The *Urtica Dioica* Agglutinin Prevents Rabies Virus Infection in a Muscle Explant Model

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Abstract

Infection with the rabies virus (RABV) causes fatal encephalitis and paralysis in humans and animals. Post-exposure prophylaxis (PEP) consists of vaccination and the injection of anti-rabies immunoglobulins (RIGs) around the (bite) wound. This is 100% effective in preventing disease if administered in a timely manner. However, the costs, the required cold chain for storage and transport and the limited availability of RIGs makes the treatment challenging. Cheaper and easier to produce alternatives are urgently needed. To aid in the discovery and development of such alternative therapeutics, we developed a physiologically relevant infection model. Strips of freshly dissected swine skeletal muscle were placed under tension in culture medium and infected with RABV. Viral antigens were produced in the muscle explants and the virus production increased significantly over time, indicating that RABV infects and replicates in the muscle explants. Subsequently, in a search for inhibitors of RABV entry in muscle cells, we first screened a panel of 34 different lectins in a RABV / BHK-21J cell assay. The *Urtica dioica* (stinging nettle) Agglutinin (UDA; a N-acetyl-D-glucosamine specific agglutinin) was found to be able to completely inhibit infection of cells with the RABV (EC_{50} 8.2 μg/mL) by preventing binding of the virus to the host cell. When the infection of the muscle strips was carried out in the presence of UDA, infection of the tissue was completely prevented. We thus developed a physiologically relevant RABV muscle infection model and identified an easy to produce component that (i) may serve as a reference for further studies and (ii) holds promise as an alternative for RIGs in PEP.

Introduction

Rabies virus (RABV), a member of the Lyssavirus genus, is a neurotropic virus that causes an acute and invariably fatal encephalitis in humans and animals. In patients the disease is nearly 100% fatal following the onset of symptoms. Post exposure prophylaxis (PEP), in the form of vaccine and rabies immunoglobulins (RIG), can prevent fatalities when administered prior to the onset of disease(WHO, 2014). However, availability and cost of PEP means that in endemic regions, where the human burden to rabies is greatest, these tools are rarely available. Globally the high economic cost, annually US$ 8.6 billion for rabies treatment and US$ 1.70 billion for PEP(Hampson *et al.*, 2015), means that many countries have limited capacity to provide PEP when needed(Lankester *et al.*, 2014; Sreenivasan *et al.*, 2019) and as such rabies remains a significant public health threat.

Humans are most commonly exposed to rabies when bitten by rabid animals with infectious virus in their saliva. If the dermal barrier is breached, the virus infects muscle cells and gains access to neuronal cells through the neuromuscular junctions (NMJ). Once within neuronal cells the virus migrates from the peripheral nerves to the central nervous system (CNS) resulting in replication in the brain and the development of rabies. The disease is characterized by altered mental status, phobic or inspiratory spasms(Hemachudha *et al.*, 2013). To enable cell entry, the nicotinic acetylcholine receptor (nAChR), serves as one of the key receptor molecules for rabies virus binding and entry(Lentz *et al.*, 1982; Lentz, 1990; Hanham, Zhao and Tignor, 1993). However, the nAChR tends to be concentrated at the postsynaptic muscle membrane in the NMJ, which may drive accumulation of rabies virions near the NMJ enhancing the likelihood of the virus entering the peripheral neuron or motor neuron(Lentz *et al.*, 1983; Lafon, 2005). To this end, it is important to inhibit RABV accumulation or replication in muscle cells and prevent infection of the nervous system. This mechanism of action is proposed for RIGs, which are injected in the tissue near the exposure wound and prevent an ongoing RABV infection. We aim to generate an alternative to these RIGs with a lower cost, no need for a cold-chain and hence more wide availability in the areas that need it most.

To aid in the development of such strategies we first wanted to develop a physiological infection model. To this end, we explored whether muscles strips of swine, that are placed under tension in cell culture can be infected with RABV. We also explored whether lectins (a group of carbohydrate-binding proteins) can prevent infection of cells and muscle strips. Specific lectins can reduce cellular susceptibility to RABV(Marchetti *et al.*, 1995). Here we demonstrate that the *Urtica dioica* Agglutinin (UDA) can prevent infection of muscle tissue from RABV.

