 100% and the time of death ranges from 3-7 days post-infection [2, 4]. The reports also indicate that variation of the clinical expression and symptoms of ND exists among different species.

Vaccination along with strict biosecurity measures is considered as an effective way to prevent the disease. Therefore, the practice of vaccination has become routine works throughout the world to prevent the disease. However, although ND vaccination has been routinely carried out in commercial farms in Indonesia, the incidence of ND is still very common. One suspected risk factor of the ND in chickens is the high maternal antibody titer in young chickens when the first vaccination is carried out. Such high maternal antibody level can neutralize the viral vaccine, hence reduce the antibody response against ND vaccination. It was found that maternal antibody titers in commercial chickens at 2 week-age are still above the 3log 2 with a range between 3log 2 to 6 log 2 [2]. Hence for vaccine research and experimental infections purposes, especially if using commercial chickens for experimental animals, measurement of maternal antibody titers should be carried out. For experimental purposes, it is, therefore, important to select and use only chickens with undetected level of maternal antibody against NDV.

As no treatment is currently available for ND in chickens, serotherapy using hyperimmune serum (HIS) can be beneficial as an alternative treatment to protect domestic poultry from ND. Such treatment can be given before and/or after the occurrence of clinical symptoms [5]. In general, passive immunization such as treatment with HIS has beneficially proven for

numerous acute infections and it is still commonly used as emergency treatment against life-threatening diseases [6, 8]. The HIS against a particular antigen can be raised in mammalian or avian species by immunization with specific antigen, or by collecting serum or plasma from individual recovering from certain infectious diseases. The HIS can then be used in passive immunization for treatment of human and animal diseases [9]. Although it has some limitations, the use of HIS can be an option for treatment of ND in a flock of chickens where some chickens may have shown clinical symptoms but some others have not [5]. In addition to HIS treatment for chickens, this serotherapy can also be applied for treatment of birds of high economic value such as very valuable pet birds. This study was therefore conducted to determine the effectiveness of HIS in combating ND in chickens experimentally infected with virulent NDV.

## 2. Materials and Methods

### 2.1. Ethical Approval

This research has been officially agreed by the Ethical Commission for the Use of Animals in Research and Education of the Faculty of Veterinary Medicine, Udayana University, Indonesia with approval number: 309A/ KE-PH-Lit-3/VII/2017

### 2.2. Preparation of NDV hyperimmune serum in Chickens

As many as ten 3-weeks-old male layer chickens of ISA brown strain with maternal antibody (MA) against NDV of below $^{2}$logs 2 HI units were used in the preparation of hyperimmune sera against NDV. They were firstly primed with NDV LaSota commercial vaccine (Medivac, Medion, Bandung-Indonesia) at the doses of $10^{7}$ EID 50/0.2 mL, by intramuscular injection and boosted twice at two week interval with the same vaccine at the same doses and route of vaccination. HI antibody titers were examined every two week just before the boosters were performed. Four weeks after the second booster vaccination all chickens were sacrificed and whole blood was taken from each chicken. Sera of from chickens with maximum antibody titer were used for experimental serotherapy in chickens with ND.

### 2.3. Bioavailability assessment of hyperimmune sera in chickens

Two routes, subcutaneous and intraperitoneal injections, were used to asses the bioavailability (the times required to reach the maximum titer and the times of the antibody was cleared from the blood) in chickens. A total of ten male layers chickens with MDA titer of below 2 log 2 HI units, were used and were divided into two groups. Group A was injected with the HIS with dose injection of $2^{11}$ HI Unit/1mL via subcutaneous (SC) and Group B was injected with the same dose but in a different route of injection ie the intraperitoneal (IP). Sera samples were collected at 2,4,6,15,40 and 50 hrs post-injection to determine NDV antibody titers.

### 2.4. Haemagglutination Inhibition (HI) test for Determination of NDV antibody titer

The haemagglutination inhibition (HI) test was done to determine the antibody titer in sera samples. The HI test was conducted according to the procedure described by OIE [10].

### 2.5. Preparation of Experimentally NDV-infected chickens

One hundreds male day old chicks (DOCs) of ISA brown strain were purchased from a commercial hatchery (P.T. Charoen Pokphand Jaya Farm, Jembrana, Bali-Indonesia). At 2-3 weeks old, the MDA titers against NDV were examined as described previously [2]. As many as 20 chickens with MDA levels of bellow 2log2 were selected for experimentally NDV infection. All chickens were inoculated orally with 0.2 mL of $10^{5}$ TCID50 of NDV Tabanan-1/ARP/2017 isolate [11].

