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In Vivo Oncolytic Potency of Newcastle Disease Virus Gianyar-1/AK/2014 Virulent Strain Against Mice Fibrosarcoma Models

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ABSTRACT

Several viruses have oncolytic properties that can infiltrate neoplastic cells and multiply themselves in them. Through viral multiplication, neoplastic cells will be damaged and lysis. Virotherapy or using viral for alternative cancer treatments has been widely studied, one of the viruses that have oncolytic properties is the Newcastle disease virus (NDV). In this study, NDV Gianyar-1/AK/2014 virulent strain was used for the virotherapy in mice fibrosarcoma models. The purpose of this study was to determine the effect of NDV on fibrosarcoma growth and its histopathological features. Mice-bearing fibrosarcomas were divided into two groups, i.e., the control group (K) was not given virotherapy and the treatment group (P) was injected NDV intratumorally at a dose of 0.5 mL/2^7 HA units, each group consisted of three mice. Fibrosarcoma diameters in both groups were measured at the beginning and the end of the study with vernier calliper. At the end of the study, three weeks post virotherapy, all of the mice were sacrificed, and tumour tissue samples were collected. The sample was fixed with 10% formalin neutral buffer, routinely processed for histopathological examination. From the diameter results, it was found that the mean diameter of tumours at the start of the study for the K group was higher than the end of the study ($p<0.05$) while the mean diameter of the P group did not show significant changes ($p>0.05$). Microscopic changes of fibrosarcoma in the K group were a high activity of angiogenesis, high amount of anaplastic fibroblast cells with pleomorphic nucleus, and frequent mitotic figures. While in the P group were found multifocal necrosis of tumour cells with the proliferation of macrophages, collagen tissue, several giant cells, low mitotic figures and fewer anaplastic fibroblast cell. It can be concluded that NDV Gianyar-1/AK/2014 virotherapy could inhibit fibrosarcoma growth in mice.

Keywords
fibrosarcoma, mice, Newcastle disease virus, virotherapy

Introduction

Malignant neoplasm or known as cancer is one of the top ten causes of death in the world due to its capability to damage other cells and spread into other parts of the body. Cancer cells are mutated cells that genetically have undergone change, make its keep growing, dividing, and ignoring the normal cell cycle signal (Kondo, 1993). The development of cancer occurs gradually and is called stepwise tumour development or also known as multistage carcinogenesis. Cancer develops as a result of several genetic and epigenetic changes that occur over a long period of time. The cumulative effect of these alterations will establish cancer (Newkirk *et al.*, 2017). Several types of therapy options for cancer therapy are currently available. Some of them have good effectiveness but also have risks or side effects.

Oncolytic viruses (OVs) are a new class of cancer therapy. In recent years, OVs have drawn attention as a novel therapy for various types of cancers around the world. OVs are viruses that are able to infiltrate and replicate into cancer cells. Through the replication of these viruses, cancer cells will be damaged and lysed (Nejad *et al.*, 2021). Several types of viruses have been reported to have oncolytic abilities against cancer cells, including reovirus,

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myxoma virus, parvovirus, human adenoviruses, vesicular stomatitis virus, bovine herpesvirus 4, coxsackievirus A21, and Newcastle disease virus (NDV) (Motalleb, 2013). Strategies for infecting and destroying cancer cells vary between OVs. The general mechanism of oncolysis is the low expression of interferon (IFN) in several cancer cells. IFN has a central role in clearing viral particles, so its low expression makes these cells susceptible to infection (Cook and Chauhan, 2020).

NDV is one of the most well-researched OVs because it is known to affect various type of cancers. Many researchers have been widely studies the utilization of NDV for virotherapy (Cuoco *et al.*, 2021). Compared to other oncolytic agents, NDV has several advantages, including it is only pathogenic in poultry and does not have the ability to adapt in mammalian hosts, has a strong immunostimulant ability, and the nature of NDV is able to lyse various types of cancer (Zamarin and Palese, 2012).

Although several kinds of research on the oncolytic activity of NDV have often been carried out, studies of isolates originated from Indonesia are still limited. This study was conducted to evaluate the ability of the NDV virulent strain Gianyar-1/AK/2014 (Adi *et al.*, 2019; Putra *et al.*, 2016) in inhibiting the growth of mice fibrosarcoma models and to describe the microscopic feature after virotherapy.

Material and Methods

Animal tumour models

This research used six mice-bearing fibrosarcoma models induced by 0.3% benzo(a)pyrene in olive oil. The fibrosarcoma induction procedure is carried out according to Sewoyo *et al.* (2021). Mice were injected using 0.3% benzo(a)pyrene solution subcutaneously in the interscapular area. The injection was carried out 10 times gradually at intervals of once every two days. All mice were kept in the Laboratory of Veterinary Pathology, Udayana University.

