Original Article

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Evaluation of antiviral activity of Oligonol, an extract of *Litchi chinensis*, against betanodavirus

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ABSTRACT: Betanodaviruses, members of the family Nodaviridae, are the causal agents of viral nervous necrosis (VNN) in many species of marine farmed fish. In the aquaculture industry, outbreaks of betanodavirus infection result in devastating damage and heavy economic losses. Although an urgent need exists to develop drugs against betanodavirus infection, there have been few reports about anti-betanodavirus drugs. In this study, we examined the inhibitory effect of Oligonol, a purified phenolic extract from lychee fruit, on betanodavirus infection in fish cells. Oligonol significantly inhibited replication of betanodavirus (EC_{50} = 0.9-1.8 μg/mL) as shown by the reduction of the virus-induced cytopathogenic effect (CPE) and the protection of cells in the crystal violet staining assay. The inhibition was dose dependent. A time-of-addition assay indicated that Oligonol's action takes place at an early stage of the viral infection. According to an attachment inhibition assay, it is possible that Oligonol partially inhibits attachment of the virion to the cell. Our data show that Oligonol could serve as an antiviral agent against betanodavirus.

Keywords: Nervous necrosis virus, fish nodavirus, polyphenol, antiviral drugs

1. Introduction

The family Nodaviridae is composed of the genera *Alphanodavirus* and *Betanodavirus*, which predominantly infect insects and fish, respectively. Nodaviruses are small (25-30 nm in diameter), spherical, non-enveloped viruses with a genome consisting of two single-stranded positive-sense RNA molecules, RNA1 and RNA2 (*1*).

Betanodavirus is the causative agent of a highly destructive disease of marine fish: viral encephalopathy and retinopathy, also known as viral nervous necrosis (VNN). VNN devastates many species of marine fish culture worldwide (*2*). Vaccination has shown promising results in many kinds of fish viral infections but not in VNN because this disease occurs primarily in larval and juvenile fish. The developing fish are difficult to vaccinate because of their small size and immature immune system (*3*). Currently, there is no commercially available drug or vaccine against betanodavirus infection. Selection of putative virus-free spawners and disinfection procedures are used as the major control strategies to prevent the spread of the virus. Inhibitors of endosomal acidification (*4*) and gymnemagenol, a triterpene glycoside saponin (*5*), inhibit betanodavirus infection. These observations underscore the need for novel antiviral drugs, albeit with an alternative mode of action.

Several attempts have been made to use plant phytochemicals as antiviral agents against fish viral diseases (*6,7*). Numerous plant-derived polyphenols have antiviral activity (*8*). Oligonol has an anti-influenza viral activity (*9*). It is an optimized, purified, natural phenolic product from Lychee, containing catechin-type monomers and oligomers of proanthocyanidin (*10,11*), which has been approved by the Food and Drug Administration (USFDA) as a new dietary supplement. Oligonol has biological effects including antidiabetic (*12*), anticancer (*13*), and immunoregulatory properties (*14,15*). In the present study, we investigated the antiviral activity and a possible mechanism(s) of action of Oligonol against betanodavirus using an *in vitro* cell-based system.

2. Materials and Methods

2.1. *Cells, viruses, and antibodies*

E-11 cells, cloned from the striped snakehead (*Ophicephalus striatus*) cell line (SSN-1) (*16*), were

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grown at 25°C in Leibovitz's L-15 medium (Sigma-Aldrich, MO, USA) supplemented with 5% fetal bovine serum (FBS), 300 μg/mL L-glutamine, and 100 μg/mL penicillin/streptomycin. A betanodavirus, redspotted grouper nervous necrosis virus (RGNNV), used in this study was isolated and characterized as described previously (*4*). Stock viruses were prepared by harvesting the supernatant of the infected E-11 cell culture; the viruses were titrated and stored at −80°C until use. The virus titer was determined by the tissue culture infectious dose of 50% ($TCID_{50}$) assay. Anti-RGNNV coat protein antiserum was produced by immunizing a guinea pig with purified coat protein with Freund's complete adjuvant (Becton, Dickinson and Company, NJ, USA) (*17*).

2.2. *Reagents*

Oligonol (> 95% purity) was purchased from Amino Up Chemical Co., Ltd. (Sapporo, Japan), dissolved in 100% dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan) at a concentration of 100 mg/mL, and stored at 4°C. Bafilomycin A1 (Sigma-Aldrich) was dissolved in 100% DMSO and stored at − 20°C.