### 2.6. NDV hyperimmune serum treatment

NDV-infected chickens prepared as above were divided into 4 groups (I-IV) according to the treatment groups. All chickens in groups I-III were treated with NDV HIS subcutaneously at a dose of $2^{11}$ HI Unit/ 1mL per chicken, whereas those in group IV were used as control treated only with PBS. Chickens in group I were was treated twice with HIS at 6 and 15 hours after infection. Chickens in groups II were also treated twice with HIS but at 48 and 63 hours post infection. Chickens in group II were treated once with HIS at 48 hours post infection (Table 1).

The chickens were monitored twice daily to record clinical signs and mortality. Clinical symptoms were observed until day 14 post-infection. Chickens died were subjected to post-mortem examination. To confirm that those lesions due to NDV infection, antigen NDV detection using monoclonal antibody were performed as previously described [2, 12].

**Table 1:** Schedule infection and administration of HIS in each group

| Groups | NDV infection | First HIS treatment (times post infection) | Second HIS treatment (times post infection) |
|---|---|---|---|
| I | √ | 6 hours | 15 hours |
| II | √ | 48 hours | 63 hours |
| III | √ | 48 hours | None |
| IV | √ | None | none |

### 2.7 Statistical analysis

Data on the antibody titers against NDV in the each group of chickens were analyzed with a t-test. Meanwhile, the data on morbidity, mortality, clinical signs, and pathological features of chickens in all groups were compared descriptively.

## 3. Result

### 3.1 Profiles of antibody titers post-prime and booster immunizations

Although all chickens used for the preparation of hyperimmune sera against NDV were in the same strain with similar age and initial MDA titers, the antibody responses to vaccination were not uniform as there were variations in antibody titers in each chicken. The antibody titers against NDV at 2 weeks after first (prime) immunization was 3.9 $\pm$ 0.8 $\log_2$ HI units and at 2 weeks after the first booster was 6.8 $\pm$ 0.4 $\log_2$ HI units, whereas at 4 weeks after the second booster, it was 11 $\pm$ 0.7 $\log_2$ HI units, the mean antibody titer 4 weeks post the second booster was significantly higher ($p< 0.01$) comparing to those post-prime and 1st booster (Figure 1).

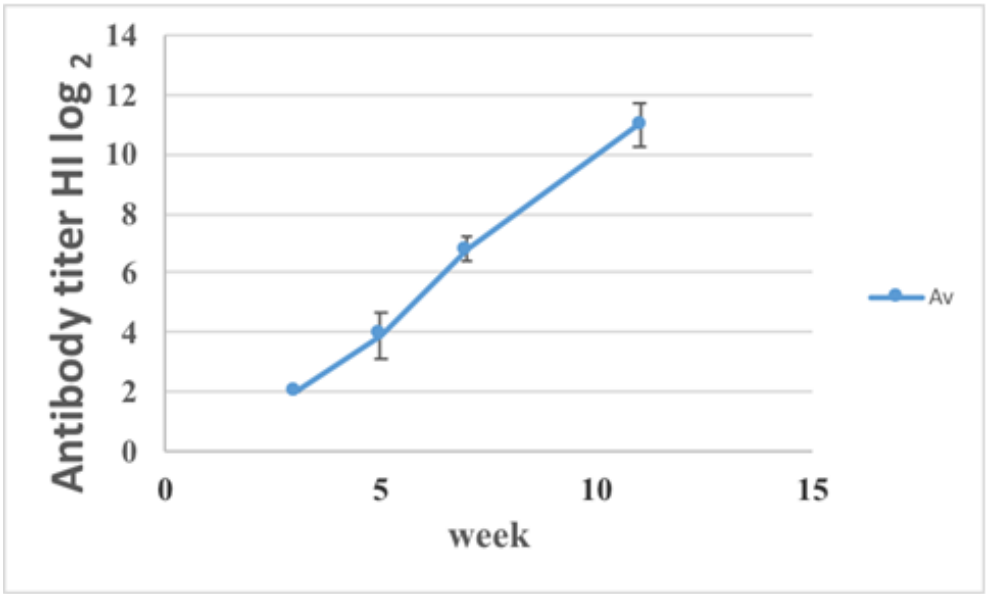


**Fig 1:** Antibody titers in sera of chickens after a single first (prime), second (first booster and third (second booster) immunizations using NDV life vaccine. A significant ($p<0.01$) increase in antibody titer against NDV was observed at 4 weeks after 2nd booster as compared to those after the prime and the first booster immunization.