Virus and viral propagation

The NDV virulent strain used in this study was Gianyar-1/AK/2014. This isolate was isolated in 2014 and originated from village chicken in Bali (Adi *et al.*, 2019; Putra *et al.*, 2016). The virus was propagated in embryonated chicken eggs at the Laboratory of Virology Animal Disease Investigation Centre Denpasar Bali. The titre of the virus was determined by haemagglutinin assay (HA) as described by OIE (2008).

Treatment group

The mice were divided using random sampling into two groups, the control group (K) and the treatment group (P), each group consists of three mice. The K group was not given any treatment while the P group was injected with NDV intratumorally with a dose of 0.5 mL/2^7 HA unit. Virus injection was performed gradually, five times with once-daily intervals. Fibrosarcoma diameter was measured twice using a vernier calliper, i.e., before treatment and at the end of study at the third-week post injection (p.i.). Upon reaching the time of third-week p.i., all mice were sacrificed by cervical dislocation. The sacrifice procedures were following American Veterinary Medical Association (AVMA) guidelines (Leary *et al.*, 2020).

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Tissue sampling and processing

After all, mice were sacrificed, the tumour tissues were collected and sampled. The tissue was immediately fixed in 10% neutral buffered formalin, routinely processed, and paraffin-embedded followed with a routine Haematoxylin Eosin (HE) staining for histopathologic examination.

Statistical Analysis

The diameter data were analysed by independent sample t-test using IBM SPSS 25, the $p<0.05$ value is considered statistically significant. Microscopic features of tumours in both groups were presented descriptively.

Results

Macroscopic feature

Fibrosarcoma form as solid nodular mass, evident in subcutis area of mice (Figure 1). The fibrosarcoma diameter before treatment for the K group and the P group was presented in Table 1. As the fibrosarcoma nodule were very variable in size, diameter data were presented in mean ± standard deviation (SD) (Table 1). The result of the independent sample t-test showed there was a significant diameter extension of fibrosarcoma in the K group ($p<0.05$) while in the P group there was no significant diameter changes between the beginning with the end of the study ($p>0.05$).

Table 1. Diameter of fibrosarcoma before and after three weeks post injection

Group	Number	Initial Diameter (cm)	Final Diameter (cm)
Control (K)	1	1.40	2.70
	2	1.14	1.00
	3	0.49	0.80
Mean		1.01 ± 0.46	1.5 ±1.04
Treatment (P)	1	0.94	1.00
	2	1.49	1.19
	3	1.69	1.35
Mean		1.37 ± 0.38	1.18 ± 0.17

Note: K group were not treated and P group were injected with NDV intratumorally

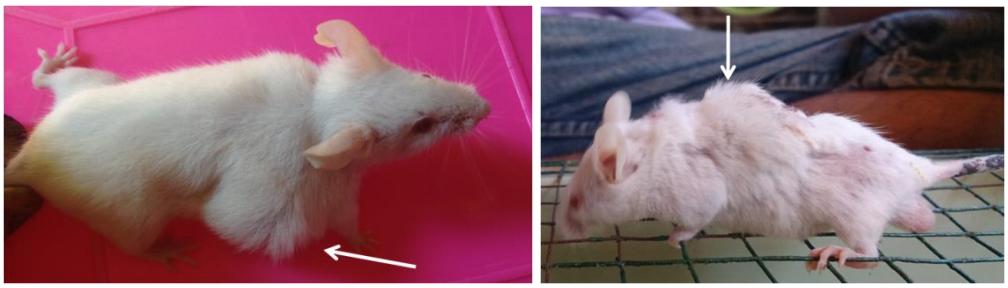

Figure 1. (Left) The macroscopic appearance of fibrosarcoma in K group (Right) The macroscopic appearance of fibrosarcoma in P group

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Microscopic feature

The histopathologic aspect of the tumours tissue in the K group was characterized by evidence of angiogenesis, a high amount of anaplastic spindle cells-shaped fibroblast with pleomorphic nucleus, frequent mitotic figure of fibroblast, collagen fibers proliferation with variation grade while in the P group were found less anaplastic spindle cells-shaped fibroblast, low mitotic figure, and collagen fibers. In the P group, the most distinct differences were macrophages proliferation in necrotizing cells and the presence of multiple giant cells with some severity variation (Table 2).