2.3. *Determination of cytotoxicity and antiviral activity*

To determine cytotoxicity of Oligonol, E-11 cells (4×10^4 cells/well) were seeded on 48-well plates and grown for 36 h. The medium was replaced with 2-fold serial dilutions of Oligonol (0.63-40 μg/mL) in the L-15 medium supplemented with 2% FBS. After incubation for 96 h, cell viability was assessed by crystal violet staining. Briefly, the cells were fixed with 70% EtOH for 5 min and then stained with 0.5% crystal violet for 6 h. After a wash with water, absorbance was measured at 560 nm using an Infinite M200 plate reader (Tecan Group Ltd., Switzerland). Cytotoxicity percentage values were calculated by the following formula: $[(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the absorbance corresponding to untreated cells and A_{sample} is the absorbance corresponding to the cells that were treated with Oligonol. The 50% cytotoxic concentration (CC_{50}) was calculated from the dose-response curve by linear regression analysis. To determine the antiviral activity, a cytopathogenic effect (CPE) reduction assay and virus titer reduction assay were performed on the supernatants as described previously (*4*). E-11 cells (4×10^4 cells/well) were seeded on 48-well plates and cultured for 96 h with 2-fold serial dilutions of Oligonol (0.63-40 μg/mL) and an equal volume of a virus suspension (0.01, 0.1, or 1.0 $TCID_{50}$/cell). The development of virus-induced CPE was observed under a microscope (Axiovert 25, Carl Zeiss, Germany), and culture supernatants were collected for measurements of virus yield. Viability of the cells was determined by crystal violet staining and calculated by the following formula: Survival rate (%) = $[(A_{virus + sample} - A_{virus})/(A_{control} - A_{virus})] \times 100$, where $A_{control}$ is the absorbance corresponding to uninfected untreated cells, A_{virus} is the absorbance corresponding to infected untreated cells, and $A_{virus + sample}$ is the absorbance corresponding to virus-infected cells that were treated with a given concentration of Oligonol. The 50% effective concentration (EC_{50}) was calculated from the dose-response curve by linear regression analysis. The selectivity index (SI) for Oligonol was calculated by the formula: CC_{50}/EC_{50}.

2.4. *Detection of the viral coat protein by Western blotting*

E-11 cells (4×10^4 cells/well) were seeded on 48-well plates and infected with RGNNV (1.0 $TCID_{50}$/cell) in the presence of Oligonol (0.63-40 μg/mL). At 96 h post-infection (h.p.i), the cells were washed twice with ice-cold phosphate buffer saline (PBS) and lysed in a buffer consisting of 4% SDS, 100 mM Tris-HCl (pH 6.8), 2.5% β-mercaptoethanol, 20% glycerol, and 0.4% bromophenol blue. The cell lysate was analyzed by a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, MA, USA). The membrane was incubated with a 1:3,000 dilution of a polyclonal antibody against the RGNNV coat protein, followed by a 1:30,000 dilution of a biotinylated anti-guinea pig IgG F(c) antibody (Rockland, PA, USA), and finally with a 1:3,000 dilution of a streptavidin-alkaline phosphatase conjugate (GE Healthcare, UK). The results were visualized in 0.0125% BCIP (Nacalai Tesque, Kyoto, Japan) and 0.0125% NBT (Wako Pure Chemical Industries, Osaka, Japan) in a 0.1 M diethanolamine solution (pH 9.5) containing 5 mM $MgCl_2$. As an internal control, actin was measured using a 1:2,000 dilution of a rabbit anti-actin antibody (Sigma-Aldrich) followed by a 1:5,000 dilution of a biotinylated anti-rabbit Ig antibody.