Materials and methods

Cells, virus and lectins

BHK-21J cells (*ATCC® CCL-10™*) were propagated in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific) with 10% fetal bovine serum (FBS) (Hyclone) and penicillin-streptomycin (P/S) (100 U/mL, ThermoFisher Scientific). When cells were infected with RABV, 2% DMEM was used. Rabies virus SAD-B19-mCherry was supplied by the Animal & Plant Health Agency, UK. Lectins were kindly provided by Professor Els Van Damme (Ghent University, Belgium), then were dissolved in PBS at 1 mg/mL and stored at -20 °C.

Antiviral assay

Compounds were added to cells by serial dilution from 100 µg/mL to 0.8 µg/mL, followed by virus infection at a multiplicity of infection (MOI) of 0.01. Antiviral activity was measured by quantification of the amount of mCherry fluorescence on day 5 post-infection (SPARK, Tecan, Belgium) ®. Cell viability was determined by an MTS assay as described previously(Jochmans, Leyssen and Neyts, 2012). The half maximal effective concentration (EC_{50}) of antiviral effect and 50% cytotoxic concentration (CC_{50}) of cell toxicity was derived from the corresponding dose–response curves.

Time of addition assay

UDA, at a final concentration of 25 µg/mL, was added to RABV infected (MOI =1) cell cultures. Depending on the condition tested the UDA was added starting 2 h before infection or immediately after virus inoculation. UDA untreated cells with virus infection were as control. At 24 h.p.i., viral RNA was extracted from cells (E.Z.N.A.® Total RNA Kit I R6834-01, Omega BIO-TEK) and cell culture supernatant (QIAamp Viral RNA Mini Kits, Qiagen) and quantified by RT-qPCR analysis.

Pre-exposure of RABV to lectins prior to infection

RABV was incubated with lectins at 37 °C for 2 h. Then, BHK cells were incubated with the mixture of virus (MOI=1) and drug at 37 °C for 2 h. After incubation, the supernatant was removed, and cells were washed with PBS 3 times. At day 5 post-infection, the mCherry

109 fluorescence was quantified (SPARK, Tecan, Belgium) ®.

110 Pre-exposure of BHK to lectins prior to infection

111 BHK cells were pre-incubated with lectins, final concentration 25 µg/mL, at 37 °C for 2 hours.
112 Then, virus was added (MOI=1) and incubated with the cells at 37 °C for 2 hours. After
113 incubation, cells were washed 3 times with PBS. At day 5 post-infection, the mCherry
114 fluorescence was quantified.

115 Swine skeletal muscle explant culture and antiviral assay

116 The biceps femoris muscle was dissected from freshly euthanized pigs (3 months, female),
117 obtained as residual tissue from ORSI Academy (Melle, Belgium). Next, the muscle biopsy was
118 dissected, parallel to the muscle fibers, in 2 cm long 2 mm thick strips using sterile scalpels.
119 Tissue strips were maintained under tension *ex vivo* using minutia pins (Entosphinx, stainless
120 steel pins, diameter 0.25 mm) on sterile sylgard coated wells of a 6-well plate. Tissue strips
121 were submerged in DMEM with 10% FBS, P/S (100 U/mL), and in 2% FBS DMEM with P/S
122 during antiviral experiments at 37°C incubator with 5% CO_2. UDA was diluted to a final
123 concentration of 25 µg/mL in the supernatant and incubated with muscle explants at 37 °C for
124 2h. Then the muscle explants were inoculated with 2 × 10^4 $TCID_{50}$ RABV. After 4 h incubation,
125 muscle explants were washed 5 times with PBS, and maintained with 25 µg/mL UDA for 6
126 days. A sample of 300 µL of the culture SN was collected every day for RT-qPCR or titration.

127 Muscle culture supernatant titration

128 The titer of virus presents in swine muscle explant supernatant at day 6 was determined by 10-
129 fold serial dilution titrations in a 96-well plate with confluent BHK cells. On day 7, virus
130 expressed fluorescence was observed under a fluorescent microscope. $TCID_{50}$ of virus titer was
131 calculated by Spearman–Kärber method.

132 Fluorescent immunostaining and microscopy of muscle

133 On day 6 p.i. swine skeletal muscle explants were washed 5 times with PBS and transferred
134 into 1.5 mL Eppendorf tubes. Samples were fixed in 4% formaldehyde for 1 h and stored at
135 4 °C in PBS until staining. Before imaging, they were permeabilized with ice-cold 80% Acetone
136 at room temperature for 1 h and washed 5 times in PBS. Afterwards, muscle explants were

137 incubated 1 h at 37 °C with FITC Anti-Rabies Monoclonal Globulin (800-092, Fujirebio)
138 diluted 1:10 in PBS. After 5 times PBS washing, muscle explants were incubated in 5μg/mL
139 Hoechst (H1399, Invitrogen) for 15 min at room temperature. Imaging was performed using
140 the Leica DMi8 S Platform with Leica Application Suite X software (Leica, Heidelberg,
141 Germany). All pictures were obtained with a 10 x objective and z position between 1820 μm to
142 1950 μm.