Table 2. Comparison of microscopic feature of mice fibrosarcoma in three weeks post treatment

Lesion	Control (K)			Treatment (P)		
	K1	K2	K3	P1	P2	P3
Angiogenesis	+++	+++	++	-	-	-
Spindle cell with pleomorphic nucleus	+++	++	+++	++	++	+
Epithelioid cell	++	-	-	-	-	-
Mitosis	++	++	+++	++	+	+
Collagen fiber	+++	++	+++	+++	++	++
Necrosis	-	-	-	+++	+++	+++
Macrophages	-	-	-	+++	+++	+++
Giant cell	-	-	-	-	-	+

Histopathologic examination of the K group suggested there were expansion of the lesion as indicated by a high rate of mitotic figure (Fig. 2) whereas that in the P group seemed to be showed such a kind of growth inhibition, characterized by multifocal necrosis followed by macrophages proliferation (Fig. 3). No capillary vessels were found suggesting the lesion become less active.

Discussions

NDVs are non-pathogenic to mammals and possess naturally oncolytic properties, which make them highly promising cancer therapeutic agents. The more virulent NDV is, the stronger its oncolytic activity (Buijs *et al.*, 2014). Several reports on the *in vitro* and *in vivo* oncolytic activity of this virus have been reported and suggest various mechanisms in inhibiting cancer growth. Several oncolytic mechanisms of NDV have been described, including direct cytopathic effects due to viral replication on tumour cells, indirect effects of innate and adaptive immune cells of the host, and a combination of both (Tayeb *et al.*, 2015). Several studies have also demonstrated other mechanisms such as the involvement of NDV with the apoptotic cascade (Elankumaran *et al.*, 2006) and suppression of angiogenesis through inhibition of the expression of proteins involved in it (Al-Shammary *et al.*, 2020).

In this study, NDV Gianyar-1/AK/2014 isolate was used which were obtained from village chickens in Gianyar, Bali in 2014 (Putra *et al.*, 2016). This virus belongs to genotype VII which is virulent (Adi *et al.*, 2019). Tumour diameter in the K group had a significant increase at the end of the study ($p<0.05$). Meanwhile, the P group did not experience a

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significant increase in diameter ($p>0.05$). The mean tumour diameter in the P group decreased after NDV virotherapy, even it was insignificant ($p>0.05$). Histopathologically, the K group fibrosarcoma's were still found with anaplastic fibroblast cells with pleomorphic nucleus, high mitotic figures, many reactive blood capillaries, and epithelioid cells suggested that the tumour was still reactive (Fig. 2). On the other hand, in the P group, many tumour cells were found with necrosis accompanied by the proliferation of macrophages, collagen tissue, several giant cells, and no capillaries were found. The tumours on this group also have less anaplastic fibroblast cells. Necrosis that occurs and the decrease in tumour diameter is closely related to the success of NDV replication in tumour cells. As previously mentioned, tumour cells generally have low expression of IFN, making them prone to infection (Cook and Chauhan, 2020).

This study is in line with the report of Al-Shammary *et al.* (2020) and Sewoyo *et al.* (2021) who showed that NDV was able to reduce the angiogenic activity of tumours. During growth and development, for solid tumours to grow larger, a massive blood supply is required. Cells that are actively developing need oxygen and nutrients to survive (Lugano *et al.*, 2019). The absence of blood vessels or capillaries can inhibit tumour growth. Without blood vessels, the supply of oxygen and nutrients will be gone. This can lead to cellular death or necrosis.

Infiltration of macrophages may indicate the clearance of cellular debris or necrotic cells to maintain homeostasis. The clearance of these damaged cells is generally carried out by macrophages and neutrophils through phagocytosis, a phenomenon that involves the ingestion and degradation of necrotic particles (Westman *et al.*, 2020). Activated macrophages can be used as immunotherapy, they can kill cancer cells through the recruitment of other immune cells, such as cytotoxic T-lymphocytes. Macrophages have an antitumorigenic activity that can inhibit the process of tumour cell development. Macrophages kill target cells through the secretion of tumour necrosis factor-alpha (TNF-α), nitric oxide, interleukin-1β, and reactive oxygen intermediates (Forsell *et al.*, 2007). Based on Figure 3, we can see that there were several extensive macrophage proliferations in the treated group of fibrosarcoma-bearing mice. Some NDV isolates infection in tumour cells can result in upregulation of major histocompatibility complex (MHC) class I molecules, which is leading to tumour cells destruction mainly by natural killer (NK) cells and CD8+ cytotoxic T lymphocytes (Tayeb *et al.*, 2015). The NDV Gianyar-1/AK/2014 may induce the activation of macrophage through this, but it needs studied more deeply to know the further mechanism.