2.5. *The time-of-addition assay*

E-11 cells (4×10^4 cells/well) were seeded on 48-well plates and infected with the virus at 1.5 $TCID_{50}$/cell, nearly equal to multiplicity of infection (M.O.I) of 1.0, for 1 h (viral adsorption). Following that, the cells were washed twice with the L-15 medium without FBS to remove any unbound virus. The cells were incubated with the L-15 medium containing Oligonol (10 μg/mL) and 2% FBS from − 3 h to 0 h (*Pre-adsorption*), 0 h to 1 h (*Co-adsorption*), or 0 h to 96 h (*During infection*). For virus treatment, the virus (6×10^6 $TCID_{50}$) was incubated with 10 μg/mL of Oligonol at 25°C for 3 h. Following this, the mixture of virus and Oligonol was diluted 1:25 with the L-15 medium and then added to the cells and incubated at 25°C for 1 h. The cells were then washed twice and cultured in a fresh medium for 96 h (*Pre-treatment of virus*). The supernatants were harvested at 96 h.p.i, and the viral titers were determined by the $TCID_{50}$ assay.

2.6. *The attachment inhibition assay*

E-11 cells (5×10^5 cells) were seeded on 35-mm culture dishes and then pretreated with Oligonol (2.5 and 10 μg/mL) in the L-15 medium free of FBS at 25°C for 3 h. Following this, the cells were infected with the virus at an M.O.I of 1.0 in a medium containing various concentrations of Oligonol at 4°C for 1 h. After washing twice with the medium containing the same concentration of Oligonol, and twice with ice-cold PBS, the cells were lysed by TRIzol reagent (Life Technologies Corporation, CA, USA), total RNA was isolated as described previously (*4*), and analyzed by reverse transcription PCR (RT-PCR).

2.7. *Detection of viral RNA by RT-PCR*

RT-PCR was performed as described previously (*4*) with some modifications. Briefly, for the detection of (+) RNA1 and 18S ribosomal RNA (rRNA), the RNA samples extracted from the infected cells (5×10^4 and 5×10^2 cells, respectively) were used as a template. For the detection of (+) RNA1, the RNA samples were reverse transcribed with M-MLV reverse transcriptase (Life Technologies Corporation) using a primer, RGRNA1-2490R, (5′-GTCAGTGTAGTCTGCATACTG-3′) at 37°C for 50 min. Aliquots (one-tenth volume) of the reverse-transcribed samples were used for PCR amplification. PCR was performed using Pfu polymerase (Bio Academia, Inc., Osaka, Japan) and primers RGRNA1-2490R and RGRNA1-1868F (5′-TGCGTGAGTTCGTCGAGTTT-3′) with 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. For the detection of 18S rRNA, 18S rRNA-R primer (5′-GCTGGAATTACCGCGGCT-3′) and 18S rRNA-F primer (5′-CGGCTACCACATCCAAGGAA-3′) were used. Reverse transcription and PCR amplification for 15 cycles were performed as described above. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Intensity of the bands was quantitated using the Image J software (National Institutes of Health, MD, USA).

3. Results

3.1. *Oligonol inhibits betanodavirus replication*

To determine the cytotoxicity of Oligonol, serial concentrations of Oligonol (0.63-40 μg/mL) were added to near-confluent E-11 cells (monolayer). In the viability assay, the cells treated with Oligonol concentrations lower than 10 μg/mL did not show any significant morphological changes (Figure 1A). The CC_{50} value of Oligonol was 27.0 ± 1.0 μg/mL.

To evaluate the antiviral potency of Oligonol against RGNNV, E-11 cells were treated with various concentrations of Oligonol and inoculated with RGNNV (0.01, 0.1, or 1.0 $TCID_{50}$/cell) for 96 h. Cell morphology was observed under a microscope (Figure 1A), and the cells were stained with crystal violet to calculate the EC_{50} value (Figure 1B and Table 1). We used bafilomycin A1 as a positive control drug. In the presence of bafilomycin A1 (10 nM), CPE development was completely suppressed, as reported previously (*4*). Similarly, CPE development was completely suppressed in the presence of 10 μg/mL (1.0 $TCID_{50}$/cell) as well as 2.5 μg/mL (0.01 $TCID_{50}$/cell) of Oligonol. The relative survival rate of the cells was calculated from the absorbance of the culture plates stained with crystal violet (Figure 1B). At the concentration of 10 μg/mL, almost a 100% survival rate was observed. As shown in Table 1, EC_{50} and SI of Oligonol depend on the amount of the virus used during inoculation. The effect of Oligonol on virus production was evaluated by the $TCID_{50}$ assay (Figure 1C). Oligonol treatment significantly inhibited virus production in a dose-dependent manner. The influence of Oligonol on expression of the viral coat protein was determined by Western blotting (Figure 1D). Ninety-six hours after the infection, cell lysates were prepared and subjected to Western blotting using an anti-coat protein antibody. The intensity of coat protein bands was significantly decreased by Oligonol (> 1.25 μg/mL). These results suggest that Oligonol inhibits betanodavirus replication in a dose-dependent manner with minimal cytotoxicity.

