RECOVERY OF RABID DOGS BY INTRATHECAL INJECTION OF RABIES VIRUS NEUTRALIZING ANTIBODIES

By

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(Under the Direction of Zhen Fu)

ABSTRACT

Rabies is a fatal neurotropic zoonotic disease. Although rabies is preventable, there is no cure once the virus invades in to the CNS. In this study, dogs were infected with rabies virus DRV-NYC stain and treated with intrathecal injection of canine Ig with or without rabies VNA after onset of clinical signs. Dogs without treatment or treated with canine Ig without VNA all died of rabies while 66% of dogs treated with canine Ig with VNA survived. Both rabies N gene and antigen were detected in the dead, but not in the survived dogs. The inflammation and neuronal damage is extensive in the dead, but minimal in the survived dogs. These results indicate that the intrathecal injection of rabies VNA can clear the virus from the CNS and save dogs from clinical rabies. The intrathecal treatment with VNA may be a good method to treat clinical rabies in the future.

Key Words: Rabies, Rabies Therapy, Immune Therapy, Virus Neutralizing Antibody (VNA)

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DEDICATION

I dedicate this work to my family and friends for all their unconditional support, your support and love make this work possible.

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CHAPTER 1

INTRODUCTION

Rabies

Rabies is an ancient fatal neurotropic zoonotic disease caused by a lyssavirus that has been described for centuries in many different literary records with the characteristic of transmission by bite and neurological symptoms (Organization 2013). Historically, there have been several major epizootic outbreaks worldwide that took the lives of many human and animals. Although there were only scattered cases recently in the US, rabies is still an urgent threat for humans and animals worldwide because of the difficulty to eliminate the reservoir and incurability. (Monroe et al. 2016; mondiale de la Santé and Organization 2017).

Etiology

Rabies virus belongs to the order Mononegavirales, with only one non-segmented, negative-stranded RNA genome. Within this order, viruses with a bullet shape envelop are classified in the Rhabdoviridae family, which includes Lyssavirus, Ephemerovirus, and Vesiculovirus (Organization 2013). The genus Lyssavirus, based on the WHO Expert

Consultation on Rabies Second report, includes 12 Lyssavirus species divided into two phylogroups because of the genetic distance and serological cross-reactivity: Phylogroup I contains the classical rabies virus (RABV), European bat lyssavirus type 1 and type2, Duvenhage virus, and Australian bat lyssavirus, Aravan virus, Khujand virus and Irkut virus. Phylogroup II contains Lagos bat virus, Mokola virus, and Shimoni bat virus. However, the West Caucasian bat virus should be considered as the Phylogroup III (Organization 2013; Delmas et al. 2008).

Rabies virus has two major structural part: the internal helical neucleocapsid and the external envelop with a 10 nm glycoprotein spike. The internal nucleocapsid contains a 12-kb non-segmented negative RNA genome that encodes five viral proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase, also known as large protein (L). The ribonucleoprotein core (RNP) is for transcription and replication and is comprised of the negative RNA genome encased by N protein and associating with L protein and its cofactor, P protein. The M protein functions as the connection between the G protein spike and the RNP and plays a significant role in virus assembly and budding (Mebatsion, Weiland, and Conzelmann 1999; Harty et al. 1999).

N protein consists 450 amino acids (NCBI Reference Sequence: NP_056793.1) and is the most conserved protein in all the lyssaviruses which is responsible for the board antigenic cross-reactivity. Since it is conserved, N protein usually is used as the target of viral genome detection (Kulonen et al. 1999; Smith et al. 1992; Marston et al. 2007). Instead of completely consistency, N protein is confirmed to have polymorphism among its 1350 base pair (Kissi, Tordo, and Bourhy 1995). Furthermore, anti-N antibody is

considered a good tool not only to detect virus particles but also may have potential to become a strategy of vaccine development (Fu et al. 1991; Yan et al. 2000; Lafon and Wiktor 1985).

P protein contains 297 amino acids (GenBank: ABW97210.1) and it has been demonstrated by an *in vitro* RNA synthesis assay that L protein alone can initiate synthesis on naked RNA. While P protein can enhance the initiation and processing of the L protein (Morin et al. 2017). P protein also can interact with the N protein by binding on the carboxy terminal *in vivo* (Chenik et al. 1994). P protein shows a diverse potential to bind to different proteins such as dynein which may indicate P protein is involve in the retrograde transportation of rabies virus transmission (Raux, Flamand, and Blondel 2000). The most important potential function of P protein may be its interaction with STAT (signal transducers and activator of transcription). P protein can inhibit the alpha interferon (IFN-α), IFN-γ-stimulated Jak-STAT signaling and Gp130-dependent signaling by affecting the distribution of STAT1, STAT2 and STAT3 (Brzózka, Finke, and Conzelmann 2006; Vidy, Chelbi-Alix, and Blondel 2005; Chelbi-Alix et al. 2006; Lieu et al. 2013). The P-STAT interaction being the STAT antagonist is crucial for rabies virus infection in brain (Wiltzer et al. 2014).

Among these five viral genes, G gene has the lowest homogeneity. The G gene produces a single transmembrane glycoprotein which contains 524 amino acid (NCBI Reference Sequence: NP_056796.1) and is assembled as a trimeric spike (Gaudin et al. 1992). The spike can bind to the muscular form of the nicotinic acetylcholine receptor (nAChR) of the postsynaptic muscle membrane of motor neurons and the neuronal cell adhesion molecule (NCAM) (Lafon 2005; Thoulouze et al. 1998). Furthermore, low pH

can change the conformation of G protein which has provided a way to illustrate merging of the viral particle into host cell membrane (Gaudin et al. 1993). Profound evidence shows that G protein is also related to rabies virulence and it is responsible for inducing host immunity with virus neutralizing antibodies (VNA) (Cox, Dietzschold, and Schneider 1977; Dietzschold et al. 1990; Prehaud et al. 1988).

The fusion of the rabies virus envelope to host cell membrane, also called adsorption, initiates the entire process. After adsorption, clathrin-coated pits internalize rabies virus particle (Piccinotti, Kirchhausen, and Whelan 2013). The virions are transported through cytoplasmic endosomal vesicles (Superti, Derer, and Tsiang 1984; Klingen, Conzelmann, and Finke 2008; Schnell et al. 2010). In the vesicles, changes in pH will induce changes of the viral glycoprotein structure and release of the viral capsid into the cytoplasm (Gaudin et al. 1999). The G protein then goes through a three-step conformational change to release the viral genome into the host cell cytoplasm: At first, when the virions transported by vesicles in neutral pH, the G protein stays in the native state; then when the pH gets down to 6.4, there will be viral aggregation at the binding site and the glycoprotein becomes sensitive to proteases; and finally, with the pH value decreasing to 6.1, the viral membrane fuses with endosomal membranes and releases the naked viral genome to the cytoplasm (Gaudin et al. 1991; Gaudin et al. 1993). The L protein then transcribes the genomic strand of rabies RNA into leader RNA which leads to sequential synthesis of 5' end-capped and polyadenylated mRNAs that encode the five viral proteins (Poch et al. 1990; Schnell et al. 2010; Albertini, Ruigrok, and Blondel 2011). The intracellular ratio of leader RNA to N protein then activates viral replication to generate more virions. When enough viral genome and proteins are produced, the viral protein N,

P, and L complex encapsulates negative-stranded genomic RNA to form the RNP core, and the M protein forms a capsule, or matrix, around the RNP (Albertini, Ruigrok, and Blondel 2011). The RNP and M complex migrates to an area of the plasma membrane containing glycoprotein inserts, and the M-protein initiates coiling. The M-RNP complex binds with the glycoprotein, and the completed virus buds from the plasma membrane (Chenik et al. 1994).

The entire rabies virus life cycle therefore includes into 9 steps: 1: Adsorption (receptors and virion interaction). 2: Penetration (virus entry). 3: Uncoating (envelope removal). 4. Transcription (synthesis of mRNAs). 5. Translation (Synthesis of structural proteins). 6. Processing (G-protein glycosylation). 7. Replication (production of genomic RNA from intermediate strand. 8. Assembly. 9: Budding (complete virions).

Signs and Symptoms

In 1826, Krugelstein was the first one to publish an extensive 640-page book on every phase of rabies (Krugelstein 1826). Today, we have a thorough picture of all the clinical signs of rabies. In humans, the incubation period of infection can vary from 5 days to several years, but is usually 60 to 90 days (Organization 2010, 2013). Similar to humans, for dogs, the incubation can be vary from 7 to 125 days (Fekadu 1988). The classical development of canine rabies can be divided into three periods: the prodromal, the excitative, and the paralytic. The first symptoms of rabies, also called prodromal phase, may be non-specific to any disease with general neuropathic pain including weakness or discomfort or abnormal sensation or fever and in dogs, may be demonstrated as the

behavior changes. For instance, active and high-strung dogs may become more easygoing than usual while ordinarily friendly dogs may become shy and hide from humans or become irritable and snappy. These symptoms may last for days. As the disease progresses, the patient will develop two types of rabies: furious and paralytic rabies (Hemachudha et al. 2013; Hemachudha et al. 2005; Bennett, Dolin, and Blaser 2014).

The classical signs of the furious type include spasms in response to tactile, auditory, visual or olfactory stimuli (aerophobia and hydrophobia) alternating with periods of lucidity, agitation, confusion and signs of autonomic dysfunction; while the atypical ones do not present phobic spasms or autonomic hyperactivity (Consales and Bolzan 2007). The average illness period of disease in furious type is 6 days and in paralytic type is 11 days (Hemachudha et al. 2005). In most cases, there is a typical change in the bark, an altered phonation, caused by paralysis of laryngeal muscles. Spasms or even paralysis of the pharyngeal muscles make it difficult to swallow leading to drooling. Sometimes, frothing of the drooling salivation occurs due to heavy, rapid respiration. The paralytic type (dumb rabies) can happen after short or absent excitative phase. In this type, the disease usually progresses to muscular incoordination, paralysis, coma, and death. The most characteristic sign is the so-called dropped jaw, resulting from paralysis of the masseter muscles, making it impossible for the dog to eat or drink. No matter in which species or type of disease, once the characteristic encephalomyelitis of rabies appears, the disease is nearly always fatal, and treatment is typically supportive (Jackson et al. 2003).