Based on these findings in this study, the occurrence of necrosis in the P group is most likely caused by direct cytopathic effect due to the virus replication, and/or the host immune system infiltration towards tumour cells and/or inhibition of angiogenesis. In summary, it was found that intratumoral injection of NDV Gianyar-1/AK/2014 leads to inhibition of fibrosarcoma progression, which is reflected in the tumour diameter and necrosis that occurred microscopically. Further studies need to be carried out to further investigate more deeply the mechanism of this virus in inhibiting tumour cells growth.

Conclusion

Virotherapy using NDV Gianyar-1/AK/2014 could inhibit fibrosarcoma growth in mice. There is a distinct microscopic feature of fibrosarcoma between the non-treated and treated

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group with the NDV. In the mice-treated group with NDV, it was found multifocal necrosis of the tumour cells accompanied by macrophages proliferation, some giant cells, and the absence of immature capillaries that reflects low angiogenesis activity.

Conflict of Interest

Authors declares that there is no competing interest exist.

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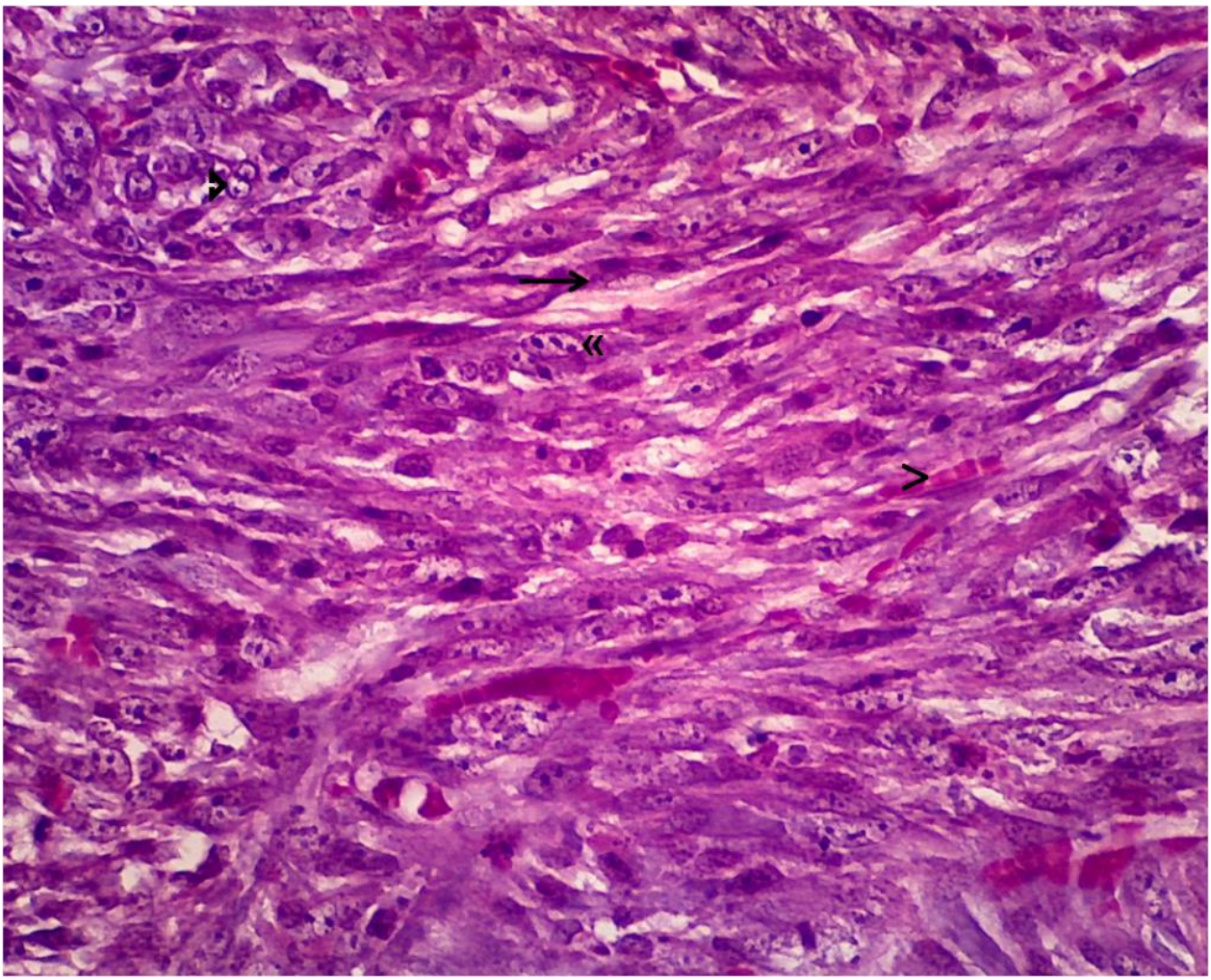

Figure 2. Microscopic feature of the K group. There was found a typical features of fibrosarcoma. The neoplastic cells are not well-demarcated with proliferation of collagen fiber (→), spindle cell («) and epithelioid cell (◗). Unclear cell boundaries, irregular arrangement, and capillary vessels (>) appear to be very clear and reactive (HE, 400x).