143 **Real-time RT-qPCR**

144 Rabies virus N gene was amplified by real-time quantitative PCR. The primers are as follow:
145 5'-TGGGCACAGTTGTCACTGCTTA-3' (forward) and 5'-CTCCTGCCCTGGCTCAA-3'
146 (reverse). iTaq™ Universal SYBR® Green One-Step Kit (BIO-RAD) was used for qPCR. A 20
147 μL qPCR reaction contains 4 μL extracted sample RNA or standard, 10 μL iTaq Universal
148 SYBR® Green reaction mix, 0.25 μL reverse transcriptase and 600 nM of each forward and
149 reverse primer. qPCR was performed in a Roche LightCycler 96 with the procedure: 10 min at
150 50 °C for reverse transcription, 1 min at 95 °C for polymerase activation and DNA denaturation,
151 40 cycles of 95 °C 15s and 62 °C 30s for PCR amplification. Viral copies were calculated based
152 on a standard curve using control material.

153 **Statistics**

154 Statistical analysis in this study was done by GraphPad Prism 9. A P-value less than 0.05 was
155 considered as statistically significant. P values associated with each graph are indicated as: *,
156 P value < 0.05; **, P value < 0.01; ***, P value < 0.001; ****, P value < 0.0001.

157 # Results

158 ## Plant lectin antiviral activity screening and confirmation

159 We tested the potential effect of a panel of 34 different lectins on infection of BHK cells with
160 RABV using a reporter virus. Several lectins with selective antiviral activity against RABV
161 were identified (Table 1) of which UDA was taken forward for further assessment. Activity of
162 UDA was confirmed in additional tests (EC_{50} 8.2 µg/mL and CC_{50} 65 µg/mL) (Figure 1). To
163 explore at which step of the virus replication cycle UDA exerts its activity, it was added at
164 different time points after infection. The lectin retained full activity when present during
165 infection, but not when added at a later stage (Figure 2A); indicating that UDA blocks RABV
166 infection at the entry step. In a second experiment it was shown that also pre-treatment of the
167 cells results in an inhibition ($p < 0.0001$) of infection, which indicates that UDA can block viral
168 entry by binding to the cells (Figure 2B).

169 ## RABV replicates in swine skeletal muscle explants

170 Swine muscle explants were inoculated with 2×10^4 $TCID_{50}$ virus and the culture supernatant
171 was collected every day for 6 consecutive days. On day 5-6 post-infection, viral RNA in the
172 culture supernatant was >10 fold higher as compared to day 0 (Figure 3A). To confirm that
173 infectious virus is produced, BHK cells were infected with the culture supernatant obtained
174 from the muscle cultures on day 6. The mCherry red fluorescence expressed by the virus was
175 monitored by fluorescent microscopy (Figure 3B). These results demonstrate that swine skeletal
176 muscles are susceptible to RABV *ex vivo* and that infectious viral particles are produced.

177 ## UDA inhibits RABV infection in swine skeletal muscle

178 To investigate whether UDA can prevent RABV infection of muscle tissue, the muscle strips
179 were pre-incubated with UDA (25 µg/mL) for 2 h followed by 4 h virus inoculation. Afterwards,
180 the virus inoculum was removed and new medium containing UDA (25 µg/mL) was added to
181 the tissue cultures. On day 6 post-infection, the culture supernatant was collected for titration
182 and the muscle tissues were fixed for immunofluorescent staining. UDA treatment resulted in
183 a 600-fold decreased in virus production (Figure 4A), which is consistent with the results
184 obtained from the immunofluorescence staining (Figure 4B).

