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Effect of different selenium sources and levels on porcine circovirus type 2 replication in vitro

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Abstract

Porcine circovirus type 2 (PCV2) has been linked to several disease syndromes during the last decade. A deficiency in selenium has also been associated with the increases of virulence of some viruses and severity of infectious disease. In order to evaluate the effect of different selenium sources and levels on PCV2 replication in PK-15 cells, three selenium sources, i.e. sodium selenite, kappa-selenocarrageenan and DL-selenomethionine at concentrations of 0, 2, 4, 8, and 16 μmol/L were used throughout this experiment. PCV2 loads in PK-15 cells were measured by a newly developed real-time quantitative PCR. A significantly inhibitive effect of DL-selenomethionine on PCV2 replication in vitro was demonstrated and the inhibition was concentration dependent within the range of 2–16 μmol/L. The inhibitive effect of DL-selenomethionine on PCV2 replication may be caused by enhanced activity of glutathione peroxidase. Our results may serve as a basis for further studies of the biological function of selenium and control of PCV2 infection.
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Introduction

Selenium is a nutritional essential trace element for all mammalian species including humans. A substantial body of research has also defined an important role for selenium in both antioxidant defense and immune function [1]. Selenium is found in varying quantities in the rocks and soils of different regions of the world. In these areas, this is reflected in the differing amounts of selenium in forage crops and diets and in the animals and humans that consume locally produced foods. There is much debate as to whether the modification of selenium status of humans can be associated with an altered incidence and/or increased susceptibility to many diseases [2]. For example, the intensity of infection with influenza is also enhanced by selenium deficiency [3], and selenium deficiency can alter chemokine and cytokine expression in viral infections [4]. Selenium supplementation suppresses TNF-α-induced HIV replication in culture [5]. More recently, selenium has been shown to inhibit activation of HIV-1 in cell culture through oxidative stress [6].

Porcine circovirus type 2 (PCV2) is associated with postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis nephropathy syndrome (PDNS) [7,8], pregnant sow reproductive obstruction [9], porcine respiratory disease complex (PRDC) [10], and congenital tremor type A2 (CT) [11]. Some researchers suggested that PCV2 infection might cause immunosuppression [12–15].

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A deficiency in selenium has also been associated with the increases of virulence of some viruses and severity of infectious disease [2,16]. However, so far an effect of selenium on PCV2 infection has not been reported. In addition, there have been few papers published about effects of different selenium sources including organic and inorganic selenium on viral replication. The objective of this study was to evaluate the effect of selenium sources and levels on PCV2 replication in PK-15 cells by a newly developed real-time quantitative PCR.

Materials and methods

Cells and viruses

PK-15 cells (porcine kidney) and BHK-21 cells (baby hamster kidney) were provided by China Institute of Veterinary Drug Control and propagated at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium and MEM, respectively, supplemented with 10% FBS, penicillin (20 µg/mL), streptomycin (20 µg/mL), referred hereafter as the culture medium.

The wild-type PCV2 (PCV2NJ2002) used in the experiment was isolated originally from a kidney tissue sample of a pig with naturally occurring PMWS. The determination of PCV type was performed by sequencing (TaKaRa Biotech Co. Ltd., Dalian). Porcine circovirus type 1 (PCV1) used in the study was isolated from PK-15 cells contaminated with PCV1. Pseudorabies virus (PRV) and porcine parvovirus (PPV) used in the study as negative reference were supplied by China Institute of Veterinary Drug Control.

PCV2 culture

PCV2 stocks were generated from PK-15 cells infected with PCV2 according to the following procedure: PK-15 cells were infected with PCV2 at a titer of $10^{3.5}$ $TCID_{50}$/mL, when cells had reached approximately 40–50% confluence. After 1 h absorption, the inoculum was removed, and the cell monolayer was washed three times with phosphate-buffered saline (PBS). RPMI 1640 medium including 2% FBS, penicillin (20 µg/mL) and streptomycin (20 µg/mL), was subsequently added, and incubation was continued at 37 °C with 5% CO_2 for 72 h. Then the infected cells were subcultured at a 1:3 ratio in RPMI 1640 medium. PCV2 was then serially passaged in PK-15 cells. The virus harvested at each passage was stored at −80 °C. PPV and PRV were propagated, respectively, in PK-15 cells and BHK-21 cells.

