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The Potency of Virulent Newcastle Disease Virus Tabanan-1/ARP/2017 as Virotherapy Agents in Rat Fibrosarcoma Models

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ABSTRACT

Newcastle Disease Virus (NDV) has oncolytic activity and has been promoting as a virotherapy agent. The objective of this study was to evaluate the potency of NDV Tabanan-1/ARP/2017 as a virotherapy agent. This study was used the white rat *Rattus norvegicus* as an animal fibrosarcoma model. Benzo(a)pyrene solution at 0.3% (w/v) was used to induce fibrosarcoma. After fibrosarcoma appeared, rats were treated as follows: Rats in group P0 were injected with 0.5 mL phosphate buffer saline (PBS), while those in group P1 were injected with the NDV, intratumorally at a dose of 0.5 mL of 2^9 HA titer of NDV Tabanan-1/ARP/2017. The dynamic of tumor growth was evaluated. Upon the starting point of the treatment, the mean volume of rat tumors mass of P0 and P1 were 1,769.83±1,103.58 mm^3 and 1,194.29±592.82 mm^3, respectively. At the end of the treatment, the mean tumor volume of P0 was 8,549.38±5,347.51 mm^3, while at P1 was 3,848.25 ±3,539,189 mm^3. From the observation of microscopic images, it was found that the number of blood vessels at P1 was 44.67±19.348, significantly lower ($p<0.05$) than that of the P0 (121.33±34.530). From this study, it can be concluded that virotherapy with NDV Tabanan-1/ARP/2017, can inhibit fibrosarcoma growth and reduce the number of blood vessel in the tumor. The effectiveness of Tabanan-1/ARP/2017 as a virotherapy agent is discussed.

Introduction

Virotherapy is the use of viruses for cancer therapy. Viruses used for virotherapy are viruses that can infect and damage cancer cells. The virus is able to multiply in cancer cells, as well as kill them without damaging normal cells around them. Alice Moore is the first scientist to try virotherapy on experimental animals (Patil *et al*., 2012). Many reports and studies have shown that several types of viruses show oncolytic activity, one of those is the Newcastle disease virus (Motalleb, 2013., Fountzilas *et al*., 2017., Schwaiger *et al*., 2017; Yurchenko *et al*., 2018).

Newcastle disease virus (NDV) or Avian orthoavulavirus 1 (AOAV-1) is belong to the genus orthoavulavirus family Paramyxoviridae (Amarasinghe *et al*., 2019). Based on the results of the mean death time in chicken eggs, NDV can be classified as a highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic) (Beard & Hanson, 1984). Virulent NDV is the causative agent of Newcastle Disease (ND) in poultry. Currently, ND is still endemic in several regions in the world including Bali-Indonesia (Adi *et al*., 2010; Adi *et al*., 2019a). Although virulent NDV is highly pathogenic in poultry, the virus is non-pathogenic in mammals so it can be promoted as a cancer therapy agent (Schwaiger *et al*., 2017). NDV grows well on mammalian cancer cells selectively and induces oncolysis (Schwaiger *et al*., 2017; Yurchenko *et al*., 2018). Compared to other oncolytic virus members, NDV can lyse various types of cancer (diverse oncolytic) without causing sequelae (Motalleb, 2013).

According to the authors knowledge, a study to determine the virotherapy potency of NDV isolates from Indonesia has never been reported. One of the NDV field isolates originating from Indonesia is Tabanan-1/ARP/2017. The virus is genotype VII based on the phylogenetic analysis of the Fusion (F) gene (Adi *et al*., 2019b). Based on that, this study was carried out to evaluate the potency of NDV Tabanan-1/ARP/2017 as a virotherapy agent by evaluating the effectiveness of the virus in suppressing the growth of fibrosarcoma and blood vessels proliferation.

Materials and methods

Ethical Approval

This study has been officially approved by the Ethical Commission for the Use of Experimental Animals, the Faculty

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of Veterinary Medicine, Udayana University. With the approval number 57/UN.14.29/PT.01.04/2020. The procedures carried out in this study are under the provisions and requirements of the ethics committee.

Experimental Animal

The study used male white rats (*Rattus norvegicus)* Sprague Dawley strain aged 3 months with body weight 115-130 gram. The rats were obtained from the Laboratory of Pharmacology, Faculty of Medicine and Health Sciences, Udayana University. These rats were then kept in the Laboratory of Veterinary Pathology, Faculty of Veterinary Medicine, Udayana University. The maintenance was carried out in a humane and ethical manner. Clean tap water and standard pellet diet were provided throughout the study period.