Discussion

Rabies is a fatal disease of animals and humans and remains a public health risk in large parts of the world. Whereas there is an effective PEP procedure, there are no therapeutic options for patients that present clinical symptoms. Despite initial promise, the treatment of rabies disease using the induction of a therapeutic coma termed the “Milwaukee Protocol” was proven to be ineffective(Appolinario and Jackson, 2015). Ribavirin, Favipiravir, interferon-alpha (IFN-α) have shown antiviral efficacy *in vitro* but no benefit in either patients or mice (Merigan *et al.*, 1984; Warrell *et al.*, 1989; Banyard *et al.*, 2019). Dermaseptins have been described as potential anti-rabies compounds *in vitro* and with 50–60% of survival gain in mice(Mechlia *et al.*, 2019). Drug cocktails containing Favipiravir, Ribavirin, Interferon α/β, a Caspase-1 inhibitor, a TNF-α inhibitor, a MAPKs inhibitor and human rabies immune globulin (HRIG) have been explored in mouse RABV models (Smreczak *et al.*, 2019) but no clear therapeutic effect was noted. A cocktail of two human monoclonal antibodies RVC20/RVC58 delivered directly into the brain could cure half of the symptomatic rabid mice in the research(de Melo *et al.*, 2020). One part of the current PEP protocol involves the injection of RIG around the exposure wound that can prevent the transport of the virus via the neuromuscular junction to the peripheral nervous system and subsequently to the CNS. However, RIG is costly, not readily available and it needs a cold chain for storage and transport. To aid in the discovery of novel PEP strategies, we wanted to develop a physiological RABV infection muscle model. We demonstrate that swine muscles strips can be productively infected with RABV.

In parallel we demonstrated that some lectins could inhibit RABV entry in cell culture and in the swine skeletal muscle explant culture. Lectins are characterized by their type of reversible binding to a specific mono- or oligosaccharide(Peumans and Van Damme, 1995), they can be found in many biological functions including protection against bacteria and virus infection(Sharon and Lis, 1989; Lis and Sharon, 1998; Meiers *et al.*, 2019). For enveloped viruses, the viral glycans play an important role for processes such as envelop formation, immune evasion or virus release(Watanabe *et al.*, 2019). Studies have demonstrated that lectins bind with viral glycans of the enveloped viruses, such as HIV and coronavirus(Hansen *et al.*,

1989; Balzarini, 2006; Keyaerts *et al.*, 2007; Lam and Ng, 2011). We screened a library of lectins and identified UDA among the most active. We demonstrated that UDA interacts with BHK cells and in that way protects the cells from RABV infection, most likely by blocking virus receptor binding. Typically, when people are exposed to RABV infection, HRIG provides passive protection by neutralizing the virus in the wound and muscles before vaccine-induced antibodies appear (Warrell, 2014). We demonstrate that UDA can protect muscle cells from rabies infection indicating that lectins may be an alternative for the RIGs.

In conclusion, we developed a novel, physiologically relevant replication assay for RABV by using swine skeletal muscle explant cultures and demonstrate that the plant lectin UDA can prevent RABV infection in this model. UDA may serve as a reference for further studies and may hold promise as an alternative for RIGs in PEP.

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Author contributions

L. T. and L. T. isolated and dissected the muscle stripes from the freshly euthanized pigs. G. W. provided the rabies virus-mCherry. E. V. D. provided the library of lectins. A. R. F. and A. C. B. advised suggestions for writing. X. W., D. J. and J. N. wrote the manuscript.

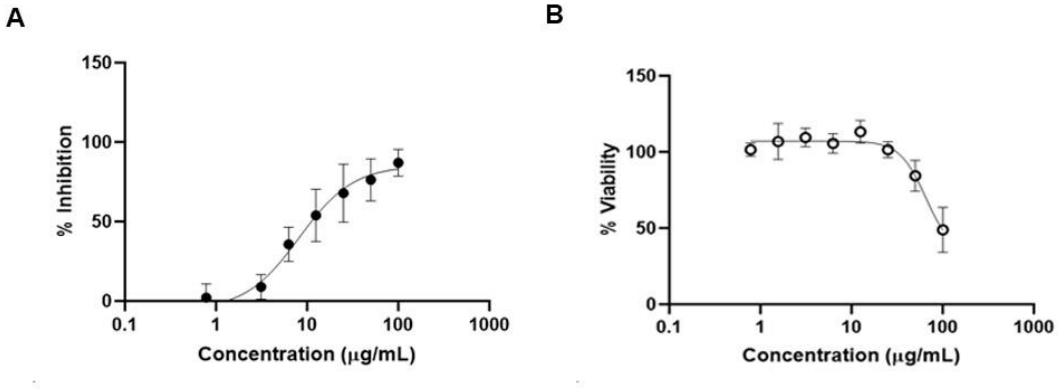
241 **Table 1. Antiviral activity of lectins against RABV**