DNA extraction

Infected cells were centrifuged for 5 min at 10,000g in a table-top centrifuge, the supernatant was collected and extracted using a Genomic Mini-prep Kit (Generay Biotech Co. Ltd., Shanghai) according to the manufacturer's instructions. Briefly, 200 µL extract was supplemented with 20 µL QIAGEN protease, 4.0 µL RNase and 200 µL buffer AL. After vortexing for 15 s, the mixture was incubated at 56 °C for 10 min. Then, it was supplemented with 200 µL absolute ethanol and was carefully applied onto a QIAamp spin column within a 2 mL collection tube. It was sequentially supplemented with 500 µL buffer AW1 and AW2 after each centrifugation. Then, the mixture was supplemented with 200 µL buffer AE (elution buffer) after centrifugation at full speed (20,000g). It was incubated at room temperature for 5 min and then centrifuged at 6000g for 1 min.

Real-time PCR for quantification of PCV2

The real-time PCR for quantification of PCV2 [17] was performed in a PTC-100 system (MJ Research) using SYBR Green 1 (TakaRa, Dalian). The following primers were used for PCV2 quantitation: PCV2f, 5-TAGTATTCAAAGGGCACAGAG   -3; PCV2r, 5-GTAGTCAAAACGCCCTCCTG -3. A region of 108 bp was amplified from the PCV2 ORF2 gene. The best results were obtained with 25 µL reaction mixture containing 2 µL DNA template, 2.4 µL $MgCl_2$ (4 mmol/L, TaKaRa, Dalian), 0.2 µmol/L forward primer, 0.2 µmol/L reverse primer, and 1 µL SYBR Green I mixture. The thermal profile for real-time PCR was 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, 56.3 °C for 5 s and 72 °C for 10 s. In each run, negative reference (DNA of PPV, PCV1 and PRV) and blank samples were tested along with the positive samples.

For a standard curve, 10-fold dilutions of recombinant pMD19-ORF2 plasmid [17] were made in order to give 10^6–10^1 copies/µL. The dilutions were stored at −20 °C, while stock plasmid was stored at −70 °C.

Selenium treatment and assessment of cell toxicity

In order to assess whether any inhibitive effect of selenium on PCV2 replication was the result of selenium-induced host cell toxicity, we examined the effect of selenium on PK-15 cells. The experiment was conducted in a 3×5 factorial arrangement in a randomized complete block design. Three selenium sources, i.e. sodium selenite, kappa-selenocarrageenan, and DL-selenomethionine (Sigma) were used throughout this experiment as selenium supplement, respectively. Before the three selenium sources were added, they were

first dissolved in RPMI 1640 medium and sterilized by double filtration (0.8 and 0.2 μm pore sizes), and then diluted with RPMI 1640 medium including 2% FBS, penicillin (20 μg/mL), and streptomycin (20 μg/mL) to reach a final concentration of 0, 2, 4, 8, and 16 μmol/L, respectively.

PK-15 cells were added to 96-well culture plates at a density of 1×10^5 cells/well. When reaching approximately 40–50% confluence, cells were washed three times and the culture medium was replaced by fresh medium containing selenium sources and levels as described above. Cells were then further incubated for 72 h. The viability of cells was determined by MTT (Sigma) assay.

Effect of selenium on PCV2 replication in PK-15 cells

PK-15 cells were added to 24-well culture plates at a density of 1×10^5 cells/well. When reaching approximately 40–50% confluence, the cells were infected with PCV2 at a titer of $10^{3.5}$ TCID$_{50}$/mL. After 1 h absorption, the inoculum was removed, and the infected cells were washed three times with PBS, and then supplemented with fresh culture medium containing selenium sources and levels as described above. Wells added with RPMI 1640 medium but without PCV2 served as non-infected controls. All cells were continuously incubated at 37 °C with 5% CO$_2$. After 72 h post-inoculation, the infected cells were harvested by freeze-thawing three times and the DNA was extracted as described above. PCV2 load was determined by quantitative real-time PCR.