Diagnosis and Histopathological changes

It is shown by a study using different detection assays using samples from a rabies outbreak in 1988: diagnosis using the direct fluorescent antibody (DFA) test, detecting the presence of rabies virus antigens in frozen brain tissue was the most sensitive and accurate test and is currently recommended as the gold standard for rabies diagnosis (Kulonen et al. 1999; Organization 2013). The test may require that the animal be euthanized and brain stem and cerebellum are preferably tested. Before current diagnostic methods were invented, histologic examination with hematoxylin and eosin staining was used in the diagnosis. Encephalomyelitis, with perivascular cuffing of lymphocytes or polymorphonuclear cells, lymphocytic foci, Babes nodules consisting of glial cells, or Negri bodies are often found as evidence of rabies (Kristensson et al. 1996; Tangchai and Vejjajiva 1971). Immunohistochemistry (IHC) methods is a sensitive and specific way to detect rabies in formalin-fixed tissues as well, but with chromogenic staining rather than fluorescence. Just like the DFA test, it uses specific antibodies to detect rabies virus inclusions and it is more sensitive than conventional histopathological examination (Sinchaisri et al. 1992; Jogai, Radotra, and Banerjee 2000). Virus isolation in cell cultures by murine neuroblastoma cells (NA) or baby hamster kidney (BHK) cells provides an excellent environment for amplification of rabies virus without the use of animals (Smith et al. 1978; Kawai, Matsumoto, and Tanabe 1975; Rudd and Trimarchi 1987).

On the other hand, in clinical practice, fast and accurate laboratory diagnosis of rabies is extremely essential for timely administration of post-exposure prophylaxis. Recommended tests are performed on samples of saliva, serum, spinal fluid, and skin biopsies of hair follicles at the nape. Saliva can be tested by virus isolation or reverse

transcription followed by polymerase chain reaction (RT-PCR) while serum and spinal fluid are tested for antibodies to rabies virus by Enzyme-Linked Immunosorbent Assay (ELISA) or rapid fluorescent focus inhibition test (RFFIT). Skin biopsy specimens are examined for rabies antigen in the cutaneous nerves at the base of hair follicles (Meslin, Kaplan, and Koprowski 1996; Organization 2013).

Transmission and Epidemiology

Rabies virus enters the body through wounds or by direct contact with mucosal surfaces (Organization 2013). Most commonly, rabies virus transmits through rabid dog bite with virus particle in saliva (Organization 2013; Ichhpujani et al. 2008). When the rabies virus is introduced into a muscle through a bite from another animal, it will travel from the site of the bite to the brain by moving within nerves, from the endplate to the peripheral nerves system (PNS), and from the motor axon to the central nervous system (CNS) (Ugolini 2007; Klingen, Conzelmann, and Finke 2008). The animal does not appear ill during virus traveling. The time between the bite and the onset of symptoms is called the incubation period and it may last for days to months. (Hemachudha et al. 2013) Later, the virus reaches the brain and replicates causing inflammation of the brain. Then, the virus moves from the brain to the salivary glands and saliva. At that time, virus has infected the neurons of brain and animals will begin to show the signs of prodromal phase. Most of these signs are non-specific, but within a very short period of time, the virus has caused enough damage to the brain that the animal begins to show signs of rabies. The viral particles may be found intermittently in the saliva, and the relative amount of excreted

virus may vary greatly over time, before and after the onset of clinical signs (Fekadu, Shaddock, and Baer 1982; Nagaraj et al. 2006).

Nevertheless, there are occasional cases showing that the virus can be transmitted by unusual ways, for example, airborne (Winkler 1968; Winkler et al. 1973; Johnson, Phillpotts, and Fooks 2006), milk excretion (Control and Prevention 1999), or organ transplantation (Hellenbrand et al. 2004; Srinivasan et al. 2005). All mammals are susceptible to rabies virus infection, but several species can be the viral reservoirs, such as, raccoons, skunks, foxes, and bats. Raccoons were the most frequently reported rabid wildlife species in the CDC annual rabies surveillance report in the US (Monroe et al. 2016).

Rabies in the U.S.

Over the last 100 years, rabies in the United States has changed dramatically. According to CDC annual surveillance report, since 1980, wildlife has accounted for over 90% of all rabid animals reported in the United States and the principal reservoirs are raccoons, bats, skunks, foxes, and mongooses (in Puerto Rico) (Monroe et al. 2016). The annual rabies-related human deaths in the United States has declined from more than dozens annually to one or two per year (Noah et al. 1998; Monroe et al. 2016; Dyer et al. 2014; Reid-Sanden et al. 1990). Most of the human rabies cases have been associated with rabies virus from bats, particularly the silver-haired bats (Monroe et al. 2016). Rabies has been diagnosed in a total of 37 persons in the United States since 2003. Moreover, bats were implicated as the source of infection in 17 patients: 7 cases associated with bat bite,

6 cases of bat contact without a bite, and 4 cases associated with bats without a known exposure (Monroe et al. 2016).

Rabies around the world

Rabies virus claims an estimated 59,000 human lives annually, mostly among underserved populations in Africa and Asia (Organization 2015; Hampson et al. 2015). The dog bite has been implicated as the main source in human rabies infection worldwide (mondiale de la Santé and Organization 2017). In 2015, the World Health Organization (WHO) started a project called "Global Elimination of rabies", to achieve zero human deaths from dog transmitted rabies by increasing dog vaccination coverage to 70%, timely pre- or post-exposure prophylaxis for the population at risk, robust human and dog disease surveillance and community mobilization (Hampson et al. 2015)

In Europe, canine rabies has gradually disappeared from most countries since the 1930s (excluding the European bat rabies), and wildlife was also the most important reservoir, especially foxes which accounted for 83% rabid animals (Finnegan et al. 2002; Cliquet and Aubert 2003; Schatz et al. 2013). Thanks to the all the effort, oral vaccine implementation, foxes-mediated rabies from vast areas of Western and Central Europe have been eliminated (Freuling et al. 2013).

In developing countries in Asia and Africa, rabies is endemic and is a major public health problem. In Asia, estimates suggest that in 2010, human rabies deaths occurred between 15,900 to 34,500 which is significantly decreased due to vaccine program implementation and improvement of post-exposure prophylaxis; unfortunately, despite

surveillance systems are too highly variable to have more accurate case numbers (Organization 2013; Taylor and Knopf 2015). India has been shown to have the highest incidence of rabies worldwide, despite the fact it is not a nationally notifiable disease (Taylor and Knopf 2015; Organization 2013). It has been reported that rabies exists in almost all regions of the country and canine rabies appears to be the major reservoir (Hampson et al. 2015; Cherian et al. 2015; Reddy et al. 2014). Compared to rabies in the US or Europe, only 3% of cases were from cats and wildlife (Sudarshan et al. 2006). Similarly, in China, rabies cases are reported in almost all provinces, but most human rabies cases come from rural areas and stray dogs were responsible for more than 95% of the human rabies cases (Organization 2013; Zhang et al. 2005; Song et al. 2009). The main reasons that the developing countries do not have good control of rabies was because of the substantial economic burden and also the lacking of the knowledge of the public health impact of rabies (Hampson et al. 2015; Organization 2013; Zhang et al. 2005). A well-organized rabies control programs with effective rabies vaccine and prompt proper prophylaxis is extremely needed in these developing countries in order to eliminate rabies globally (Organization 2013) .

Prevention

Although rabies is a fatal disease, it can be controlled by vaccination of domestic animals and prevented by a promptly proper medical care: pre-exposure prophylaxis or post-exposure prophylaxis (PEP) (Jackson et al. 2003; Gnanadurai, Huang, et al. 2015). Pre-exposure vaccination is suggested for people who are at risk of exposure to rabies virus

because of their job, residence or travel. . Post-exposure prophylaxis consists of immediate wound cleaning and vaccine immunization, together with administration of rabies immune globulin for people who have been bitten or exposed to rabid animals (Paul and O'Connell 2002; Noah et al. 1998; Organization 2013).

Nowadays, there are multiple commercial rabies vaccines available for domestic animals in the market. The vaccines can be classified into several types, including 1) inactivated and modified-live virus vectored products, 2) products for IM, SC and/or oral administration, 3) products with durations of immunity for periods of 1 to 3 years, and 4) products with various minimum ages of vaccination. Among these, inactivated vaccine is the most widely used licensed rabies vaccine and sold in the USA for domestic animals (Brown et al. 2016). For humans, human diploid cell vaccine (HDCV) or purified chick embryo cell vaccine (PCECV) are two vaccines that are recommended by CDC guideline to use in prophylaxis (Manning et al. 2008). HDCV is prepared from the Pitman-Moore strain of rabies virus grown on human diploid cell culture and is inactivated with beta-propiolactone (Wiktor, Plotkin, and Koprowski 1977). On the other hand, PCECV is prepared from the fixed strain, Flury LEP, of rabies virus grown on chicken fibroblasts (Dreesen et al. 1989). The recommended approach for pre-exposure primary vaccination involves three 1.0-mL injections of HDCV or PCECV administered intramuscularly on days 0, 7, and 21 or 28 for people that haven't received any immunization (Manning et al. 2008). However, every vaccine may also have its side effects. In a 3-year length vaccine efficiency study in dogs, 217 cases with adverse effect were reported and 4 cases of lack of efficacy (Frana et al. 2008). Neurological adverse events, such as fever, headache, dizziness, gastrointestinal symptom, encephalomyelitis or Guillain-Barré-like syndrome

and systemic hypersensitivity reactions, such as urticaria, pruritic rash, and angioedema have been reported in human cases (Pait and Pearson 1949; Redewill Jr and Underwood 1947; Tizard 1990; Varricchio et al. 2004; Fishbein et al. 1993). Besides the risk of complications from the exposure of primary vaccine or booster, the cost of a course of standard prophylaxis including rabies immune globulin and four doses of vaccine given over a two-week period typically exceeds $3,000 according to the CDC website latest updated in 2015. Furthermore, unware of exposure and non-specific early signs of rabies will make effective prophylaxis more difficult to achieve (Messenger, Smith, and Rupprecht 2002).