Benzo(a)pyrene-induced fibrosarcoma in rat

Benzo(a)pyrene (Sigma-Aldrich, St. Louis, MO, USA) can induce local malignant tumors when administered mixed with *oleum olivarum* (Kallistratos & Fasske, 1980; Vegad, 2008). Before being used it was dissolved in *oleum olivarum* (0.3% w/v), then stirred until it became homogeneous. Once homogeneous, this solution was sterilized by heating in an autoclave at a temperature of 115°C for 15 minutes. To induce fibrosarcoma, rats were injected with 0.1 mL benzo(a)pyrene solution 0.3% (w/v), ten times every two days via the subcutaneous route in the interscapular area (Sukardiman *et al*., 2015).

Virus and viral propagation

The NDV Tabanan-1/ARP/2017 (Fusion gene accession no MH215997) is an Indonesian field isolate with the motif of the Fusion protein cleavage site -^{112}RRQKRF^{117}- typically for virulent NDV (Adi *et al*., 2019b). The virus was propagated in 9- to 11-day old embryonated chicken eggs (ECEs) via the chorioallantoic membrane and was incubated at 37 °C for 48–72 h. The infective allantoic fluid was then collected and the titer of the virus was determined by the hemagglutinin assay (HA) as described by OIE (2008).

Treatment group

All rat with benzo(a)pyrene-induced fibrosarcoma tumors were then divided into two treatment groups P0 and P1 and treated as follows: Rats in P0 groups was injected with 0.5 mL phosphate buffer saline (PBS) while those in P1 group were injected with the NDV, intratumorally into the solid node at a dose of 0.5 mL of 2^9 HA titer of NDV Tabanan-1/ ARP/2017. Both Group P0 and P1 received four doses of injection for four consecutive days as previously performed by Yurchenko *et al*. (2018). The injection is carried out from various sides of the tumor so that the material injected can spread across the tumor. Each treatment used three rats as replications. The administration of tumor treatment was carried out on the 93rd day after injection of benzo(a)pyrene solution, considering that macroscopically the fibrosarcoma mass could be observed (Figs. 1a-c).

Measurement of tumor growth

Tumor growth was monitored and plotted on days 0, 5, 7, 9, 11, 13, and 15 post-injection (p.i). Tumor length and width were measured using calipers, and the tumor volume was calculated using a standard formula: Tumor volume (mm^3) = 0.5 × length × $(width)^2$ (Kersemans *et al*. 2012; Yurchenko *et al*. 2018). Note that the largest longitudinal diameter is the length, and the largest transverse diameter is the width.

Tissue collection and Histopathological assessment

Upon reaching the time of 15 days p.i., the rat was then euthanized, follow the procedures recommended by the American Veterinary Medical Association (AVMA) as described in Leary *et al*. (2020). The rats were injected with the anesthetic ketamine (Agrovet Market SA, Lima, Peru) and xylazine (Interchemie, Metalweeg, Netherlands) five times the normal dose (normal dose for rats 50 mg/kg and 5 mg/kg BW, respectively) via the intraperitoneal route. The whole fibrosarcoma tissue was excised and weighed, and the tumor sample was then put into 10% neutral buffered formalin for fixation before proceeding to be a paraffin-embedded tissue block, sectioned 4 µm thickness were stained with Hematoxylin and eosin (HE). The slides were then examined for the presence of histopathological changes. The blood vessel counts were performed in five independent microscopic fields per tissue section for each rat. All histopathological slides were examined under a bright-field microscope (Nikon, Tokyo, Japan).

Statistical Analysis

The data of means tumor volume and the number of blood vessels were analyzed by analysis of variance values of *$p<0.05$, **$p<0.01$, and ***$p<0.001$ were considered significant. Analysis of the dynamic tumor growth was performed using correlation and regression analysis and presented in the graph. Additional pathological lesions were examined grossly and microscopically, and then were compared descriptively. The statistical analysis software used is SPSS for Windows version 25.

Results

Several NDV isolates have been extensively studied regarding its oncolytic ability in tumor cell lines. In this study, field isolates NDV from Tabanan Bali, Indonesia was evaluated for their effectiveness as a virotherapy agent in an experimental animal (Fig. 1).

At the starting point of the treatment, the mean tumor volume of rats at P0 and P1 were 1,769.83±1,103.58 mm^3 and 1,194.29±592.82 mm^3, respectively. Tumor volume and the position in the two groups upon the starting treatment not uniform but statistically did not show a significant difference ($p>0.05$).