3.2. *The mode of action of Oligonol against betanodavirus*

To investigate the mode of action of Oligonol, the time-of-addition assay was performed (Figure 2A). Either Oligonol (10 μg/mL) or DMSO (0.04%) was added to E-11 cells before or during viral infection at an M.O.I of 1.0. Compared with DMSO treatment, pre-treatment of the virus with Oligonol showed no significant inhibitory effect on virus yield, suggesting that Oligonol lacks a virucidal activity (Figure 2A, right). On the other hand, a significant reduction (over 99%) in virus yield was observed when the cells were pre-incubated with Oligonol before virus adsorption (*Pre-adsorption*). Similarly, during the analysis of cell morphology (Figure 2B), pre-treatment of the virus with Oligonol did not significantly inhibit CPE development. In contrast, CPE development was substantially suppressed when the cells were pre-incubated with Oligonol before virus adsorption. When Oligonol was present during the full replication cycle (*During infection*), the titers of the progeny virus and CPE development were strongly reduced. In fact, substantial inhibition of virus yield and CPE development were observed when the cells were incubated with Oligonol before and during virus adsorption (*Pre-adsorption* and *Co-adsorption*). In summary, Oligonol showed significant antiviral activity against RGNNV when added before or during viral adsorption. These results suggest that Oligonol may interfere with the viral adsorption.