The case reports of rabies survivors

Until now, there have been about 8 case reports of survival from clinical rabies with good follow-up data. The first report about how medical care can help the patient was reported in 1969: A 2½-year-old boy was severely bitten by a rabid bobcat in San Diego. He was sent to the hospital and was timely treated with duck embryo rabies vaccination but not with rabies antiserum. There was no virus isolated but rabies VNA was high and viral antigen was detected on brain biopsy. The boy had survived 133 days after onset of symptoms (Emmons et al. 1973). Following previous evidence, rabies therapy was improved since then. In 1972, there was a 6-year-old boy presented clinical rabies 20 days after he was bitten on the left thumb by a bat. Rabies virus was isolated from the bat but not from the boy. The serum sample and CSF tested positive for anti-rabies antibody while the direct inflorescent antibody (DFA) tested negative. He received a course of duck

embryo rabies vaccine and was recovered after 6 months of illness (Hattwick et al. 1972). A laboratory infection with rabies virus was reported in 1977. The patient was infected by a fixed strain through the respiratory route and was diagnosed by the rise in serum and CSF antibody titer. The patient was reported with remarkable recovery (Control and Prevention 1977; Control 1977). In 1976, there was a 45-year-old women who was bitten by an aggressive dog and developed cerebellar striatal syndrome within 21 days of the bite. She received a 14 daily doses of suckling mouse brain vaccine and also two booster doses. The serum and CSF sample tested positive for anti-rabies antibodies. The patient had a remarkably recovery after 13 months (Porras et al. 1976). Another case report was documented in 1992. A 9-year-old boy was bitten by a rabid dog (confirmed by direct fluorescent assay) and the wound was cleansed with antiseptic immediately after bite. The boy received a course of VERO rabies vaccine and a dose of human diploid vaccine a day after the bite. The boy developed symptoms of rabies 18 days after the bite. His serum and CSF samples tested positive for anti-rabies antibodies but none of the samples nor biopsy had detectable virus antigen or RNA. The patient survived but with serious neurologic sequelae (Alvarez et al. 1994). Another case happened in India in 2002. A six-year-old girl was admitted to hospital because of a 4-day history of fever, inability to swallow liquids, photophobia and visual hallucinations, and altered sensorium for 2 days after she was bitten by a stray dog on the face and hand. She received three doses of purified chick embryo cell vaccine right away and also one dose of human diploid cell vaccine (HDCV). No local wound treatment was given, and rabies immune globulin (RIG) was not administered. The rabies antibody titers of serum and CSF increased from day 8 to day 90. However, skin biopsy from the nape of the neck was negative for rabies antigen and so was a cornea1 test.

The girl recovered after 5 months of illness but with mild neurologic sequelae (Madhusudana et al. 2002).

In the US, center of disease control and prevention (CDC) has a great surveillance system to monitor rabies. In the US, from 1960 to 2013, there were 117 cases and only 5 survivors on the record (Petersen and Rupprecht 2011; Monroe et al. 2016). A case in 2004 was a 15-year-old girl who had a history of bat bite a month previously and was submitted to hospital because of generalized fatigue and paresthesia of the left hand. Although her nuchal skin and saliva were negative for virus by virus isolation and direct fluorescent antibody staining, rabies virus-specific antibodies were detected in the patient's serum and CSF. The patient received supportive care with intravenous ribavirin and neuroprotective measures, including a drug-induced coma (Ketamine) and ventilator support. After 36 days of illness, the patient was extubated and able to act normally. This is the first documented recovery from clinical rabies by a patient who had not received either pre- or post-exposure prophylaxis for rabies (Willoughby Jr et al. 2005; Control and Prevention 2004).

In 2009, there was a 17-year-old girl who went to hospital because of persistent frontal headache, photophobia, emesis, neck pain, dizziness, and paresthesia of face and forearms. The girl was first treated with intravenous ceftriaxone and dexamethasone for 3 days. The patient was reported to have had contact with a bat but hadn’t noticed a bat bite nor scratch. No rabies virus antigens or RNA were detected in saliva and nuchal skin biopsy sample but serum and CSF samples tested positive for rabies virus antibodies by IFA. The patient received 1 dose of rabies vaccine and 1,500 IU of human rabies immune globulin. The girl was discharged after 16 days of hospitalization and was reported with recurrent

neurologic symptoms. This is a case where there was lack of animal bite and the patient survived without intensive care (Control and Prevention 2010).

The latest case of survivor from rabies was an 8-year-old girl in California who was brought to hospital because of progressive sore throat, difficulty swallowing, and weakness. The patient was sedated with ketamine and midazolam and started on amantadine and nimodipine to prevent cerebral artery vasospasm, and fludrocortisone and hypertonic saline for supportive care. No human rabies immune globulin nor rabies vaccine was administered. Neither rabies virus antigens nor RNA were detected in any sample. The patient was discharged from the hospital 37 days later and showed no signs of cognitive impairment (Control and Prevention 2012).

Treatment

Although rabies is preventable with prophylaxis, there is no proven cure after the onset of symptoms (Manning et al. 2008). Moreover, if rabies is not treated timely, patients typically will die within five to seven days after the onset of symptoms (Gode et al. 1976). Therefore, it is urgently needed to develop therapeutics for clinical rabies. A proper medical care may prolong survival period up to 133 days but the majority of patients with rabies do not survive more than 3 weeks (Noah et al. 1998; Emmons et al. 1973). A lots of review have been done to find out the potential therapy. However, from the previous case reports of survival from rabies, there was no one treatment protocol that has been identified as the remedy. Also, in view of the poor prognosis and the severe neurologic sequelae in the survivors, routine management of patients with rabies should be palliative

with use of sedatives, narcotic analgesics, antiepileptic medications, and neuromuscular blockers (Jackson et al. 2003). A complete course of rabies vaccination was doubtful because of the possibility of post-vaccination encephalomyelitis was described in one case report (Porras et al. 1976). But a single dose of rabies vaccine combined with immune globulin and intensive medical care seems appropriate (Control and Prevention 2012, 2010). A regimen of combined therapy was suggested including administration of rabies vaccine (multiple-site intradermal route), HRIG (intramuscular), ribavirin (intravenous and intraventricular via Ommaya reservoir), IFN-α (intravenous and intraventricular via Ommaya reservoir), and ketamine (intravenous infusion) (Jackson et al. 2003). However, several therapies have been attempted in the past without much of a success (Fooks et al. 2014). Most of the documented survivors of rabies received post-exposure prophylaxis with one or more doses of rabies vaccines (mondiale de la Santé and Organization 2017; Monroe et al. 2016).

A clinical case report in 2004 was a success for an induced-coma therapy and it was the first case of a survivor who did not received any prophylaxis. This protocol of treatment was named the "Milwaukee Protocol" which included an N-methyl D-aspartate (NMDA) receptor antagonist therapy (Control and Prevention 2004; Willoughby Jr et al. 2005; Zeiler and Jackson 2016). Because of the success of the first clinical survivor, Milwaukee protocol was implemented as a clinical trial worldwide and also was updated with a combination of therapeutic coma, ketamine infusion, amantadine, and the screening/prophylaxis/management of cerebral vasospasm (Zeiler and Jackson 2016). However, the protocol hasn't proved to be effectiveness and at least 31 documented failures reported in the literature to date including Asia, Europe, Africa and north/south America

(Jackson 2011; van Thiel et al. 2009; de Souza and Madhusudana 2014; Jackson 2013; Zeiler and Jackson 2016). Although there were two case reports from Colombia and Peru claimed to be considered survivors of Milwaukee protocol because they survived the initial phase of acute illness, they eventually died (Aramburo et al. 2011; Caicedo et al. 2015). Therefore, unfortunately, Milwaukee protocol is still questionable of its true effectiveness.

In reviewing all the case reports of survivors from clinical rabies, we found a commonality between all the 8 survivors: they were all tested positive for the anti-rabies antibodies in serum or CSF. The similar observation about the presence of rabies virus neutralizing antibodies in the CSF after experimental infection was noted in rabid dogs as well (Gnanadurai et al. 2013). By contrast, early studies have shown that more than 70% rabies patients do not develop VNA by the time of death (Hemachudha 1994). It is clearly suggested that VNA in the central nervous system plays a vital role in the recovery from rabies. However, it is difficult for any molecules to deliver to the central nervous system effectively because of first-pass metabolism on entering the systemic circulation and, also the uptake of the drug by other non-target body tissues and the blood–brain barrier (BBB) and the blood–spinal cord barrier (Appolinario and Jackson 2015; Stockwell et al. 2014; Bartanusz et al. 2011). The blood-brain barrier (BBB) consists of a layer of endothelial held together by tight junctions to prevent small molecules from blood vasculature throughout the brain (Neuwelt 2004). It is clearly that not only the immunoglobulin administrated, but also the VNA that developed by patient himself may have the difficulty to enter the central nervous system to clear the virus (Hemachudha et al. 1989). Nevertheless, it was proven that inability to enhance blood-brain barrier permeability in rabid animal may lead to animal death (Roy et al. 2007). Also, it has been shown that the

enhancement of BBB permeability is required for lymphocytes and other immune factors to enter into the CNS to clear RABV and prevent the occurrence of rabies (Hooper et al. 2011; Roy and Hooper 2007).

Previous studies of VNA treatment

It was reported that administrating VNA to the brain after the disruption of blood-brain barrier (BBB) may increase the survival rate in rats (Liao et al. 2012). To further illustrate the alteration of BBB permeability may be the key to develop potential rabies therapy, a mice model was used. Our previous studies revealed that intracerebral administration of recombinant rabies virus (rRABV) expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) or a combination of inactivated rRABV and chemoattractant protein-1 (MCP-1, also termed CCL2) both can prevent mice from developing rabies and clearing RABV from the CNS by enhancing the innate and adaptive immunity as well as the BBB permeability (Wang et al. 2011).

Another study was done to support the relationship between BBB and VNA to the clinical rabies in mice by using the RABV-antibody positive serum which was prepared from blood of ICR mice immunized with rRABV-GMCSF vaccines (Wen et al. 2011). The ICR mice was infected with Street RABV (Dog Rabies Virus from Mexico, DRV-Mexico) (Zhang and Fu 2012). At 5 days post infection (dpi), mice were treated intravenously from the tail vein with mouse serum containing VNA. Recombinant murine MCP-1 was given intracerebrally to enhance the BBB permeability. Mice were observed for 3 weeks. A 60% of survival rate was observed in the mice treated with serum containing VNA together with

MCP-1. On the other hand, only 10% of the mice survived after treatment with the combination of MCP-1 and RABV-negative serum or with the positive serum alone without MCP-1. These results showed exogenous VNAs is crucial in the clearance of RABV from the brain and prevent the development of rabies if the BBB permeability is enhanced (Huang et al. 2014).

Despite the success in mice and rabbits, those animal models are not the best one to reflect the possible outcome in human. Dogs are proved to be the natural rabies hosts and also, the incubation period (7 to 125 days) which is similar to the average 60 to 90 days in humans (Organization 2010, 2013; Fekadu 1988). Therefore, dogs may be the best suited animal model especially for the development of rabies therapy for humans (Gnanadurai, Huang, et al. 2015). These studies indicate that it is feasible to develop an intrathecal administration of VNA therapy for rabies.

Goals and objectives

We thus proposed to administer VNA directly into the CSF in dogs after developing clinical rabies. The main objective of this study is to treat the rabid dogs by intrathecal injection of canine Ig with VNA. The goal of this study is to provide evidence that rabies virus can be cleared from the CNS of dogs by IT administration of VNA, which will provide a solid foundation for developing rabies therapy in humans.

CHAPTER 2

MATERIALS AND METHODS

Viruses and animals

New York Street rabies virus, also known as DRV-NYC, was used in this study. The virus was acquired from Center of Veterinary Biologics, United States Department of Agriculture and is approved for rabies challenging. According to the information provided by USDA, the NYC strain rabies was recovered from the salivary gland of sero-negative cats challenged in the three-year duration immunity study.