### 3.2. Bioavailability of antibody in serum following two different sites of injection

After the injection of HIS into chickens subcutaneously, the mean antibody titers in blood examined at 2,4,6,15,40 and 50 hrs after injections were 2.6, 2.8, 3.8, 3.2, 2.2, and 0 $\log_2$HI unit respectively which were generally higher than those injected via intraperitoneal route which were 2.0, 2.0,2.8, 2.6, 2.0 and 0 $\log_2$ HI unit respectively (Figure 2). It was found that the highest mean of antibody titer ($3.8 \pm 0.8$ $\log_2$ HI units) was observed at 6 hours post-injection via subcutaneous routes. The mean antibody titers declined after 15 hours post-injection and was undetectable after 50 hours post injection (Figure 2). Although the patterns of antibody levels in blood following subcutaneous and intraperitoneal injections were similar, the subcutaneous injection method gave a higher antibody level in blood as compared to intraperitoneal injection (Figure 2).

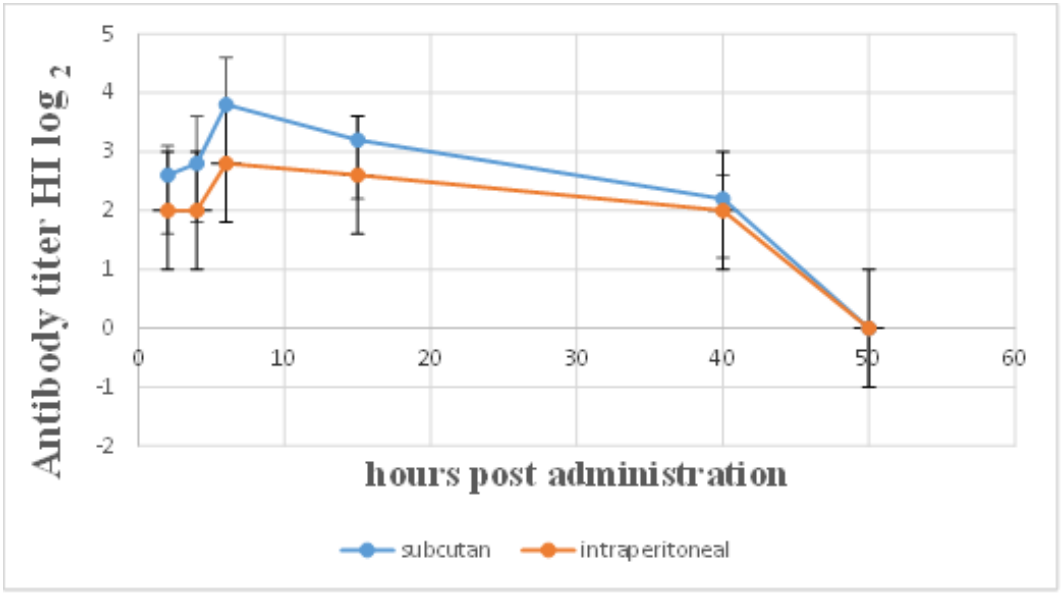


**Fig 2:** Bioavailability of antibody in blood following injection hyperimmune sera against NDV via subcutaneous and intraperitoneal routes. The titer of antibody against NDV in hyperimmune sera injected to chickens was $\log_2^{11}$ HI units. In blood, it peaked at 6 hours post-injection, then declined gradually and became undetectable after 50 hours post injection. Note: SC injection resulted in a higher peak antibody titers than the route per IP ($p<0.05$).

### 3.3 Chicken performance, survival rate, and pathological lesions

ND-chicken performances after receiving HIS treatments varied according to the times and repetitions of treatments (Table 2). Nor clinical sign and mortality was observed in chickens of group I until the end of the study. In group II, chickens still showed symptoms of illness although they have received a repeated doses of HIS at 48 and 63 hours and two chickens showing ND clinical symptoms died on days 4 and 5 after infections.