There was a significant difference ($p<0.05$) between the mean tumor volume in the rat in the P0 and P1 groups through 15 days post-injection. In the P0 treatment group the tumor volume increased until the end of the observation however in the P1 treatment group there was a tendency for tumor volume to decrease after the virotherapy. At the end of the treatment, the mean tumor volume in groups P0 and P1 was 8,549.38±5,347.51 mm^3 and 3,848.25±3,539.189 mm^3 respectively. The mean volume number based on the estimated interval of the regression line was significantly ($p<0.05$) lower on day 11th to day 15th post-injection (Fig. 2b).

In the gross pathology of tumor, redness was predominant in the P0 group, which 2 out of 3 exhibited redness (Table 1). Paleness appearance indicated that the cells undergoing necrotic, which was more dominant in the P1 tumor mass.

When palpated, tumors from both groups had a firm consistency, which implied the presence of connective tissue, collagen, and fibroblasts. The tumor weights of P0 and P1 were 6.3±5.1 g and 4.3±3.6 g, respectively. The mean tumor weight of group P1 was lower than that of P0, however it was not significantly lower statistically ($p>0.05$).

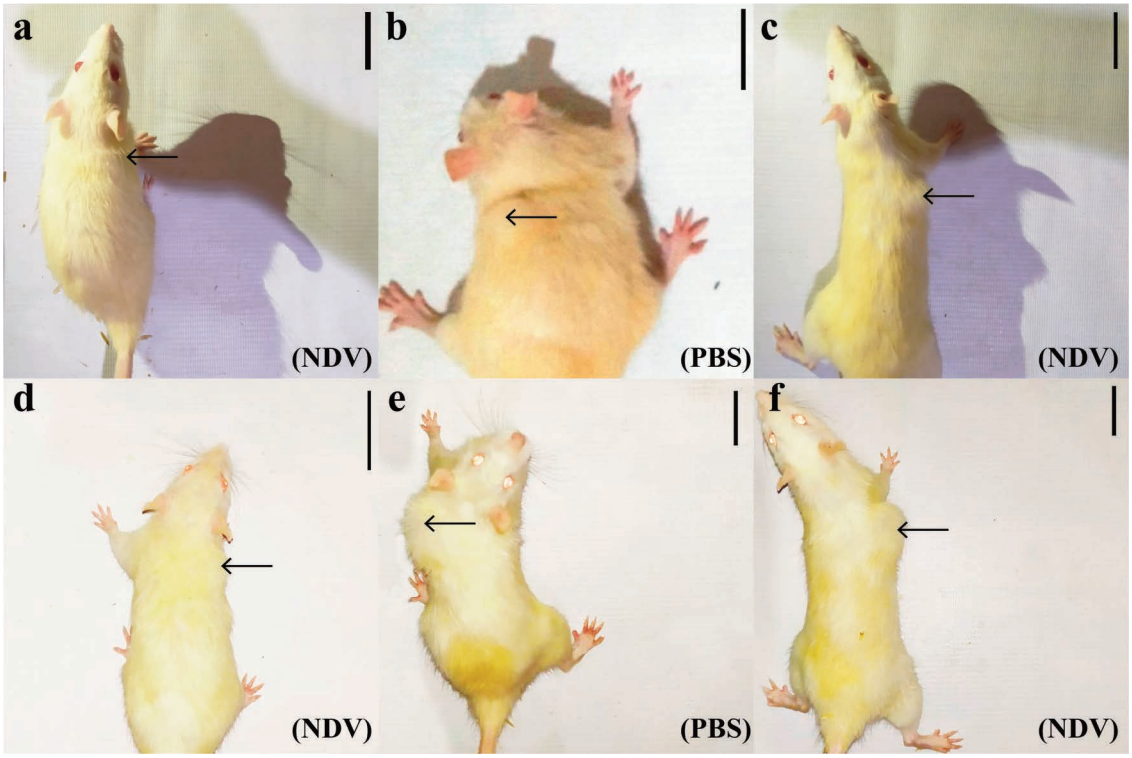

Fig. 1. The tumor mass was visible on day 93 after induction of 0.3% (w/v) benzo(a)pyrene, 3 out of 6 rats with fibrosarcoma at the first injection with NDV (a and c) and with PBS (b). d,e,f Tumor mass features on day 9 post-injection. Type of the treatment indicated at the figure. Scale bar 3 cm.