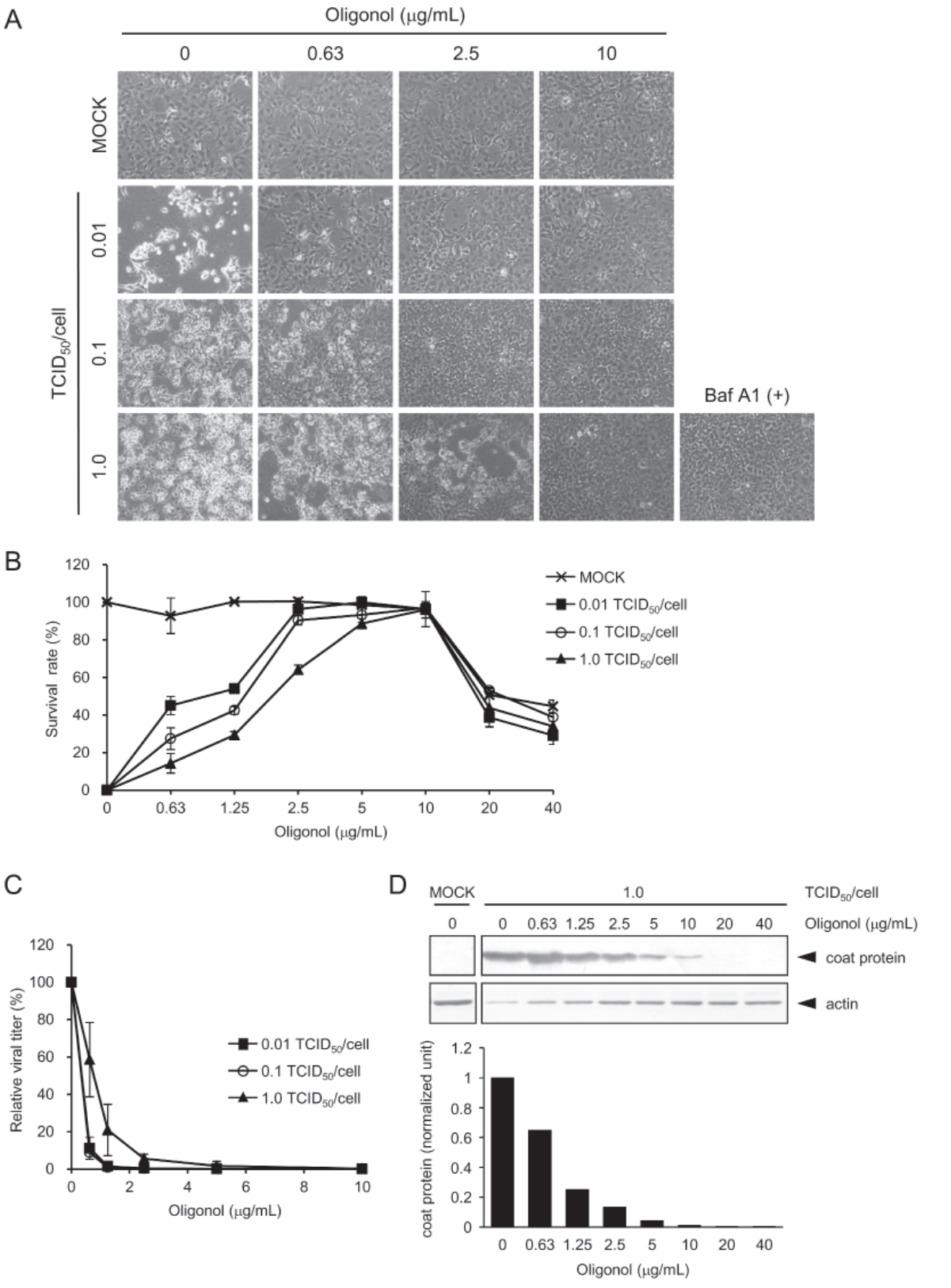

Figure 1. Anti-betanodavirus effects of Oligonol. E-11 cells infected with RGNNV (0.01, 0.1, and 1.0 $TCID_{50}$/cell) were treated with Oligonol for 96 h. **(A)** Effect of Oligonol on betanodavirus-induced CPE development observed under a phase-contrast microscope. Baf A1 (+): bafilomycin A1 (10 nM). **(B)** Effect of Oligonol on the cell survival rate. After staining with crystal violet, absorbance at 560 nm was determined and the percentage of survival rate was calculated. **(C)** Effect of Oligonol on betanodavirus production. Culture supernatants were collected and a viral titer was measured by the $TCID_{50}$ assay. The relative viral titer was calculated as the percentage of the viral titer to that of the cells infected in the absence of Oligonol. The data represents the mean of three independent experiments, and the error bars show standard deviation. **(D)** Effect of Oligonol on viral coat protein expression. Total cell lysates were prepared with lysis buffer and subjected to SDS-PAGE, and Western blotting was carried out using an anti-RGNNV coat protein antibody. Band intensity of the coat protein was normalized to actin and expressed as a ratio of the amount of coat protein in the treated cells to that in untreated cells.

3.3. *Oligonol partially inhibits attachment of betanodavirus to the cell*

To investigate the mechanism of the inhibitory effect of Oligonol on betanodavirus infection, we examined the influence of Oligonol on the attachment of betanodavirus to E-11 cells by the attachment inhibition assay. It has been reported that endocytosis of betanodavirus does not occur at 4°C (*18*). Cells were inoculated with the virus at an M.O.I = 1.0 in the presence of either 2.5 or 10 μg/mL of Oligonol for 1 h at 4°C. When E-11 cells were infected with the virus in the absence of Oligonol, the viral (+) RNA1 band was detected (Figure 3A). However, when the cells were treated with either 2.5 or 10 μg/mL of Oligonol, the intensity of these bands decreased. Relative band intensity of (+) RNA1 was calculated in normalized units (Figure 3B). In the presence of either 2.5 or 10 μg/mL of Oligonol, the relative band intensity decreased. These results suggest that Oligonol may inhibit the attachment of betanodavirus to the cell.

Table 1. *In vitro* cytotoxicity and anti-betanodavirus activity of Oligonol [a]

$TCID_{50}$/cell	EC_{50} (μg/mL)[b]	CC_{50} (μg/mL)[c]	Selectivity index (SI)[d]
0.01	0.87 ± 0.07	27.0 ± 1.0	31.1 ± 3.1
0.1	1.17 ± 0.06	27.0 ± 1.0	23.2 ± 2.0
1.0	1.81 ± 0.09	27.0 ± 1.0	15.0 ± 1.3

The values represent mean ± standard deviation of three independent experiments. [a] Evaluation after 96 h of treatment [b] 50% effective concentration [c] 50% cytotoxicity concentration [d] Selectivity index = CC_{50}/EC_{50}.