**Table 2:** Clinical signs and survival rate of chickens in each group following treatments with hyperimmune sera against NDV

| | Time of HIS injection | | Clinical sign (day post infection) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | 1st dose | 2nd dose | 2 | 3 | 4 | 5 | 6 | 11 | ,14 |
| I | 6 hours pi | 15 hours pi | None | None | None | None | None | None | None |
| II | 48 hours pi | 63 hours pi | sneezing | | prostration before died (1/5) | died (1/4). Sleepines (3/3) | near recovered (3/3) | recovered (3/3) | recovered (3/3) |
| III | 48 hours pi | none | sleepines ,sneezing | diarrhea, depression, tremor (1/5) | prostration before died (1/5) | died (1/4) | died (1/3) | died (1/2) | torticolis (1/1) |
| IV | None | None | sleepines ,sneezing | diarrhea, depression, tremor, prostration | diarrhea, depression, tremor, prostration (died 2/5) | died (2/3) | died (1/1) | | |

The remaining three chickens in group II gradually recovered and at the end of the experimental, all these 3 chickens finally recovered to normal. The chickens in group III exhibited severe diarrhea and two chickens died at days 5 and 6 pi respectively. Two chickens left showed torticollis which was exhibited in the 10th-day post-infection and finally one out of two died at day 11th pi. All chickens in groups IV died, time of death during 4-56 days post-infection. The survival rates of chickens in groups 1, II, III, and IV were 100%, 60%, 20%, and 0% respectively (Figure 3)

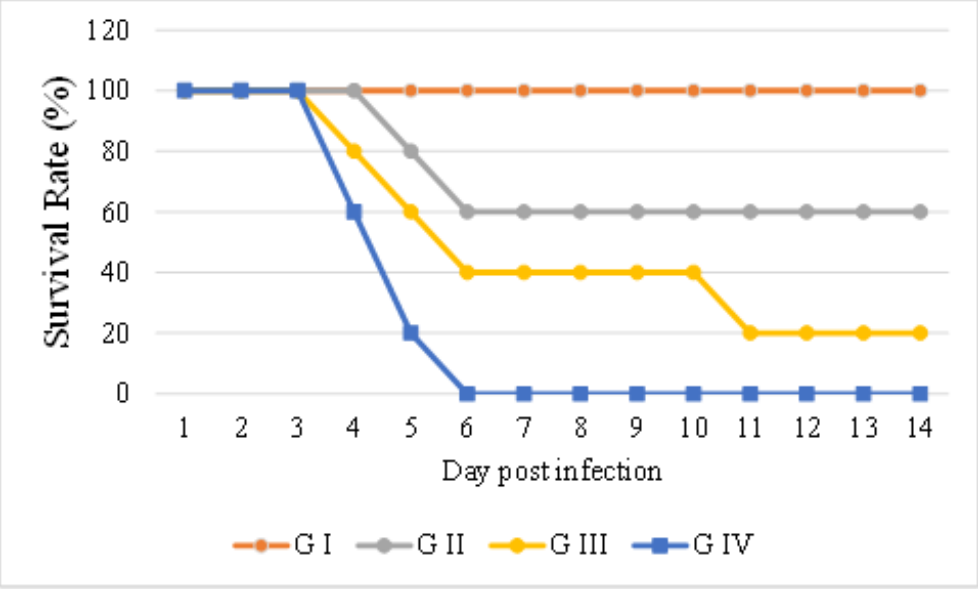


**Fig 3:** Survival rates of chickens in each group until the end of the experiments. In Group I: all chickens (100%) survived (5/5) without clinical sign when repeated HIS treatment were given. Group II: 60% chickens (3/5) survived when repeated HIS treatment were given after all chickens showing clinical symptoms. In chickens of group III, survival rate 20% when a single dose of HIS was given after showing clinical signs. All chickens in group IV (receiving no HIS treatment) died starting from day 4 to day 6 post infection.

At the end of experimental, 14 days post infection, most chickens surviving from ND have protective antibody level ie, between HI $_2$log 4-7. When the comparisons on the average antibody titers of chickens among groups after surviving from ND, it showed that the mean antibody titers of chickens in group I (6.2 $\pm$ 0.8 log2 HI units) was not significantly different to that of chickens in groups II (5.3 $\pm$ 1.2 log2 HI units), GIII 5.0 $\pm$ 0.0 log2 HI units ), the average final titer did not differ significantly ($p > 0.05$).