The dogs used in this study were purchased from Ridglan Farms. INC., Blue Mounds, WI. Six female beagles were selected and tested for rabies maternal antibodies by RFFIT.

Rabies virus infection and animal observation

The DRV-NYC wild type rabies virus was diluted 1:250,000 with Dulbecco's Modified Eagle Medium (Gibco™) for virus infection. Naïve dogs were infected with 20 MICLD50 (50% mouse intramuscular lethal dose) of DRV-NYC virus by intramuscularly (IM) injection in the temporalis muscle on both sides with 0.5 mL after sedation with Acepromazine (AceproTabs, Henry Schein®). Infected dogs were monitored once a day

before infection and three times a day after infection. During the observation period, once suspicious clinical signs of rabies were observed such as depression, loss of appetite, anorexia or behavior change, the dog would be checked every three hours and treated. All signs and conditions were recorded. The appearance of hind limb paralysis of one or both limbs was considered the experimental endpoint at which time animals were humanely euthanized by intraveous Beuthanasia®-D (Schering-Plough Corporation). Blood, CSF and brain samples were collected before treatment and/or at end-point for further analysis.

Purification of Canine immunoglobulin (Ig)

Canine Ig containing VNA was purified from dogs that were immunized with TriGAS from a previous study (Gnanadurai, Yang, et al. 2015). Whereas the canine Ig without VNA was purified from the serum of naïve dogs. The canine immunoglobulin (Ig) was isolated by following the procedure of the Thiophilic Adsorption Kit (Pierce®). After antibody purification, the 10 mL solution was put into a G2 dialysis cassette (Slide-A-Lyzer™) to remove any chemicals and salt in the antibody solution. The 10 mL desalted solution was concentrated to 1 mL by using an Amicon Ultra-15 centrifugal filter 3k device (Millipore™). The same procedure was conducted with naive canine serum. The purity of canine immunoglobulin were confirmed by SDS electrophoresis and stained by GelCode® (Thermo scientific®). Further, the purified canine immunoglobulin solutions were tested for pyrogens by LAL chromogenic endotoxin quantitation test (Pierce®) and VNA levels by rapid fluorescent foci inhibition test (RFFIT). After preparation, the canine immunoglobulin solutions were aliquoted into small vials and stored at 4°C.

Rapid Fluorescent Foci Inhibition Test (RFFIT)

The rapid fluorenscent foci inhibition test, as known as RFFIT, is a virus neutralization assay used for the titration of anti-rabies virus antibodies *in vitro* (Smith, Yager, and Baer 1973; Zalan, Wilson, and Pukitis 1979). Canine serum was collected at the three-different time points: before infection, prior to treatment and at the end-point of humanely euthanasia. All the serum samples were tested for VNAs by RFFIT with the steps below. First, DMEM (Gibco™) was added in a 96-well plate (Nunc®). Then, challenge virus standard 11 (CVS-11) strain suspension with 50 FFD50 (50% Focus Forming Dose) was added into each well. The canine serum samples were inactivated at 56°C for 30 minutes and then added to the wells with three-fold serial dilutions. The mixture solutions were incubated at 37°C for 90 min after which, NA cells (10^5 cells) were added to each well and the whole plate was incubated in a CO_2 controlled incubator for 20 hours at 37°C. The NA cells were fixed with 50 ul pre-chilled 80% acetone and keep at -20°C for 30 minutes. FITC-conjugated anti-RABV N antibodies (Fujirebio Diagnostics, Inc.) were diluted at 1:60 and added to each well. The staining solutions were incubated at 37°C for 1 hour in the dark. For all samples, 20 distinct microscopic fields per well were examined under a fluorescent microscope at ×100 magnification to score the rabies-infected cells with positive green fluorescence. The 50% neutralization endpoint titer was calculated by the following formula: $\log_{10}$ 50% end point dilution= $(x - d/2 + d \sum ri/ni)$. x equals to the log10 of the reciprocal of the highest dilution (lowest concentration) at which all wells are positive; d equals to the log10 of the dilution factor; ni equals to the number of wells used in each individual dilution; ri equals to number of positive wells (beginning with dilution x). A reference serum (National Institute for for Biological Standards and

Control, Herts, UK) was used as experimental control and all the values were normalized to international units (IU/mL). The lowest limit of detection is 0.1 IU/mL.

Anesthesia and Analgesia protocol

All dogs were fasted at least for 8 hours before anesthesia. Acepromazine (AceproTabs, Henry Schein®) was orally administered 30 minutes prior to the start of the procedure in order to decrease the stress. Tiletamine and Zolazepam mixture (Telazol, Zoetis®) was given 3 to 6 mg/kg intramuscularly by using a pole syringe based on the different reaction to sedation of individual animals. After animals completely lost all the involuntary muscle movement, they were moved to the surgery room. Venous catheter was placed in the cephalic vein and a blood sample was collected. Normal saline (Hospira®) was administered at 5 mL/kg/hr intravenously during treatment and around 100-200 mL subcutaneous fluid was given after treatment. Propofol (PropoFlo™, Zoetis®) was given intravenously at 2 mg/kg for induction. Atropine (Vet One®) was given intramuscularly at 0.22 mg/kg. After the dog was put under anesthesia, 2 to 3% concentration of Isoflurane was used by intubation to maintain the depth of anesthesia with 2 L/min Oxygen flow. Heart rate, respiratory rate, body temperature, blood pressure and oxygen level were monitored by a monitor (CMS8000VET, Contec®) during anesthesia and recorded. A heating pad (T/Pump, Stryker®) was provided at 42°C to help control body temperature. Artificial tears and ophthalmic ointment (Puralube®, Dechre®) were applied to protect the eyeball from drying. Buprenorphine (Buprenex®, Reckitt Benckiser Pharmaceuticals, Inc.) was given intramuscularly at 0.005-0.1 mg/kg after treatment to prevent acute pain. After

treatment, animals were examined for pharyngeal reflex, muscular tone and palpebral reflex. Once the animal showed response to stimulation, it would be placed in a cage in sternal recumbency and closely monitored until fully awake.

Treatment protocol

The DRV-NYC infected rabid dogs were monitored three times per day for the appearance of any rabies clinical signs. The initial rabid clinical sign is usually non-specific and mild, such as hyperactivity, depression, loss of appetite and behavior change (hiding, quietness, lethargy, abnormal hyperactivity, nervousness and shivering). Once clinical signs were present continuously and constantly, the dog would be selected to start the treatment. The rabid dogs were alternatively selected based on the onset of symptoms to be treated by purified canine Ig containing 10 IU VNAs or with canine Ig with similar protein concentration but without VNA.

Once it was decided that the dog was to be treated, it was anesthetized, positioned in right lateral recumbency, and the area around external occipital protuberance was clipped and cleaned with 10% iodine solution and 70% alcohol wipes. A 22-gauge disposable spinal needle with a stylet was then used for a cerebellomedullary cisternal tap. The cerebrospinal fluid was collected in a 2 mL screw-cap microcentrifuge tube. For intrathecal injection, 1080 µL of cerebrospinal fluid was mixed with 120 µL purified Ig solution with or without VNA. The mixture was mixed thoroughly and then slowly injected back at the same site of the cisternal tap. Accompanying the intrathecal treatment, all the dogs received subcutaneous normal saline fluid and a/d diet® for supportive therapy.

Brain, blood and serum collection protocol

For blood collection, dogs were sedated and about 10 mL blood was collected from the jugular or saphenous vein at day 0, before treatment, and at termination. The whole blood sample was placed at 37°C for 30 minutes to 1 hour and then put at 4°C for overnight. After the sample was fully clotted, it was spun down at 10,000 g for 10 min and the supernatant was isolated for serum antibody analysis.

At termination, the entire brain was collected at necropsy and washed with 1 × PBS (Phosphate-buffered saline: 8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g KH2PO4, for 1 L). The brain was separated into right and left hemisphere. The right hemisphere was stored at -70°C while the left one was fixed in 10% neutral buffered formalin solution (100ml formalin stock solution, 4g NaH2PO4 and 6.5g Na2HPO4 for 1L) for further analysis.

Rabies virus detection by Reverse transcription polymerase chain reaction (RT-PCR)

The frozen left brain hemisphere sample was used to extract RNA within a laminar flow hood. Around 100 mg of brainstem from each sample was taken and mixed with pre-cold 1 mL TRIzol^{TM} reagent. The mixture was ground with a pestle and incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex and then vortexed. Then 0.2 mL of pre-cold chloroform was added and the sample incubated for 2 to 3 minutes at room temperature. The sample was then centrifuged at 12,000 ×g for 15 minutes at 4°C and the colorless aqueous phase containing the RNA was transferred to a new tube and 0.6 mL of pre-cold 100% isopropanol was added. Following incubation at room temperature for 10 minutes the solution was centrifuged at 12,000 ×g

for 10 minutes at 4°C. After removing the supernatant, the RNA pellet was washed with 1 mL of pre-cold 75% ethanol, vortexed briefly, then centrifuged at 7500 ×g for 5 minutes at 4°C. After removing the wash solution, the RNA pellet was air dried. The RNA pellet was suspended in RNase-free water and stored at -70°C for further cDNA synthesis.

The RNA sample extracted from brainstem tissue was used for cDNA synthesis. First, 500ng RNA was mixed with 1μL oligo dT18-20 primer, and 1μL 10mM dNTP, and distilled water for a total 13μL mixed solution. After heating the mixture to 65°C for 5min, incubate on ice for 2mins. Then, 4μL 5× First-Strand buffer, 1μL 0.1M DTT, 1μL RNase inhibitor (Roche®), 1μL Superscript II (Invitrogen®) was added and incubated at 55°C for 60mins, and inactivated by heating at 70°C for 15mins. The cDNA of brainstem was collected can be kept at -20°C.

Polymerase chain reaction (PCR) was performed by using a pair of primer: F-N127, 5'-ATG TAA CAC CTC TAC AAT GG-3' and R-N8m, 5'-CAG TCT CYT CNG CCA TCT-3'. This primer pair were made for rabies virus N gene and the expected PCR product 1.5kp. 0.5μL Ex Taq (Takara®), 2μL Primer F, 2μL Primer R, 4μL dNTP, 4μL 10X buffer, 4μL sample cDNA, and H2O added to 40μL was prepared for the PCR reaction and incubated at 94°C for 10 min. Cycling parameters were 35 cycles of 94°C for 30s, 56°C for 30s, 72°C for 40s and a final incubation at 72°C for 10 min. The final PCR product was separated by gel electrophoresis with 1.5% agarose gel at 100 volts for 20 minutes.

