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All-trans retinoic acid regulates intracellular redox status in MARC-145 cells infected with porcine reproductive and respiratory syndrome virus

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Abstract: A relationship between oxidative stress, and apoptosis and/or death has been suggested for many years. The objective of this study was to determine the influence of all-trans retinoic acid (ATRA) on the cell survival rate and the antioxidant defense system. MARC-145 cells were treated with ATRA after infection with porcine reproductive and respiratory syndrome virus (PRRSV). The cell survival rate, total antioxidant capability (T-AOC), glutathione peroxidase (GSH-P_X) activity, superoxide dismutase (SOD) activity, lactate dehydrogenase (LDH) activity, and glutathione (GSH) content were determined in samples collected from cells incubated with ATRA for 24 or 48 h. The results show that PRRSV infection induced a marked decrease in the cell survival rate, SOD activity, GSH-P_X activity, GSH content, and T-AOC, and increased hydrogen radical and LDH activity. ATRA supplementation significantly ($P < 0.05$) increased the cell survival rate, T-AOC, SOD activity, GSH-P_X activity, and GSH content, and decreased hydrogen radical production and LDH activity, indicating that ATRA obviously strengthened the cellular antioxidant defense system, improving radical scavenging activity and regulating the intracellular dynamic equilibrium between the oxidation and antioxidation systems. Enhanced antioxidant capacity may explain why the cells treated with ATRA had increased resistance to PRRSV infection. Lower cell survival rates after PRRSV infection might have been due to impaired antioxidant capacity. The present findings suggest that the redox state may play a crucial role in the cell survival rate, and that exogenously administered ATRA may be of value in the treatment of PRRSV infection.

Key words: All-trans retinoic acid, PRRSV, antioxidant capacity

Abbreviations: PRRSV: porcine reproductive and respiratory syndrome virus; ATRA: all-trans retinoic acid; FCS: fetal calf serum; T-AOC: total antioxidant capability; SOD: superoxide dismutase; GSH: glutathione; GSH-P_X: glutathione peroxidase; LDH: lactate dehydrogenase; MDA: malonaldehyde

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a pathogen of swine that emerged in the late 1980s as the causative agent of severe reproductive failure in sows, and respiratory disease in piglets and growing pigs (1). PRRSV is a member of the family Arteriviridae in the order Nidovirales (2), and swine is its only known host. To date, it is one of the most economically important swine pathogens.

Vitamin A is one of the most widely studied nutrients in relation to immune function and disease. Vitamin A and its active metabolites can increase an

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organism's immunity. Recent evidence indicates that vitamin A supplements can enhance resistance against canine distemper virus, HIV, and measles virus, and reduce the infection and mortality rates (3). Retinoic acid (RA) is an active metabolite of vitamin A and regulates a wide range of biological processes, including cell proliferation, differentiation, and morphogenesis, as well as cell apoptosis.

An increasing body of evidence indicates that viral replication is regulated by the redox state of the host cell. It is suggested that oxidative stress induced by reactive oxygen species (ROS), especially hydrogen radicals, could play a role in the stimulation of viral replication and in the development of immunodeficiency. Excessive production of ROS can attack nucleic acid bases, amino acid side chains in proteins, and the double bonds in polyunsaturated fatty acids, thereby compromising cell integrity and function. However, cells are protected against oxidative damage by defense systems. The first line of defense is enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-P_X). The second defense system includes molecules that interact directly with free radicals to neutralize them (e.g. retinol, ascorbic acid, and α-tocopherol). Several studies have reported involvement of intracellular redox balance in the pathogenesis of viral diseases (4). Intracellular and body fluid redox alterations have been observed in mice infected with the influenza virus (5). Antioxidant substances, such as β-carotene, vitamin E, and glutathione (GSH) inhibited the replication of different types of viruses, both in vivo and in vitro (6). All these reports indicate that important steps in viral infection may be directly or indirectly dependent on the redox state of the host cell.

The objective of the present study was to elucidate the role of the intracellular redox state in the pathological progression of PRRSV infection. The study was carried out to evaluate 1) whether or not ATRA could reduce PRRSV-infected cell mortality, 2) the intracellular levels of SOD, GSH-P_X, and GSH for determining the protective effect of ATRA on cells by increasing cell antioxidation activity, and 3) the role of ATRA in hydroxyl radical production, lipid peroxidation, and cell membrane integrity following PRRSV infection based on an in vitro infection model. Based on the results, this is the first in vitro model to demonstrate that ATRA could increase the cell survival rate by enhancing antioxidant capacity following PRRSV infection.