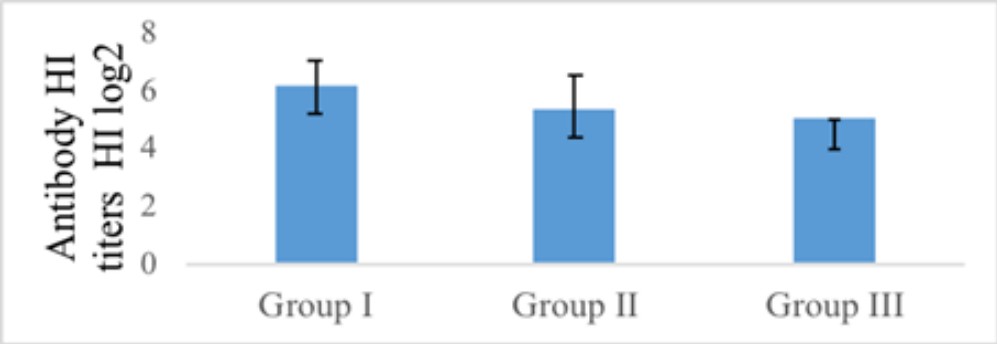


**Fig 4:** Profiles of anti-NDV antibody titers of chickens survived from ND after receiving HIS treatments. The highest HI antibody titer was in chickens of Group I as compared to those of group II and III.

Pathological examination of chickens that died during 4th to 6th day pi from groups III and IV showed lesions of blood circulatory disorders in several organs such as lung ( Figure 5 a) and intestine. In microscopic examination was found necrosis of lymphoid tissue such as payer patches, spleen, bursa of Fabricius and caecal tonsil. A prominent microscopic change perivasculitis was found at day 14th pi in the chicken group that injected with HIS after showing clinical symptoms and received no booster (Figure 5b). This microscopic lesion support clinical symptoms of torticollis that have appeared since day 10 pi.

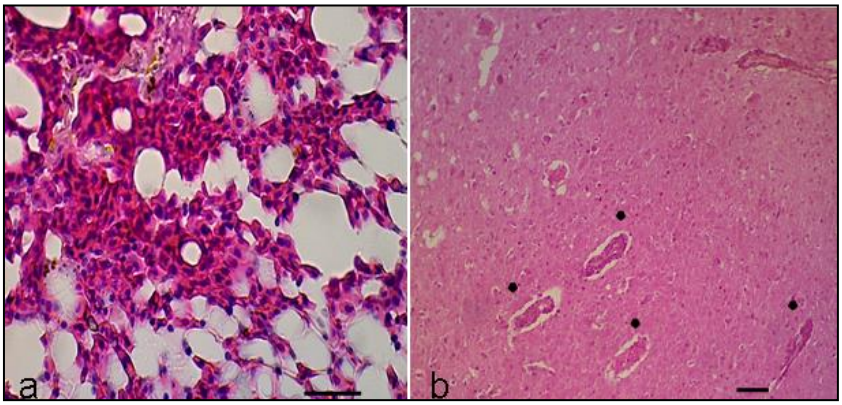


**Fig 5:** Microcscopic changes of several tissue organs of chicken after infected with NDV and treated by HIS after clinical symptom commence. Chicken that died 4-6 dpi prominently have vascular disorder HE staining bar 30 mM. (a).Chicken with neuronal sign have mild to moderate proliferative perivasculitis, and edema perivascular in its cerebrum (arrow).HE staining,bar 50 mM(b).

## 4. Discussion

Factors that contributing to the success of antibody preparation in experimental animals are genetic, age and nutritional status the hosts, and the types of antigens [13]. Although a single injection has been able to induce antibody production in animals, repeated injections of such antigen were still required in order to achieve a high antibody titer [14, 15]. Many recommendations and guidelines for the preparation of hyperimmune sera are described in the literatures. However, modifications of procedures and protocols from those recommendations and guidelines are needed in order to obtain hyperimmune serum with high titer of antibody. In this study, a prime vaccination followed by single booster was unable to produce high titers of antibody against NDV in chickens. When a boost was given at 2 weeks after the prime immunization, the antibody titer was still low, but it increased when a second booster dose was given at 4 weeks after the prime immunization which is in agreement with the previous reports that high antibody titers can be obtained following the 3rd and 4th inoculation antigens [9, 16].

A preliminary trial to evaluate the bioavailability of HIS in bloodstream was conducted via subcutaneous and intraperitoneal injection. Following subcutaneous injection, antibody titer peaked at 6 hours after injection, then decreased gradually and disappeared from blood circulation at 54 hours after the injection. Compared to IP injection, SC injection resulted in a significantly higher titer of antibody in blood at 6 hours post-injection ($p<0.05$). The mechanism of why the HIS injection per SC resulted in a higher antibody titer in the blood is not yet understood.