Lectin	Species	[1]EC_{50} (µg/mL)	CC_{50} (µg/mL)	SI
Gal/GalNAc-specific agglutinins				
PHA-L4	Phaseolus vulgaris	47 ± 13	>100	>2.1
RPA	Robinia pseudoacacia	5.4 ± 2.5	11 ± 1.4	2.1
RSA	Rhizoctonia solani	>100	>100	Na
SJA	Styphnolobium japonicum	32 ± 19	>100	>3.2
GalNAc-specific agglutinins				
BPA	Bauhinia purpurea	3.2 ± 1.0	9.0 ± 2.1	2.8
CAA	Caragana arborescens	95 ± 5	>100	>1.0
DBA	Dolichos biflorus	>100	>100	Na
SBA	Soybean	>100	>100	Na
WFA	Wisteria floribunda	>100	>100	Na
Gal-specific agglutinins				
Jacalin	Jackfruit	>100	>100	Na
Morniga G	Morus nigra	>100	24 ± 8.6	<0.2
PHA-E	Phaseolus vulgaris	47 ± 1.5	86± 18	1.8
PNA	Arachis hypogaea	>100	>100	Na
GlcNAc-specific agglutinins				
DSL	Datura stramonium	57 ± 2.2	69 ± 12.4	1.2
GS II	Griffonia simplicifolia	>100	>100	na
Nictaba	Nicotiana tabacum	29.0 ± 3.9	>100	>3.4
UDA	Urtica dioica	8.1± 1.3	54 ± 6.8	6.7
UEA II	Ulex europaeus	>100	>100	na
WGA	Wheat germ	21 ± 7.0	17± 6.3	0.8
Man/GalNAc-specific agglutinins				
AMA	Arum maculatum	7.1 ± 2.9	9.1 ± 2.2	1.3
TLC I	Tulipa hybrid	32± 5.7	>100	>3.2
Mannose-specific agglutinins				

APA	Allium porrum L.	36 ± 11	>100	>2.9
Banana lectin	Musa acuminata	6.2 ± 2.6	40.7 ± 11.5	6.5
Calsepa	Calystegia sepium	>100	>100	na
Con A	Canavalia ensiformis	13 ± 3.7	22 ± 4	1.7
ConarvA	Convolvulus arvensis	90.7 ± 9.3	>100	>1.1
GNA	Galanthus nivalis	>100	>100	na
HHA	Hippeastrum hybrid	29 ± 2.3	>100	>3.4
Morniga M	Morus nigra	>100	27.4 ± 14.1	<0.3
NPA	Narcissus pseudonarcissus	16 ± 5.4	42± 5.8	2.6
PSA	Pisum sativum	13 ± 7.5	>100	>7.7
Neu5Acα (2.6) Gal/GalNAc-specific agglutinins				
SNA I	Sambucus nigra	14 ± 7	>100	>7.4
Sialic-acid-specific agglutinin				
ACA	Amaranthus caudatus	>100	>100	na
MAA	Maackia amurensis	>100	>100	na

Man: mannose, GlcNac: N-acetyl glucosamine, GalNAc: N-acetyl galactosamine, Gal: galactose, Neu5Ac: N-acetylneuraminic acid, SI: selectivity index

[1]EC_{50} and CC_{50} are reported as the average and SD of 3 independent experiments



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248 **Figure 1. Anti-RABV activity and dose-response curve of UDA.** Lectins were serially
249 diluted and added together with mCherry-RABV (MOI=0.01) into plates containing BHK cells.
250 On day 5 p.i. the antiviral activity was determined by quantification of the mCherry
251 fluorescence (A). In a parallel experiment the effect of UDA on cell viability was determined
252 using a viability staining (MTS) (B). Averages and standard deviations of 3 independent
253 experiments are presented. Fitting the dose-response curves indicated an EC_{50} of UDA of 8.2
254 μg/mL and a CC_{50} of 65 μg/mL.