Histopathological evaluation

The right brain hemisphere collected at termination was fixed in 10% buffered formalin and the cerebrum, cerebellum and brainstem were sectioned at 4 μm and stained with hematoxylin and eosin (H&E) method. Histopathological examination was performed to evaluate the degree of inflammation and neuronal damage. Severity of lesions was graded according to the methods described by Shackelford *et al*., 2012 (Shackelford et al. 2002). Degree of lesions was graded from one to five depending on severity: 0 = normal; 1 = minimal (< 1%); 2: slight (1-25%); 3 = moderate (26-50%); 4 = moderately severe (51-75%); 5 = severe/high (76-100%). Mean histopathological scores were calculated by dividing the sum of the score per grade of affected area by the total number of examined area including six different section of brain such as cerebrum, cerebellum, brainstem, thalamus, hypothalamus and hippocampus.

Rabies virus detection by immunohistochemistry

Brain tissue was trimmed to 2 mm thickness and submitted to Histology Laboratory of University of Georgia for histological slide preparation. The section of slide was deparaffinized and incubated with 10 mM sodium citrate buffer pH 6.0 at boiling temperature for 20 minutes. Then, the slide was quenched with 3% hydrogen peroxide (Fisher®) for 5 minutes, followed by blocking in 1:10 diluted power block (Biogenex®) for 5 minutes. The primary rabies monoclonal antibody provided by CDC at 1:400 dilution was incubated at room temperature for 1 hour followed by two 10-minute room temperature incubations: Biotinylated-anti-mouse antibody (Vector®) at 1:100 dilution and 4^+ HRP solution (Biocare medical®). The reaction was revealed by diaminobenzidine

peroxidase substrate (Dako®) for another 12 minutes. Counterstain was used with hematoxylin. The result of each slides was confirmed by using a light microscope.

Ethics statement

The University of Georgia's University Research Animal Resources unit is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC-I). The registration number from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Animal Care is (USDA APHIS-AC). We have an assurance on file with the NIH-Office of Laboratory Animal Welfare (NIH-OLAW), and are in compliance with the PHS Policy on Humane Care and Use of Laboratory Animals and the 8th edition of the Guide for the Care and Use of Laboratory Animals, 2011.

Statistical analysis

Statistical significance of the differences between groups was tested using student's T-test with, ** a p value< 0.001, and *** a p value< 0.0001 using Graph Pad prism software.

CHAPTER 3

RESULTS

DRV-NYC infection in beagles

Eight beagles were purchased at 3 months of age which is the latest age that can be transported across state lines without rabies immunization. Serum samples were collected before virus infection to monitor their maternal VNAs which usually declines to an undetectable level at 5 months of age. Once the maternal VNAs declines to undetectable levels, the dogs were infected with DRV-NYC rabies virus. Six out of the 8 dogs developed clinical signs of rabies after DRV-NYC infection. The morbidity of 20 MICLD50 of DRV-NYC by intramuscularly injection into the temporalis muscle was 75%.

Clinical observation

Dogs were monitored three times daily and observed for appearance of signs of rabies. The incubation period started from the virus infection to the appearance of clinical signs. Once the dogs showed any kinds of signs, it means the onset of prodromal stage and the end of the incubation period. The incubation period in this study varied from 6 to 42 days (Table 1). The early signs of prodromal stage in rabies are non-specific and mild. We observed quietness, drowsy eye contact, loss of appetite, behavior change (such as hyperactivity, licking or jumping) during this study. When the signs were constantly and

repeatedly happening, the dog was treated with intrathecal injection of canine Ig with or without VNA. Only one dog out of three dogs that were treated with canine Ig containing VNA showed severe signs of rabies. The severe neurological symptoms observed include depression or restlessness, profuse salivation and/or frothing at the mouth, difficulty in swallowing and drinking, muscular incoordination or ataxia, trembling, tremors and retching (Table 1). On the other hand, all dogs that treated with canine Ig without VNA were seen severe symptoms. The illness period started at the onset of symptoms until the dogs recovered or died. The overall illness period in this study was similar between two treatment groups. The illness period was varied from 2 to 4 days (Table 1).

In the group of canine Ig treatment with VNA, there were dog no.1, 2, and 3. Among all, dog 1 and dog 2 survived but dog 3 died. The survival rate was 66% (Figure 1). Dog 1 was found with hyperactivity such as jumping in the cage and rapid shallow breathing on the 6^{th} day post infection. Treatment with VNA was performed immediately on day 7 post infection. Dog1 did not develop severe signs and was euthanized on day 60 post infection. Dog 2 did not finish its dry feed at night shift on the 42^{nd} day post infection and started to lick the floor often and tried to bite people at the 43^{rd} day post infection. Treatment was performed on 44^{th} day post infection and Dog 2 did not develop any further severe rabies-related clinical sign. Dog 3 was found quiet, shivering and lethargic on 27^{th} day post infection and was treated right away at the same day with 20 IU VNAs mixing with self CSF. Dog 3 showed depressed, muscle shivering, lethargy and proceed to unable to walk or stand on 28^{th} day post infection and was euthanized the same day. Dog 1 and Dog 2 were euthanized at 60^{th} and 63^{rd} day post infection, respectively, because of the different date of rabies virus inoculation.

Two dogs were treated with canine Ig without VNA. Dog 4 was found quiet and dull with loss of appetite on 26th day post infection and was treated immediately on 27th day post infection with canine Ig without VNA. Dog 4 soon developed severe rabies-related symptoms such as right hind limb weakness, lethargy, salivation and ataxia. Dog 4 was euthanized on 29th day post infection. Dog 5 was found with rapid shallow breathing, quiet and decreased food intake on 38th day post infection so was treated immediately on 39th day post infection with canine Ig without VNA. Dog 5 soon developed severe rabies-related symptoms such as salivation, unwillingness to stand nor walk, and head shaking. Dog 5 was euthanized when it barely supported its self with its limbs and could not stand with its both hind limbs on 39th day post infection.

Rabies virus N gene detection by RT-PCR

Frozen brainstem samples, collected at end-point, were analyzed for the presence of rabies viral genome (N-gene) by RT-PCR. In figure 1, all the dogs that died from rabies showed positive results for the presence rabies N gene (1.5 kbp) in the respective brain samples while the survived dogs with VNA treatment showed negative results. This indicates the clearance of rabies virus from the central nervous system in survival dogs by VNAs. The positive control was the one dog died of rabies (dog no. 6) in the cage without any treatment (Fig.2).

Rabies virus N antigen detection by immunohistochemistry

Fixed brain samples, collected when the dogs were euthanized, were trimmed and submitted for histologic slide preparation. Immunochemistry was performed by using monoclonal anti-N antibody to demonstrate the presence of the virus in brain. The positive results appeared as brown signal in the cytoplasm of neural cells. All the dog which died in this study, including Dogs 3, 4 and 5, had positive immunohistochemistry signals; however, the surviving dogs including Dogs 1 and 2, were devoid of positive immunohistochemically signal in the brain sections (Figure 5). This finding further indicates viral clearance from the CNS. Dog 6 was used for positive rabies virus infection control.

Histopathological evaluation

Histopathological evaluation was performed on the different part of brain from both groups of dogs including the groups of canine immunoglobulin treatment with VNA and without VNA. The microscopic findings including presence and average severity score of each animal are summarized in Table 2. Histopathological finding of different sections of brain from the group of canine immunoglobulin treatment with VNA showed multifocal, normal (0) to slightly (2) multifocal neuron degeneration with hypereosinophilic cytoplasm and condensed chromosome, and normal (0) to moderate (3) multifocal perivascular cuffing with mononuclear cell infiltration. The histopathological finding of different sections of brain from the group of canine immunoglobulin treatment without VNA showed multifocal, normal (0) to moderate (3) neuron degeneration, and minimal (1) to

moderate severe (4) perivascular cuffing with mononuclear cell infiltration (Table 2, Figure 4). The presence of neuron degeneration in the treatment with VNA and without VNA groups were 3/3 and 2/2. The presence of perivascular cuffing in the treatment with VNA and without VNA groups were 1/3 and 2/2. The average severity score for each dog of neuron degeneration of brain in the treatment with VNA groups was 0.33 ±0.47, 0.17 ± 0.37 and 0.33 ± 0.47, respectively; The average severity score of each dog of perivascular cuffing of brain in the treatment with VNA groups was 0.00 ± 0.00, 0.00 ± 0.00 and 1.17 ± 1.07, respectively. The average severity score for each dog of neuronal degeneration of brain in the treatment without VNA groups was 1.33 ± 0.94 and 1.83 ± 1.21, respectively; The average severity score for each dog of perivascular cuffing of brain in the treatment without VNA groups was 2.67 ± 1.11 and 2.50 ± 0.96, respectively. When compared to the treatment without VNA group, there was a significant difference of decreased average severity score of both neuronal degeneration and perivascular cuffing was found in dogs treated with VNA ($p<0.001$, Figure 3). In Dog 3 there was moderate severe diffuse neutrophilic meningitis in all sections of brain which is non-rabies related so the meningitis was excluded from the statistical analysis with others. Eosinophilic intracytoplasmic viral inclusion bodies, also known as Negri body, were found in the hippocampus in Dog 4 which is a characteristic of rabies virus infection.

CHAPTER 4

DISCUSSION

Rabies is an old, fatal neurotropic zoonotic disease. Rabies is preventable if the patient received the PEP timely and promptly (Organization 2013). However, once the rabies invade to the nervous system, there is no proven therapy to treat the patients. To date, none of the therapies that have been implemented showed consistently the therapeutic efficacy (Jackson 2011, 2013; Zeiler and Jackson 2016). In 2004, a 15-year-old girl survived from rabies by therapeutic coma and the use of N-methyl D-aspartate receptor antagonist therapy which was named "Milwaukee protocol" (Willoughby Jr et al. 2005). The clinical trial of Milwaukee protocol was implemented worldwide from 2005 to 2014; however, at least 31 documented failures reported in the literature to date (Zeiler and Jackson 2016). Therefore, the effectiveness of Milwaukee protocol is questionable. Since there is no effective treatment for rabies once the clinical sign appear, there is an urgent need to develop therapeutics for clinical rabies.

The significance of VNA in the CSF has been emphasized for recovery from rabies and one of the important observations from recovered animals and humans indicates that the presence of VNA play a crucial role for recovery from rabies (Kesdangsakonwut et al. 2014; Miller et al. 1978; Hamir, Niezgoda, and Rupprecht 2011; Willoughby Jr et al. 2005). However, it is very difficult to allow VNA entering the CNS because of the BBB (Neuwelt 2004). Thus, in this study, we attempted to directly deliver rabies VNA into the CNS by intrathecal injection in dogs after appearance of clinical signs.