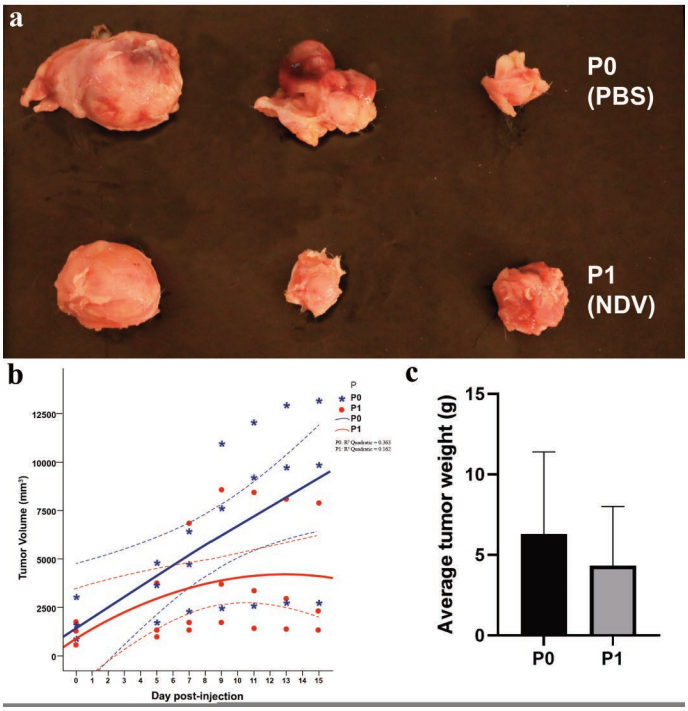

Fig. 2. a). Gross appearance of the tumor mass on the day 15 post-injection PBS (P0) and NDV-Tabanan 1/ARP/2017 (P1) noted: Tumor's size is not uniform from the starting point of treatment. b). Dynamic of tumor growth; the mean volume tumor based on the estimated interval of the regression line was significantly ($p<0.05$) lower on day 11th to day 15th post-injection c). Comparison of mean tumor weight between the two treatments at the end of the observation.

Histologically the tumor mass has been confirmed the presence of fibrosarcoma. Based on the micoscopic examination, the type of tumor was in line with expectations. A typical fibrosarcoma picture such as the massive proliferation of fibroblasts with a high level of cell density, with spindle-shaped cells, were found.

The signs of inflammation like edematous and redness of tumor mass was more prominent in P0 rather than in P1 (Table 1). Microscopically, it was found so many blood vessels engorge and filled with red blood cells in the P0 treatment group (Fig. 3a). No hemorrhage was found in either the P0 or P1 groups (Figs. 3a-b). The feature of cells undergoing mitotic was found both in the P0 and P1 group, the intensity more prominent in the P0 group. The inflammatory cells also found in the two groups.

The intratumoral vessel number for each region is hetero-

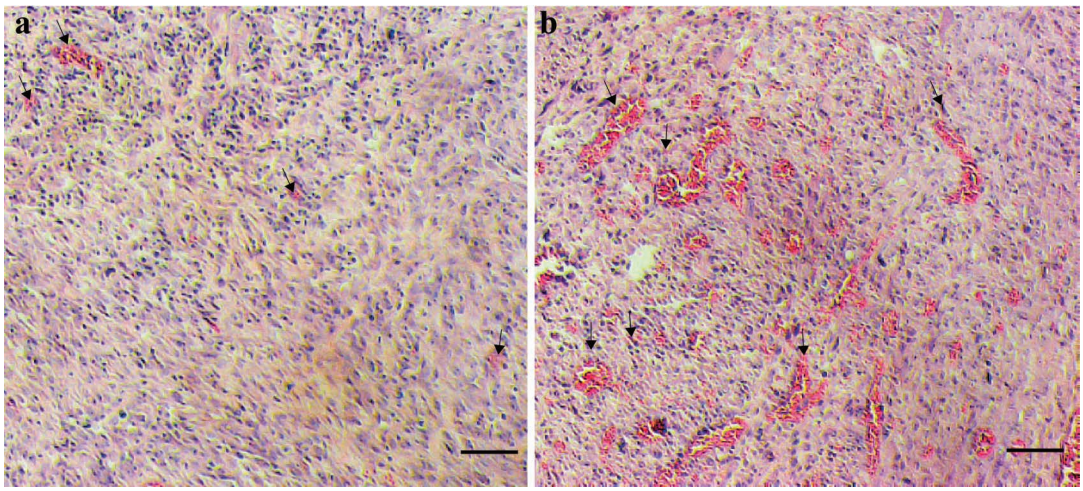

Fig. 3. Numerous blood vessel with the red blood cells in the lumen was found in the P0 treatment (b) only a few was found in the P1 treatment (a). HE Stain, bar 100 Mm.

Table 1. Macroscopic and microscopic lesion examined in the tumor tissue on the 15th days post-injection.