4. Discussion

In this study, we investigated the antiviral activity and the possible mode of action of Oligonol against betanodavirus replication. The results indicate that Oligonol effectively inhibits betanodavirus replication

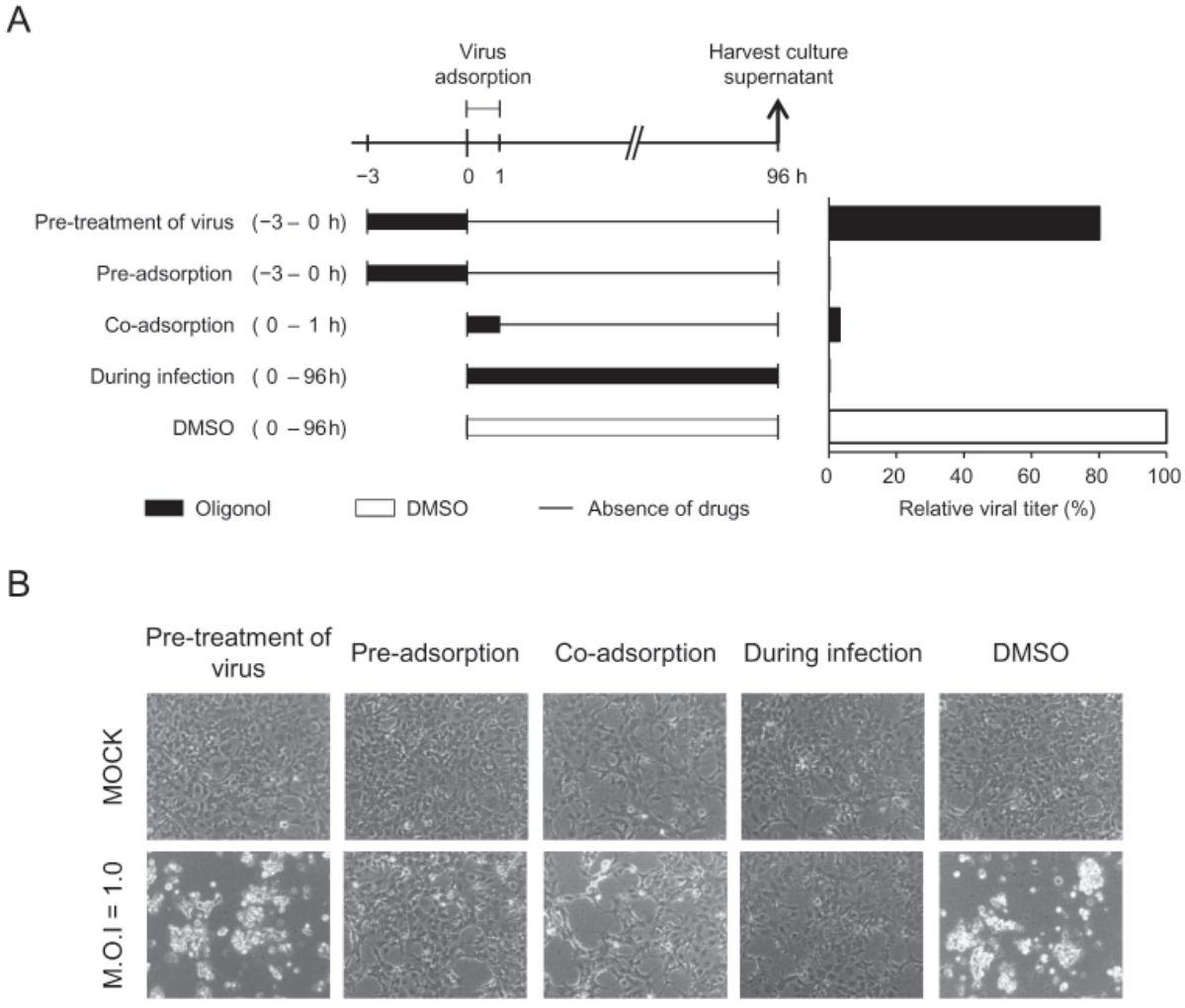

Figure 2. The time-of-addition assay for the effect of Oligonol on betanodavirus production. (A) Oligonol treatment protocols and the effect on betanodavirus production. E-11 cells were infected with RGNNV (1.5 $TCID_{50}$/cell corresponding approximately to an M.O.I of 1.0) and treated with Oligonol (10 μg/mL) or 0.04% (v/v) DMSO at the indicated periods. In the experiment with the pre-treatment of the virus, betanodavirus was incubated with Oligonol for 3 h and then diluted to achieve an M.O.I of 1.0 before viral adsorption. The culture supernatant was collected at 96 h.p.i, and virus yield was determined by the $TCID_{50}$ assay. The ratio of viral titers is presented as a mean of two independent experiments. **(B)** Effect of different Oligonol treatment protocols on the betanodavirus-induced CPE development. Pictures were taken using a phase-contrast microscope at 96 h.p.i.