We have directly administered the purified canine Ig containing rabies VNAs into rabies-infected dogs' spinal canal after onset of clinical symptoms. The intrathecal injection of VNA saved 2 out of the 3 dogs whereas 2 dogs that treated with canine Ig without VNA succumbed to rabies. A similar study has been done in rabbit which intrathecal immunization led to recovery of rabid rabbit (Kesdangsakonwut et al. 2014). In our dog treatment model, we observed the classical rabies symptoms in the dogs that succumbed to rabies including depression or restlessness, profuse salivation and/or frothing at the mouth, difficulty in swallowing and drinking, muscular incoordination or ataxia, trembling, tremors and retching. Also, the early clinical signs in prodromal phase were observed including quietness, drowsy eye contact, loss of appetite and behavior change (such as hyperactivity, licking or jumping). The incubation period varied from 6 to 42 days and the illness period was 1 to 4 days which is similar to humans (Organization 2013). Thus, we have established a canine model for rabies therapy development.

In order to confirm the clearance of rabies virus from the CNS, we performed RT-PCR and IHC in the dogs that recovered from rabies. High levels of rabies N gene and antigen were detected in the dogs that succumbed to rabies but neither rabies N gene nor antigen detected in the survived dogs. These results suggested that intrathecal injection of VNAs can clear rabies virus from CNS. For human cases recovering from rabies, all patients suffered from mild to severe neurological complication (Monroe et al. 2016; Willoughby Jr et al. 2005; Organization 2013). However, in the study, the survived dogs did not show any neurological sequelae.

Although this study clearly demonstrated the intrathecal treatment of purified canine Ig with VNA can clear RABV from the CNS and reverse the clinical course, the

number of animals used was limited. Thus larger group of dogs would be needed to confirm the effectiveness of the treatment protocol.

In summary, we have demonstrated in this study that intrathecal injection of rabies VNA can help clear rabies virus from the central nervous system and save the dogs after showing clinical signs. These studies will provide a solid foundation for developing therapeutics for clinical rabies in humans, otherwise, a lethal disease.

Tables

Table 1. Summary of observation period of different group

Group	ID	Early sign initiation[1] (dpi*)	Treatment (dpi)	Severe symptom initiation[2] (dpi)	End-point (dpi)	Illness period (days)
canine Ig treatment with VNA	Dog 1 (survived)	6	7	-	60	2
	Dog 2 (survived)	42	44	-	63	3
	Dog 3 (died)	26	27	27	28	3
canine Ig treatment w/o VNA	Dog 4 (died)	38	38	39	39	2
	Dog 5 (died)	26	27	27	29	4
w/o treatment	Dog 6 (died)	39	-	-	39	1

*dpi, day-post-infection

[1], early sign including loss of appetite, behavior change (such as hyperactivity, licking).

[2], severe symptom including depression, profuse salivation and/or frothing at the mouth, difficulty in swallowing and drinking, muscular incoordination.

Table 2. The severity score of inflammation and neuron damage in brain.

Group			*Cerebrum*	*Cerebellum*	*Brainstem*	*Thalamus*	*Hypothalamus*	*Mean*
canine Ig treatment w/ VNA	Dog 1 (survived)	Neuron Degeneration	0	1	1	0	0	0.33 ±0.47
		Perivascular cuffing	0	0	0	0	0	0.00 ± 0.00
	Dog 2 (survived)	Neuron Degeneration	0	1	0	0	0	0.17 ± 0.37
		Perivascular cuffing	0	0	0	0	0	0.00 ± 0.00
	Dog 3 (died)	Neuron Degeneration	0	1	1	0	0	0.33 ± 0.47
		Perivascular cuffing	0	0	3	2	1	1.17 ± 1.07
		Meningitis	4	4	4	4	4	4.00 ± 0.00
canine Ig treatment w/o VNA	Dog 4 (died)	Neuron Degeneration	0	3	1	2	1	1.33 ± 0.94
		Perivascular cuffing	4	1	4	3	2	2.67 ± 1.11
	Dog 5 (died)	Neuron Degeneration	0	3	3	3	1	1.83 ± 1.21
		Perivascular cuffing	2	1	4	3	3	2.50 ± 0.96

Severity of lesions was graded by the methods described by Shackelford *et al*., 2012

Figures

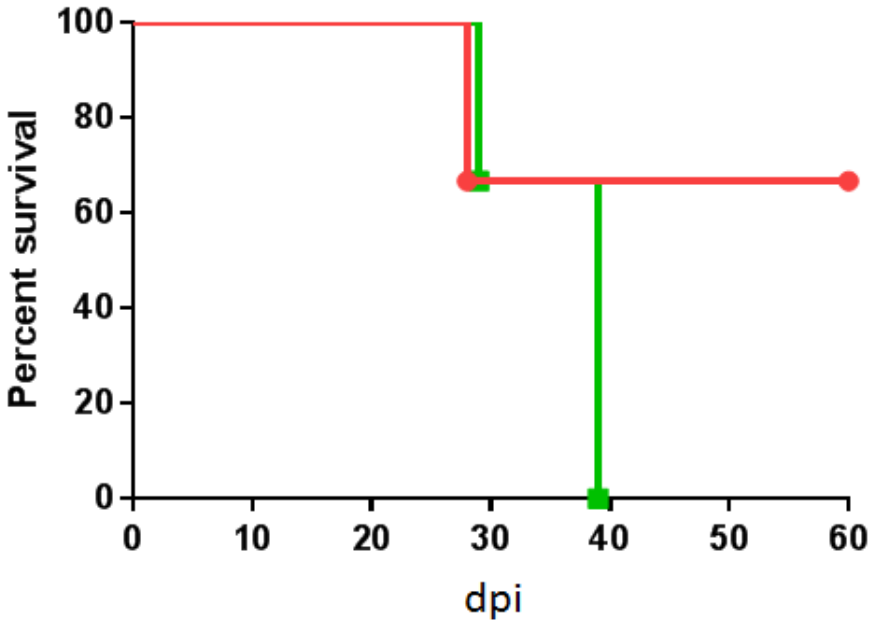

Figure 1. Survival rate of dogs treated with canine Ig w/ and w/o VNA.

The group of Ig treatment w/ VNA (red) had 66% survival rate while the group of Ig treatment w/o VNA (green) had 0% survival rate.

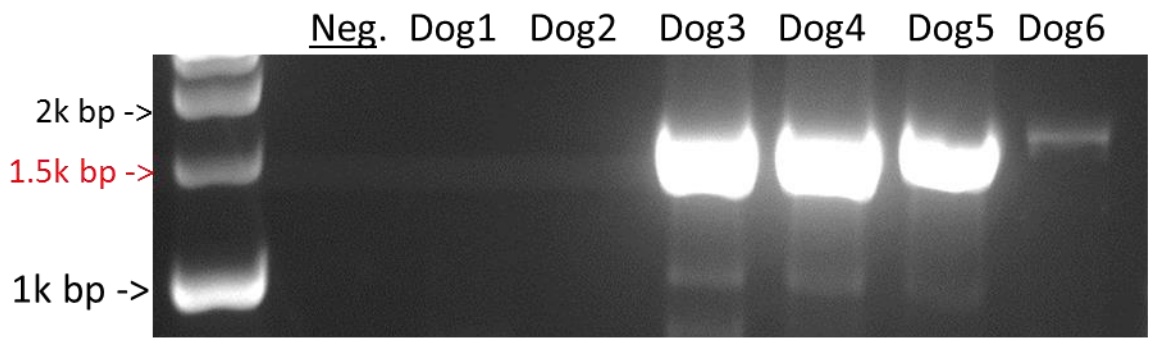

Figure 2. Rabies virus N gene detection by RT-PCR

This is the result of RT-PCR by agarose gel electrophoresis. It is showed that the survived dogs were tested negative with rabies N gene. All the dogs died of rabies were found rabies N gene in the brain tissue. Neg: negative control of PCR; Dog 1 – 3: group of canine Ig treatment with VNA; Dog 4 – 5: group of canine Ig treatment w/o VNA; Dog 6: group of no treatment (positive control of rabies NYC strain virus).

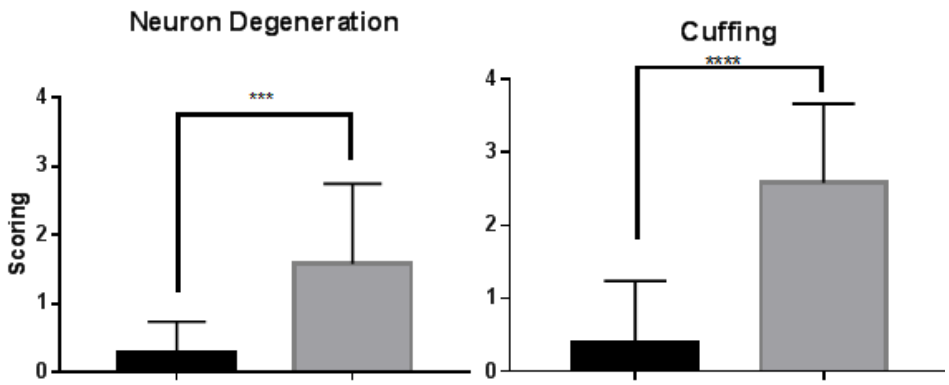

Figure 3. The average score of the histopathological changes.

The level of inflammation and neuronal damage was significantly different in group of dog treated with canine Ig treatment containing VNA (black) and no VNA (grey). The grade is calculated by a scoring system suggested by Shackelford *et al*., 2012.

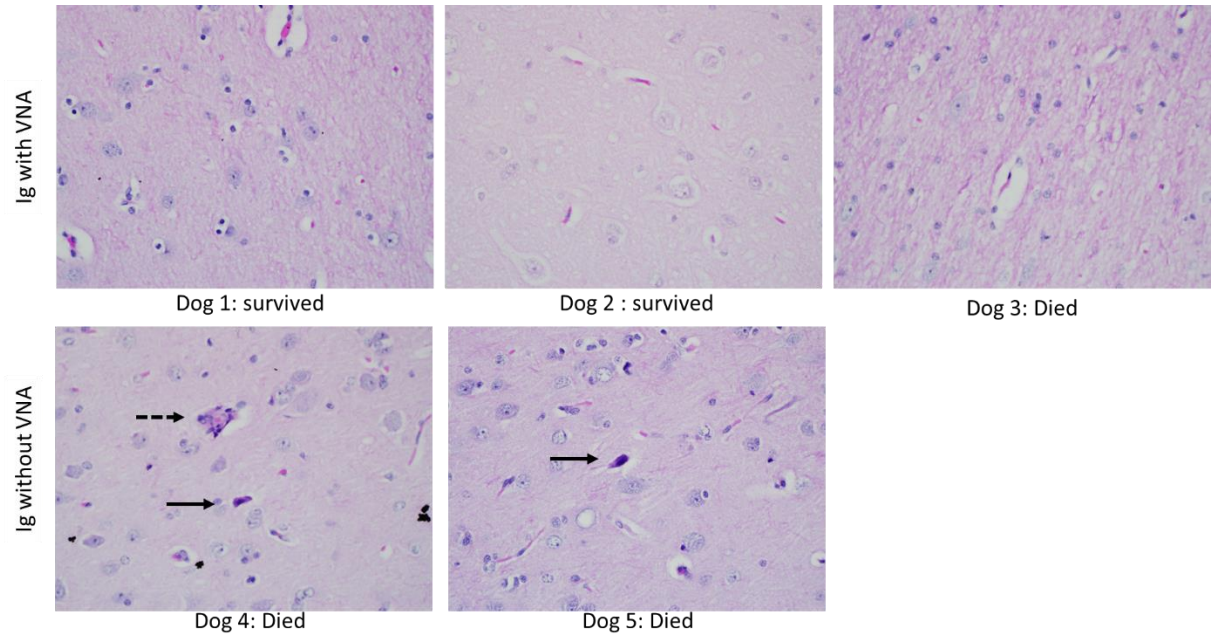

Fig 4.1. Histopathological change of cerebrum, H&E, 400×.