Cellular glutathione peroxidase activity assay

Cells were infected with PCV2 and treated with selenium-containing media as described above, and the activity of GPx was determined after 72 h of treatment. Cell extracts were prepared using cell lysis buffer for Western blot and immunoprecipitation (20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L sodium pyrophosphate, 5 mmol/L β-glycerophosphate, 1 mmol/L EDTA, 0.5 mmol/L Na$_3$VO$_4$, and 1 μg/mL leupeptin). Cell lysates were centrifuged at 12,000g for 10 min at 4 °C. GPx activity (nmol NADPH/min/mL) was measured in the supernatant using a Cellular Glutathione Peroxidase Assay Kit (Beyotime Institute of Biotechnology) that measures the coupled oxidation of NADPH during glutathione reductase (GR) recycling of oxidized glutathione from GPx-mediated reduction of t-butyl peroxide. In the assay, excess GR, glutathione, and NADPH were added according to the manufacturer's instruction.

Statistical analysis

Statistical analysis of the experimental data was performed using the statistical package SPSS 8.0 for windows. Data were analyzed for establishing their significance using one-way analysis of variance (ANOVA) followed by Tukeys HSD test. Data are expressed as means $\pm$ SE. P-values of less than 0.05 were considered significant.

Results

Real-time PCR standard curve

Ten-fold serial plasmid dilutions were tested and used to construct the standard curve by plotting the plasmid copy number logarithm against the measured CT values (Fig. 1). The generated standard curve covered a linear range of $5.2–5.2 \times 10^7$ copies/μL for PCV2. Regression analysis of the linear part of the curve gave a slope coefficient of -0.32, and the linear correlation ($R2$) between the plasmid copy number logarithm and CT was 0.997. The specificity of the real-time PCR with SYBR Green I for PCV2 was also determined by testing DNA of PCV1, PPV and PRV, and none of them but the positive control showed fluorescent reporter signal.

Assessment of selenium cytotoxicity

Table 1 shows the viability of cells after 72 h of treatment with different selenium sources and levels. During treatment with sodium selenite at a concentration of 2 μmol/L, the viability of PK-15 cells apparently increased, and was not obviously affected at a concentration of 4 or 8 μmol/L. However, during treatment with sodium selenite at concentration of 16 μmol/L,
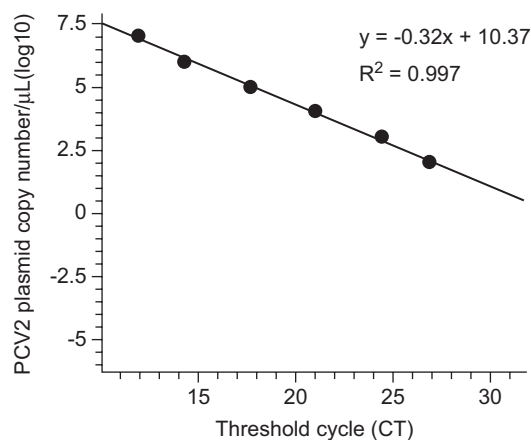

Fig. 1. Regression lines between the CT values and the input concentrations of PCV2 plasmid DNA in real-time PCR detected using SYBR Green I.

Table 1. The viability of the cells after 72 h of treatments with different selenium sources and levels

Selenium concentration (μmol/L)	Selenium sources (absorbance at OD_{570} nm)		
	Sodium selenite	Selenomethionine	Kappa-selenocarrageenan
0	1.103 ± 0.037	1.103 ± 0.037	1.103 ± 0.037
2	$1.721 \pm 0.019^*$	1.102 ± 0.03	1.118 ± 0.038
4	1.098 ± 0.032	1.063 ± 0.027	1.054 ± 0.025
8	0.792 ± 0.031	1.133 ± 0.047	1.112 ± 0.027
16	$0.352 \pm 0.098^*$	0.952 ± 0.026	0.973 ± 0.017

Values are expressed as mean $\pm$ SE, $n = 3$.
 *Indicating $P < 0.05$, versus respective control.

Table 2. Effect of different selenium sources and levels on PCV2 replication in the PK-15 cells after 72 h of treatments

Selenium concentration (μmol/L)	Viral load ($\times 10^7$ copies/μL)		
	Sodium selenite	Selenomethionine	Kappa-selenocarrageenan
0 (control)	4.2 ± 0.31	4.2 ± 0.31	4.2 ± 0.31
2	3.96 ± 0.28	3.75 ± 0.35	4.32 ± 0.38
4	3.87 ± 0.32	$2.47 \pm 0.27^*$	3.87 ± 0.33
8	3.57 ± 0.34	$1.23 \pm 0.17^*$	3.59 ± 0.36
16	3.46 ± 0.28	$0.941 \pm 0.08^*$	3.61 ± 0.27

Values are expressed as mean $\pm$ SE, $n = 3$. Control values were viral load without addition of any selenium source.
 *Indicating $P < 0.05$, versus respective control.