Lesion	Group	
Macroscopic	P0	P1
Redness/congestion	2 out of 3	1 out of 3
Edematous	2 out of 3	1 out of 3
Multifocal necrotic area	2 out of 3	3 out of 3
Microscopic		
Blood vessels with engorged lumen full of RBCs	3 out of 3	0 out of 3
Cell undergoing mitosis	3 out of 3	3 out of 3
Intensity of mitotic cells	Frequent	Rare
Infiltration of inflammatory cells on the necrotic area	Frequent	Frequent

geneous. In this study, the number of blood vessels were counted, in five independent microscopic fields under 200 x magnification. The average number of blood vessels in the P0 group was 121.33±34.53 and that in the P1 group was 44.67±19.35. It was found that the number of blood vessels at P1 was significantly lower ($p<0.05$) than the P0.

Discussion

When palpated, tumors from both groups had a firm and dense consistency, which implied the presence of connective tissue, collagen, and fibroblasts. Macroscopically redness and oedema were dominant in the P0 group. Red color possibility indicated hyperemic or hemorrhage, however, under microscope examination, no hemorrhage was found. Instead of that high number of blood vessels was found, their lumen was full of red blood cells. A high amount of blood vessels in the area of the tumor indicates angiogenesis has occurred (Folkman *et al.*, 1971). As mentioned previously tumor angiogenesis was required for tumor progression and rapidly growing tumors were heavily vascularized (Lugano *et al.*, 2020).

The volume of fibrosarcomas in the P0 group continued to increase throughout the time of observation. The dynamic of tumor growth in the P0 and P1 significantly differ ($p<0.05$). Based on the estimation interval of the regression line, tumor volume between P0 and P1 was significantly different starting from day 12 post-injection (Fig. 2b). Based on the obtained results, the time duration of virotherapy played an important role in suppressing tumor growth. In the present study, time of evaluation was only 15 days post treatment, in the future might be needed to evaluate a bit longer. Several limitations in this study were the difficulty to find rat that has tumor with a uniform volume. After the induction of benzo(a)pyrene the size of the tumor that appears was not uniform and the position varies (Fig. 1). As shown in Fig. 2a, the tumor size was not uniform because from the starting point of treatment was not uniform. However, the gross lesions were very clear, the inflammatory lesions were lighter in the P1 tumor mass. The limitation in the number of replication and the uniformity of the initial volume is likely to affect the results (Fig. 2a).

In this study, five different field under 200 x microscope magnification were assessed for counting microvessel number and the mean value was used for statistical analysis. It was clearly found that the number of blood vessels in P0 group higher than that of P1 ($p<0.05$).

The lower number of blood vessels in P1 in comparing to those in P0 can be sign of a good result, because tumor growth depends solely on the number of blood vessels. Less number of blood vessels in the tumor the better the prognosis. During tumor formation, oxygen and nutrients are required for growth, the reduction in the number of blood vessels will slow down tumor growth. As previously mentioned, the formation of new blood vessels is required in the tumor to support the rapid and progressive proliferation of cancer cells (Shrieve *et al.*, 1983., Nishida *et al.*, 2006., Lugano *et al.*, 2019)

In this study, there were many blood vessels in the tumor tissue in group P0 (Fig. 4a). Meanwhile, in the virotherapy group, administration of NDV was seen to suppress the formation of new blood vessels so that the number of blood vessels in each visual field was not as much as in P0 (Fig. 4b). The results of this study indicated that aplication of NDV isolate Tabanan-1/ARP/2017 as a virotherapy significantly inhibit the angiogenesis process and consequently suppress tumor growth. This research is in line with the study conducted by Al-Shammari *et al.* (2020), who reported that the NDV strain AMHA1 could inhibit angiogenesis in a mammary adenocar-

cinoma model.

The mechanism of how the virus can inhibit the angiogenesis process is likely closely related to the replication of NDV in the endothelial cell. NDV isolate Tabanan-1/ARP/2017 is a virulent virus and cause severe necrotic cells in several organs of chicken (Adi *et al*., 2019a). As previously reported, more virulent the virus, the stronger its oncolytic ability is (Buijs *et al*., 2014).

The virus with oncolytic activity is suitable use for a virotherapy agent. For further study, the ability of NDV Tabanan-1/ARP/2017 can inhibit the angiogenesis process needs to be studied more deeply with immunohistochemistry using monoclonal antibodies to endothelial cell antigens to visualize blood vessels more precisely.

From the results of this study, it was found that the virus is able to suppress the activity of forming new blood vessels so that the supply of oxygen and nutrients will decrease, and the tumor growth can be inhibited.

Conclusion

The results showed that NDV Tabanan-1/ARP/2017 can inhibit the tumor growth and has potency as a virotherapy agent.

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Conflict of interest

The author declares that no competing interests exist.

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