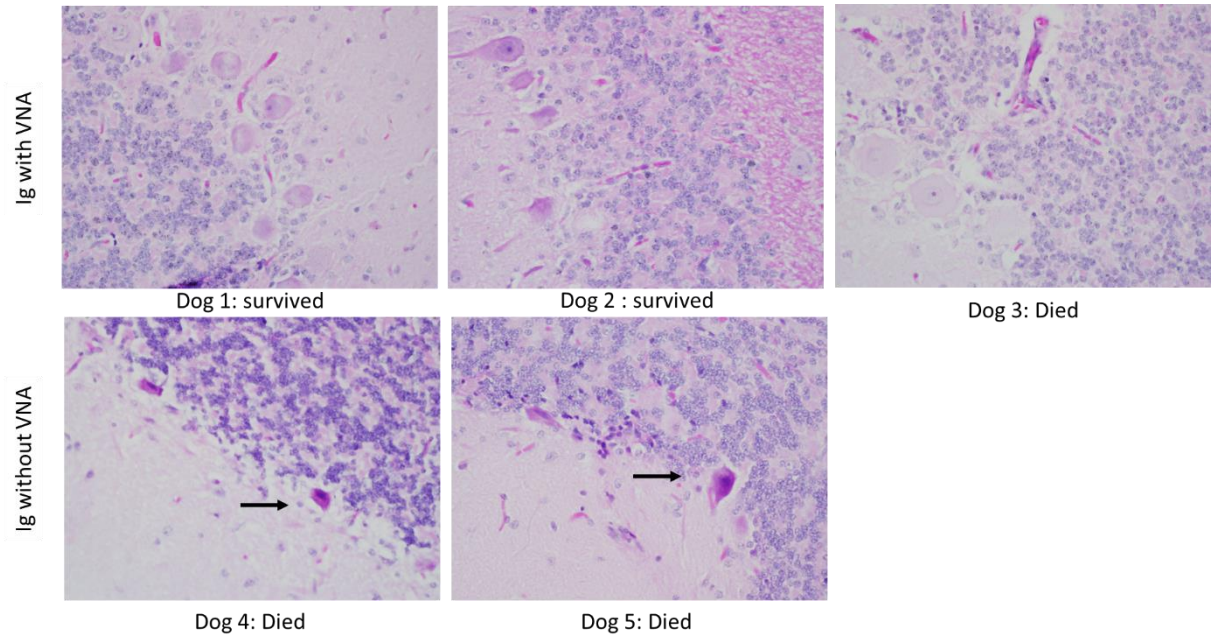

Fig 4.2. Histopathological changes of cerebellum, H&E stain, 400×.

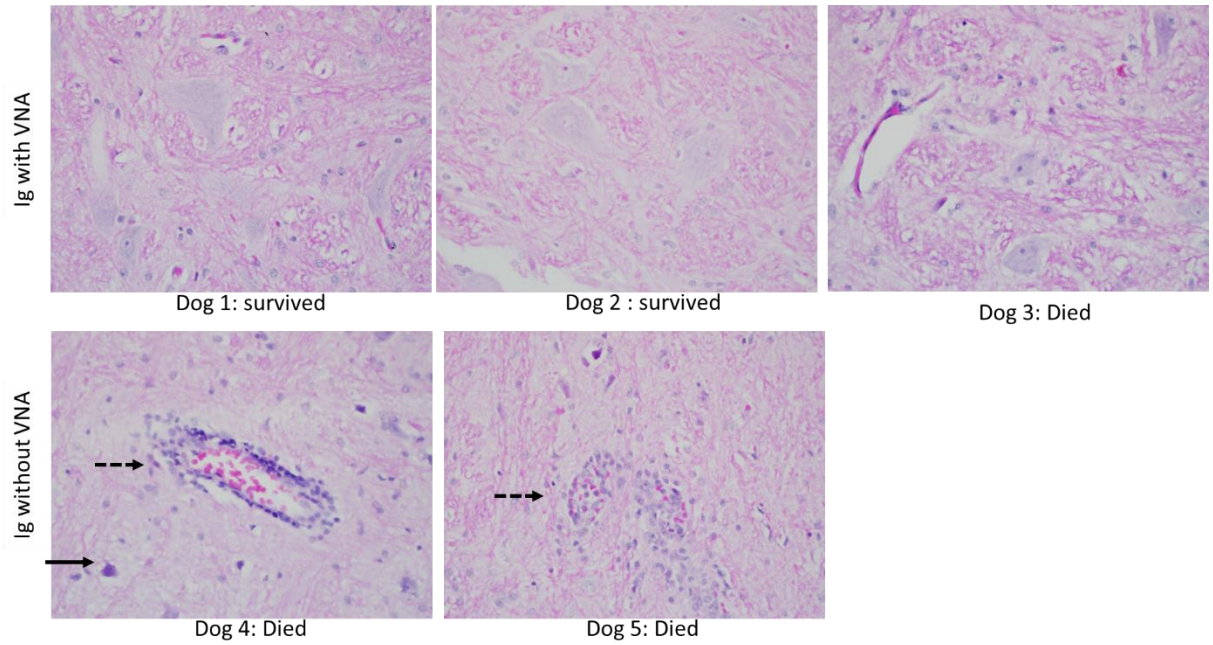

Fig 4.3. Histopathological changes of brainstem, H&E stain, 400×.

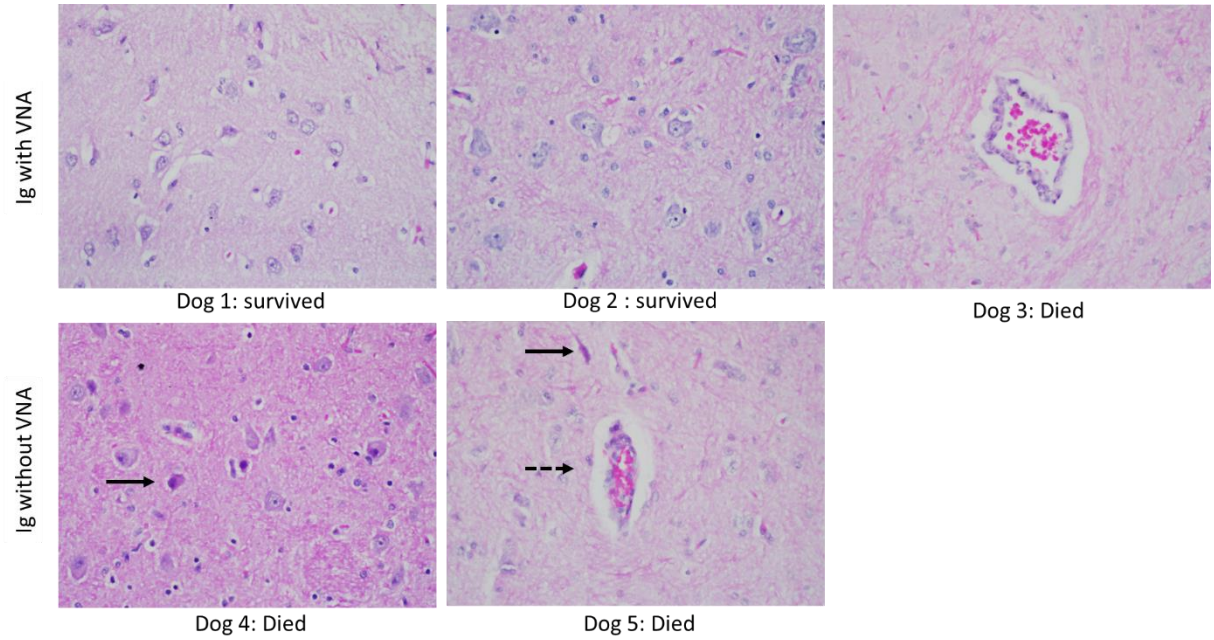

Fig 4.4. Histopathological changes of thalamus, H&E stain, 400×.

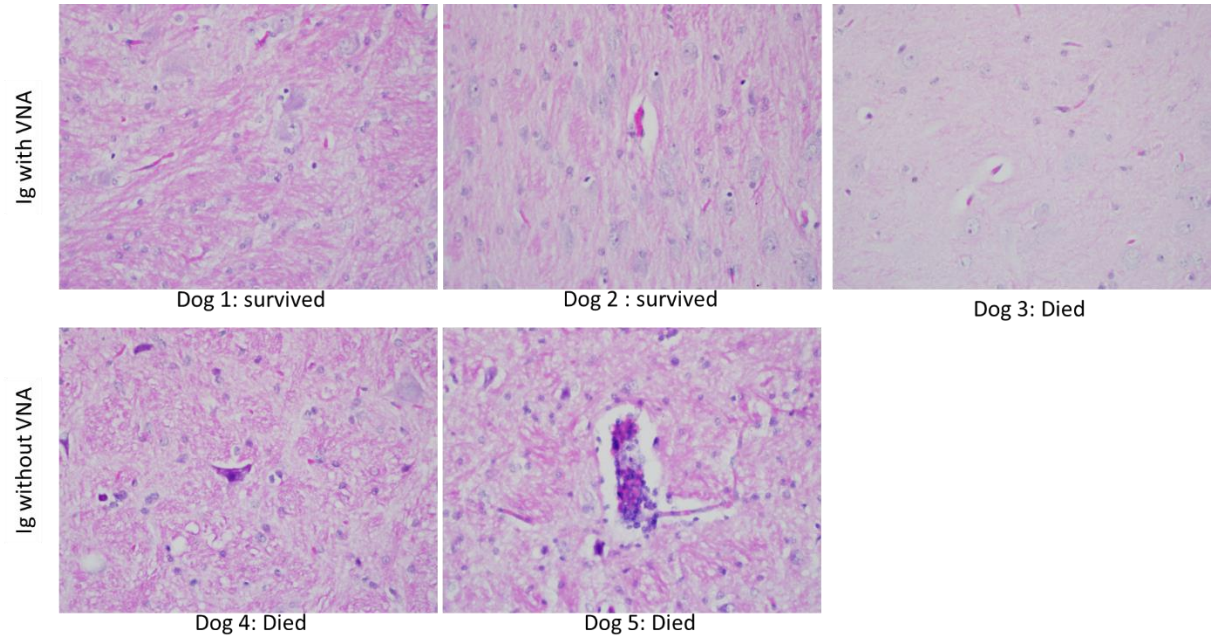

Fig 4.5. Histopathological changes of hypothalamus, H&E stain, 400×.