Table 3. Activity of GPx in the cells infected with PCV2 after 72 h of treatments with different selenium sources and levels

Selenium concentration (μmol/L)	Activity of GPx ($\times 10^2$ mu/mg)		
	Sodium selenite	Selenomethionine	Kappa-selenocarrageenan
0 (control)	1.05 ± 0.021	1.05 ± 0.021	1.05 ± 0.021
2	1.22 ± 0.18	1.98 ± 0.175	1.32 ± 0.15
4	1.65 ± 0.12	$4.30 \pm 0.35^*$	1.75 ± 0.162
8	2.01 ± 0.21	$8.16 \pm 0.52^*$	2.12 ± 0.19
16	2.21 ± 0.28	$8.89 \pm 0.611^*$	2.34 ± 0.245

Values are expressed as mean $\pm$ SE, $n = 3$. Control contains no any selenium.
 *Indicating $P < 0.05$, versus respective control.

the viability of PK-15 cells significantly decreased. During treatment with selenomethionine and kappa-selenocarrageenan in the range of concentrations used, the viability of PK-15 cells was not significantly affected.

Effect of selenium on PCV2 replication in PK-15 cells

Table 2 shows the effect of different selenium sources and levels on PCV2 replication in PK-15 cells. A concentration-dependent, significant decrease was found in cells treated with DL-selenomethionine at concentrations of 4–16 μmol/L at which no host cell toxicity was found. Sodium selenite and kappa-selenocarrageenan had only low inhibitive effects on PCV2 replication in the range of concentrations used in this study, but these differences were not statistically significant compared to controls.

Activity of GPx in cells treated with different selenium sources and levels

Table 3 shows the activity of GPx in cells treated with different selenium sources and levels. A concentration-dependent, significant increase was observed in cells treated with DL-selenomethionine at concentrations of 4–16 μmol/L. Sodium selenite and kappa-selenocarrageenan had only low enhancing effects on GPx activity, but the differences were not statistically significant compared to controls.

Discussion

The quantitative real-time PCR for PCV2 used in this study was found to be in 1- to 7-log 10 dynamic range with excellent linearity. Obviously, the quantitative real-time PCR surpassed the conventional PCR protocols, usually based on a quantitative competitive PCR, the range of which was generally limited to 4- to 6-log 10 [18]. Such a wide range of concentrations allowed us to measure viral loads in PK-15 cells treated with different selenium sources and levels.

Sodium selenite at higher concentrations (16 μmol/L) was toxic for the cells, whereas selenomethionine and kappa-selenocarrageenan (in the range of concentrations used in this study) were not. Thus, the toxicity of organic selenium (DL-selenomethionine and kappa-selenocarrageenan) was apparently much lower than that of inorganic selenium (sodium selenite), an observation consistent with previously reported data [19].

It has been reported that selenium supplementation suppresses TNF-α-induced HIV replication in culture [5], and selenium deficiency can alter chemokine and cytokine expression in viral infections [4]. The present study demonstrated for the first time that PCV2 replication in PK-15 cells can be inhibited by DL-selenomethionine in a concentration-dependent manner. The use of increased levels of vitamin E and selenium in diets of barns experiencing PCV2-associated disease has been found beneficial [20]. Our result may partly explain this observation.

A substantial body of research has also defined an important role for selenium in both antioxidant defense and immune function. Selenium is important for the control of oxidative stress and, therefore, the redox state of the cell, due to its incorporation as selenocysteine into GPx [20] and thioredoxin reductase [21]. In this study, the activity of GPx was significantly enhanced as a result of DL-selenomethionine supplementation.

Taken together, DL-selenomethionine shows a concentration-dependent anti-PCV2 activity with no or only a minor effect on host cell viability. The inhibitive effect of DL-selenomethionine on PCV2 replication is suggested to be mediated through enhanced activity of GPx that removes any hydroperoxides resulting from free radicals and protects the cell against free-radical oxidant injury [22]. These results may serve as a basis for the further study of the biological function of selenium and the control of PCV2 infection.

Acknowledgments

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