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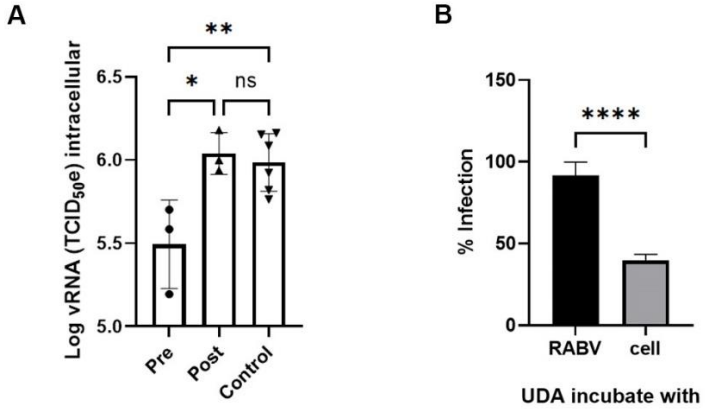

Figure 2. UDA blocks RABV entry. (A) BHK cells were incubated with UDA under pre- or post-infection conditions (see methods). After 24 h p.i., intracellular vRNA was quantified by RT-qPCR. Each condition was tested at least 3 times and averages and STDEV are indicated. (B) Pre-exposure of UDA with RABV or cells before virus infection, after 5 days, the relative number of infected cells was determined by quantification of the virus associated fluorescence. (* $P < 0.05$, ****$P < 0.0001$)

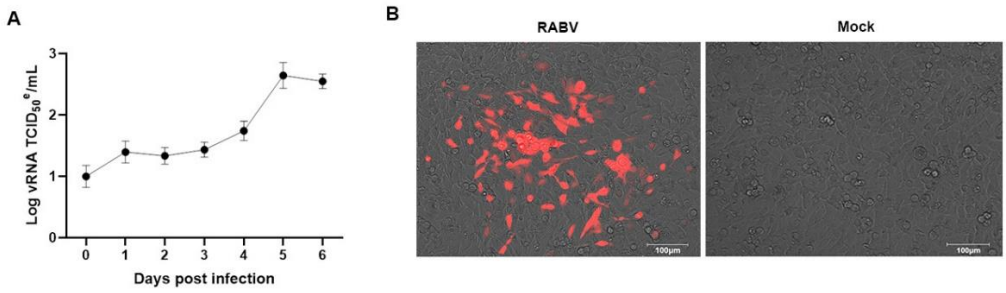

Figure 3. RABV replicates in swine skeletal muscle explants. (a) Culture supernatant was collected at each day post infection, then vRNA was quantified by RT-qPCR. (b) On day 6 p.i. the supernatant of infected (RABV) or non-infected (Mock) muscle cultures was transferred to BHK cells. After 7 days of incubation, the fluorescence of the cells was determined by microscopy.

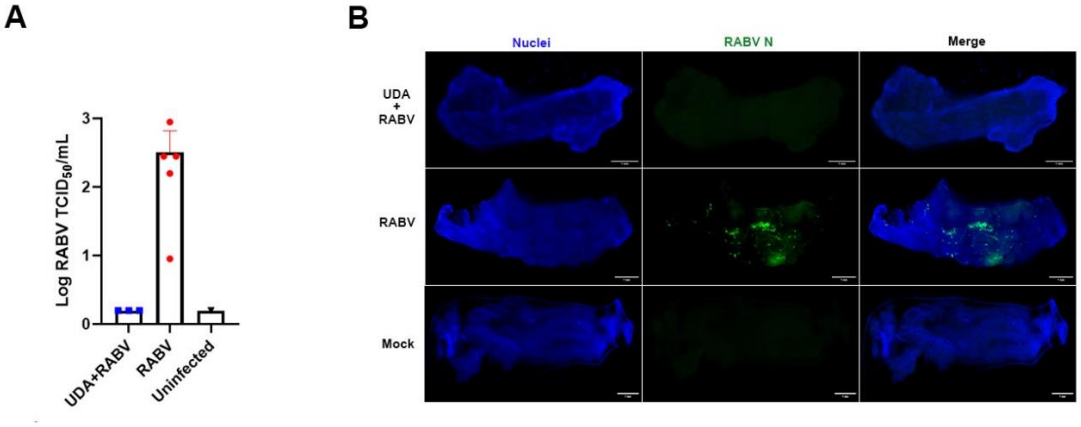

Figure 4. UDA inhibits RABV infection in swine skeletal muscle explants. Muscle explants were infected with RABV with or without UDA, at 6 d.p.i., the culture supernatant was collected, and explants were fixed. (A) Infectious virus titers in the supernatant was determined by titration as described above. (B) On day 6, muscles were fixed and stained as described above, RABV (green) replication was visualized, cell nuclei were stained with Hoechst (blue). Results shown are a representative example of 3 independent experiments.

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