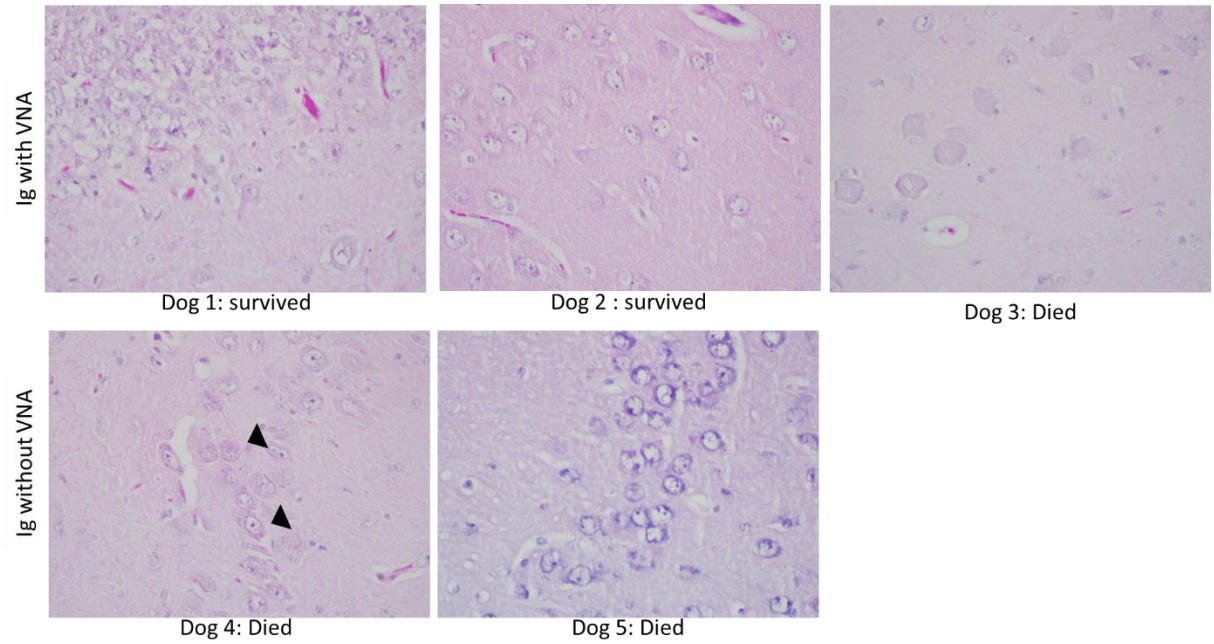

Fig 4.6. Histopathological changes of hippocampus, H&E stain, 400×.

Figure 4. Histopathological changes at the end-point.

These are histopathological evaluation in different parts of brain including cerebrum (figure 4.1), cerebellum (figure 4.2), brainstem (figure 4.3), thalamus (figure 4.4), hypothalamus (figure 4.5) and hippocampus (figure 4.6). The survived dogs showed minimal inflammation and tissue damage comparing to the died dogs (line arrow: neuron degeneration; dashed arrow: perivascular cuffing; arrow head: rabies viral inclusion body (Negri body)).

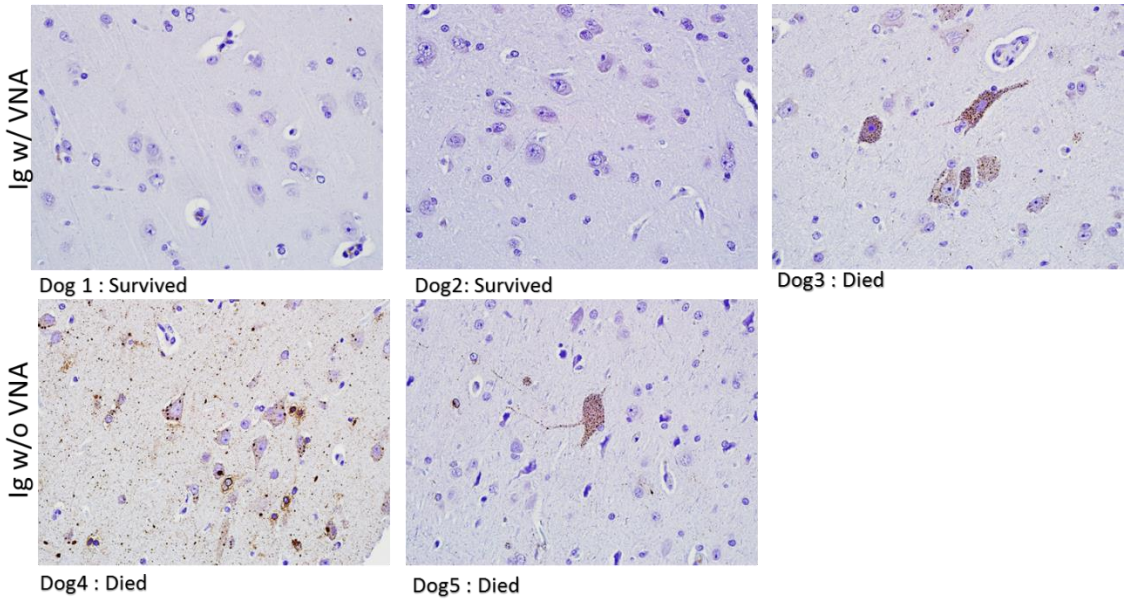

Fig. 5.1. IHC of cerebrum

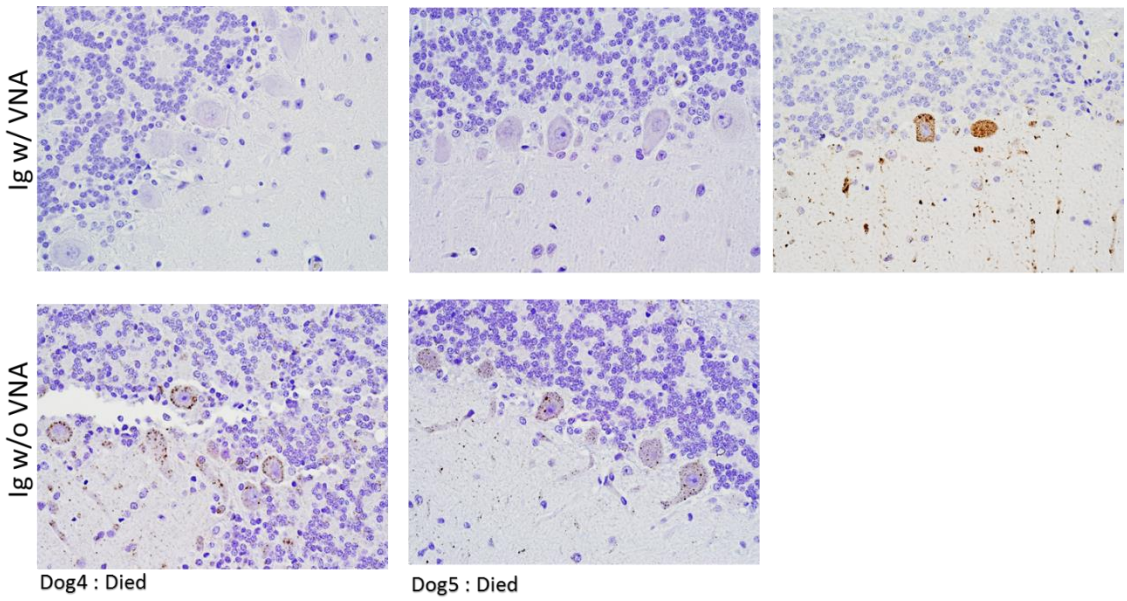

Fig.5.2. IHC of cerebellum.

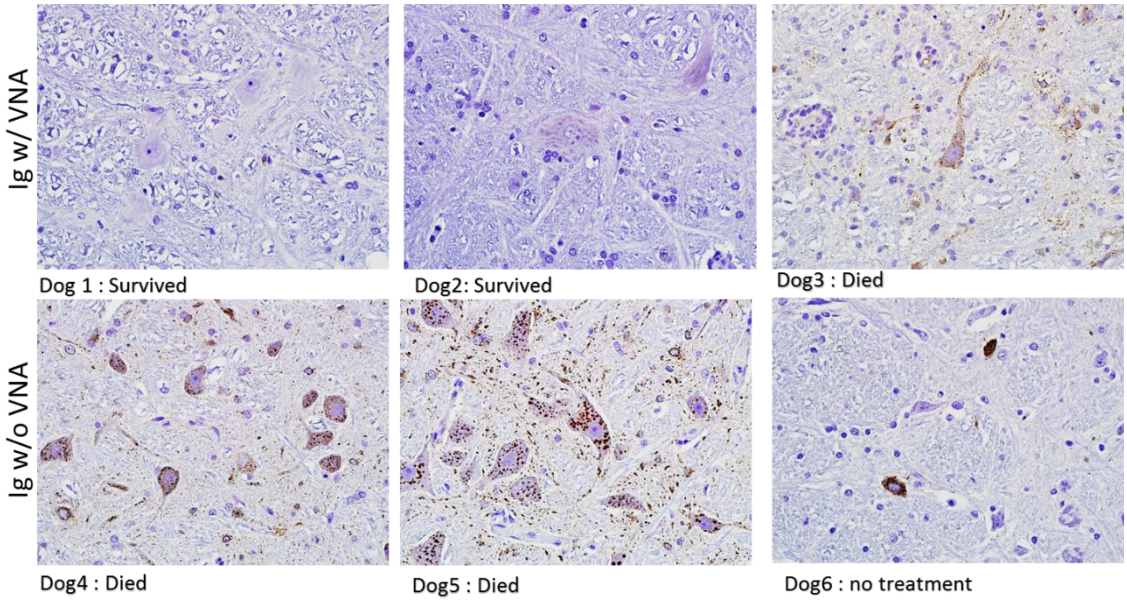

Figure 5.3. IHC of brainstem

Figure 5. Immunohistochemical detection of rabies N antigens.

The slides were stained with DAB and counterstain with hematoxylin. The intracytoplasmic brownish particle of neuron indicated the positive results of rabies N antigens. The survived dogs were tested negative in all cerebrum, cerebellum and brainstem while died dogs were found rabies N antigen positive.

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