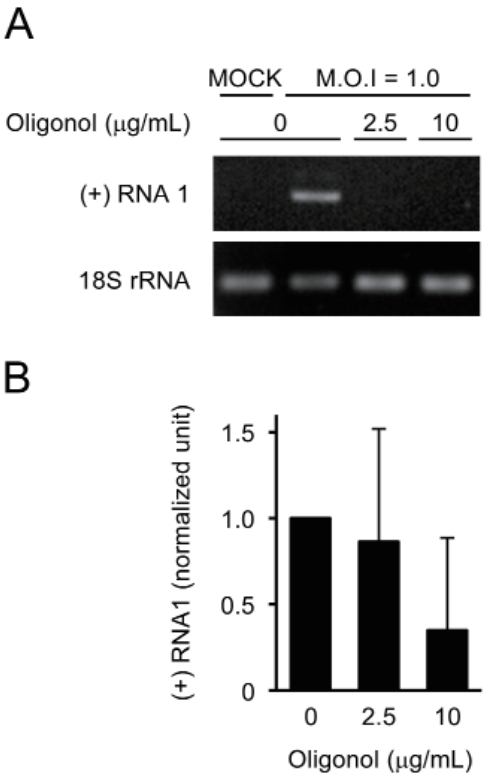

Figure 3. Effect of Oligonol on attachment of betanodavirus to the cells. (A) The attachment inhibition assay. E-11 cells were infected with RGNNV at an M.O.I of 1.0 in the presence of Oligonol (2.5 or 10 μg/mL) at 4°C for 1 h. Total RNA was isolated and (+) RNA1 and 18S ribosomal RNA (rRNA) were analyzed by RT-PCR. The data are representative of three independent experiments. **(B)** Quantification of relative band intensity of (+) RNA1. Intensity of (+) RNA1 and 18S rRNA bands was quantified, normalized to cellular 18S rRNA, and expressed as a ratio of the amount of (+) RNA1 in the treated cells to that in untreated cells. The data represents a mean of three independent experiments, and error bars show standard deviation.

in a dose-dependent manner as shown by the reduction of virus-induced CPE, virus production, and viral protein expression (Figure 1). The time-of-addition assays (Figure 2) suggested that the inhibition of betanodavirus replication by Oligonol primarily occurs at the virus adsorption step. Pre-treatment of cells before virus adsorption or treatment of cells during virus adsorption greatly reduced virus yield. However, pre-treatment of RGNNV virions prior to infection showed no inhibitory effect. These results indicate that Oligonol does not directly react with the virion.

The specific functional cell receptors for betanodavirus have not been identified yet, although it has been demonstrated that sialic acid is involved in RGNNV's binding to cells (*18*). A previous study on the antiviral effect of Oligonol on influenza A virus (*9*) showed that Oligonol affects viral surface molecules, thus interfering with the attachment step of this virus. Based on the results of the attachment inhibition assay (Figure 3), it can be concluded that Oligonol possibly inhibits attachment of the virion to the cell by interacting with cellular molecules.

Catechin, the polyphenolic compound from green tea, exhibits antiviral activity against influenza virus (*19*) and hepatitis C virus (*20*) by targeting the attachment and entry steps of the viral replication cycle. This phenomenon may be explained by the similarity of chemical structure of the different polyphenols in both Oligonol (*9*) and green tea (*19*). This observation suggests that the primary target of polyphenolic compounds is likely to be some membrane components.

In conclusion, this study demonstrated that Oligonol possesses suppressive properties against RGNNV replication. Oligonol targets an early step of RGNNV replication in E-11 cells, most likely by inhibiting the attachment of virions to cells. This inhibitory effect is accompanied by low toxicity. These results suggest that Oligonol can be considered as an anti-betanodavirus candidate drug.

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