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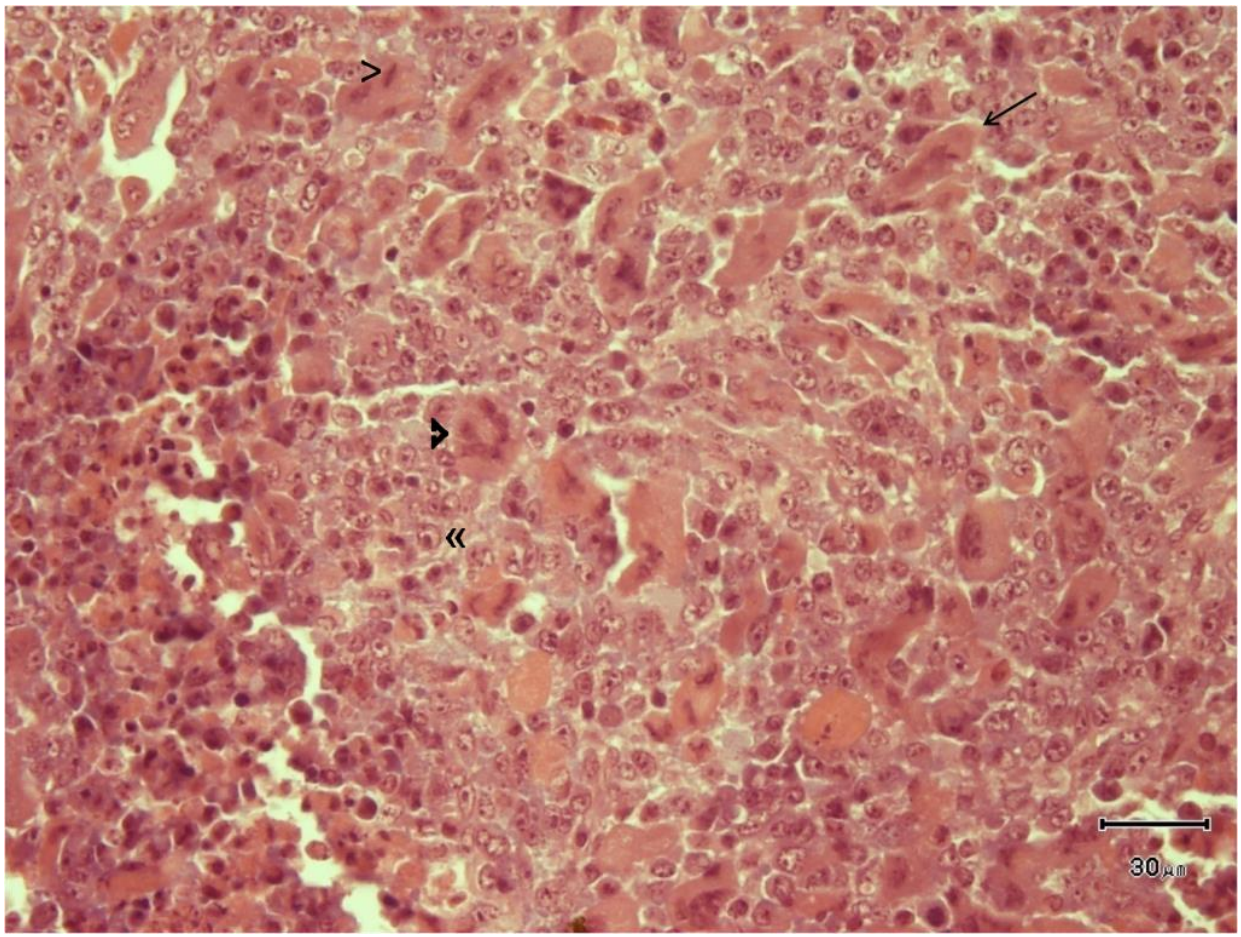

Figure 3. Microscopic feature of group P 3rd week post virotherapy. There were several multifocal necrosis regions with macrophages proliferation («), collagen tissue (→), some giant cells (◗) and less anaplastic fibroblast cells (>) (HE, 400x).