When repeated HIS treatments in NDV infected chickens was given before the onset of clinical symptom, the survival rate of the chickens was 100 %. In addition, such repeated treatment of HIS could also prevent the clinical signs and death in ND chickens. When HIS treatment in ND chickens was conducted only once and after the onset of clinical signs, survival rate decreased to 20% with one chicken showing sequelae of torticollis. In repeated HIS treatments in chickens that given after the onset of clinical symptoms, the survival rate was also 60 % but without any sequelae of those recovered from ND. It appears that repetition of the HIS dose can increase the survival rate and prevent the virus spread to the central nervous system. Of these three types of HIS treatment strategies, administration of HIS before the onset of the clinical signs followed by 2nd injection appears to yield the best result. Repeated treatments of NDV infected chickens with high doses of HIS after the onset of clinical signs were also able to decrease the mortality and to prevent neuronal sign in survived chickens. It is also interesting to note from this study that some chickens were able to survive when HIS treatment was conducted after the onset of clinical symptoms appeared. However, the emergence of sequelae in the form of

torticollis and microscopic lesion such as perivascular infiltration of lymphocyte and proliferation of endothelial cells have been observed in the cerebrum (Figure 5b). In this situation, although the chicken was able to survive, but it seem unable to prevent the spread of the virus into the brain. It appears that antibody level was insufficient to completely neutralize the virus in the bloodstream, so the infection becomes chronic and then the virus can reach into the brain.

NDV genotype VII is generally found in Indonesia. It is viscerotropic-velogenic (VV) strain of NDV which has more preference to infect and damage the digestive, respiratory, and lymphoid tissues causing high rate mortality in a short time period compared to the infect and cause damage in the brain tissues [17, 18]. The VV-NDVs infection in chickens can also result in diarrhea and respiratory disorders [17, 19] whereas the neurotropic velogenic (NV)-NDV prefers to infect neurons in the nervous system causing neurological symptoms even in the acute phase of the disease [20, 21].

As previously reported NDV is circulating in several parts of the world including Indonesia. The genotype VII of NDV found in Indonesia which is notable VV-NDV [2, 12, 22]. In the field cases, however, these VV-NDV infection in chickens can also cause symptoms of neuronal signs and microscopic lesions in the central nervous system. It seems that neurological symptoms are not merely due to viral propagation of neurons but rather due to vascular disorders. Systemic endothelial dysfunction is perhaps one of the reasons why neuronal lesions occurred in a group of chickens given HIS therapy in insufficient doses to neutralize all viruses in the blood. When the antibodies against NDV released, they should able to neutralize the virus in organs of the infected chickens. The organs will regenerate and the infected chickens will gradually be recovered. However, if the virus has damaged the brain tissue, it is difficult to recover, resulting in microscopic lesions occurred in the brain and neuronal clinical symptoms appeared. As it has been reported previously, pathological lesions in central nervous system are often found in outbreak of ND cases [12, 23]. Other studies reported that chronic infection of VV-NDV can induce neuronal signs [12, 21].

In this study chicken deaths in the untreated group were occurred on days $4^{th}$ - $6^{th}$ pi. This is in line with previous studies using viscerotropic viruses which generally turn over quickly [20]. NDV-Bali-1/7 belongs to genotype VII [2, 11]. In this study, in chicken brains showing clinical symptoms of torticollis with prominent microscopic lesions in the form of vascular and inflammatory disorders of the brain and very minimal lesions in the digestive and respiratory tract.

The mechanism of the virus reaches the brain in chickens treated with inadequate doses of HIS and administered after the appearance of the clinical signs may lead to the appearance of the sequelae. However, this matter still needs to be investigated further. It is therefore important to conduct further study in the future in regard to the beneficial aspects of HIS treatment in chickens especially when ND outbreaks occurred in a flock where some individual chickens have shown clinical symptoms whereas some others have not. In such case, HIS administration can be assessed in providing immediate immunity to stop the replication and spread of the virus which can be beneficial to prevent severe related clinical signs especially in the individuals which have not yet showed clinical symptoms, death, and wider outbreak. As it described previously, passive antibody administration is the only means of providing immediate immunity [6, 7].

## 5. Conclusion

The results of this study indicate that HIS successfully overcomes NDV infection chickens exposed to the virus. The best result was obtained with repeated HIS treatment and conducted before the onset of clinical symptoms.

## 6. Acknowledgments

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