Materials and methods

Cell culture

The MARC-145 cell line used in the present study was a gift from the pathology laboratory of Sichuan Agricultural University, Ya'an, China. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L of L-glutamine. Following virus infection the medium was switched to RPMI 1640 containing 2% FCS. Cells were seeded at 2×10^5 cells/mL and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

PRRSV strains and treatment

PRRSV strain CH-1a, the first field isolate in China, was kindly provided by Harbin Veterinary Research Institute (Harbin, China). At a titer of $10^{4.8}$, $TCID_{50}$/mL was prepared and titrated onto MARC-145 via cytopathic effect (CPE) (7). For $TCID_{50}$ assays, confluent monolayers of MARC-145 cells plated in 96-well plates were inoculated with 10-fold dilutions of the samples (8 wells per dilution) and incubated for 3 days. The total number of wells that showed a positive cytopathic effect was recorded and the $TCID_{50}$ titer was calculated.

Unless otherwise indicated, cells were infected with PRRSV at 0.1 multiplicity of infection for 90 min at 37 °C. Following infection the cells were washed and resuspended at a density of 1×10^6 cells/mL in RPMI 1640 containing a reduced concentration of FCS (2%), and 0, 0.001, 0.01, 0.1, 1, and 10 μmol/L of ATRA in a 24-well plate (1 mL/well). Infected cells were incubated for a period of 24 or 48 h in the presence of ATRA. Non-infected cells were the control in all experiments (data not shown). At 24 and 48 h after infection cells or culture supernatants were obtained and used for various assays.

Measurement of cell viability

Cell viability was determined via tetrazolium (MTT) assay. To test cell viability in the virus infection model, non-infected cells and cells infected with PRRSV were distributed at 10^4 cells/mL/well into 96-

cell plates. After 44 h of ATRA supplementation, 10 μL of the MTT labeling reagent was added to each well and incubated at 37 °C for 4 h. Then 100 μL of solubilization solution was added to each well and incubated overnight at 37 °C. The results were analyzed with an enzyme-linked immunosorbent assay (ELISA). The plates were read at a wavelength of 550 nm and a reference wavelength of 650 nm. Results are expressed as the mean of 12 different wells. The cell survival rate was calculated as follows:

OD experiment/OD control × 100%.

Measurements and analytical methods

Total antioxidant capability (T-AOC)

T-AOC was tested with commercial kits and expressed as U/mg of protein. Total antioxidative capability reflects the overall cellular endogenous antioxidative capability, including both enzymatic and non-enzymatic antioxidants. All these antioxidants can reduce Fe^{3+} to Fe^{2+}; the latter combines with phenanthroline to form colored and stable chelates.

Superoxide dismutase activity assay

SOD activity was measured in whole-cell lysate using an indirect competition assay between SOD and the indicator compound (nitroblue tetrazolium [NBT]) for superoxide produced by xanthine/xanthine oxidase, according to the method of Spitz and Oberley (8). Activity units were determined by defining 1 unit of SOD activity as the amount of sample protein capable of inhibiting the reduction of NBT by 50% of maximum inhibition. The data were normalized to protein content, as per the Bradford method.

GSH-Px activity

GSH-Px activity was measured according to the method described by Flohé and Günzler (9), based on the continuous monitoring of NADPH oxidation during the concomitant reduction of GSSG. The decrease in absorbance at 340 nm was measured. GSH-Px activity was expressed in international units per mg of cell protein, where 1 U of GSH-Px was defined as the amount of enzyme necessary to oxidize 1 μmol/NADPH/min at pH 7.0 at 25 °C.

GSH measurement

The GSH level in cell homogenates was determined using 5-5'-dithiobis (DTNB) reagent (10). The detection wavelength was 412 nm. GSH content in the sample was calculated from a standard curve obtained using known quantities of GSH normalized to protein content.

Determination of LDH activity

Released LDH activity in the culture supernatant was measured using a commercial chromatometric kit (Nanjing Jiancheng Bioengineering Institute, China). The assay was based on the modified procedure of Ulmer et al. (11). The procedure suggests using L-lactate and NAD as substrates for LDH, and monitoring the rate of production of NADH by the increase in absorbance at 340 nm. One international enzyme unit was defined as the activity of enzyme that converts 1 μmol of substrate in 1 min under standard conditions.

MDA assay

MDA content was measured using a commercial kit and the TBA method (12), which is based on the reaction of MDA with thiobarbituric acid to form a pink chromogen, expressed as nmol/mL.

Hydroxyl radical assay

Hydroxyl radical content was determined by the salicylic acid method (13). Its principle is $H_2O_2+Fe^{2+} \rightarrow \cdot OH+H_2O+Fe^{3+}$. Absorbance was measured at 510 nm and expressed as nmol/mL.

Total protein concentration was determined using the Bradford method and is expressed as mg/mL. The above parameters were analyzed in 4 independent cells, with the exception of the cell survival rate, which was analyzed in 12 different wells.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) (Statistical Analysis System [SAS] v.6.12 for Windows) and Duncan's multiple range test. A P value of less than 0.05 was considered significant. The non-infected group provided a reference for design, and the data were not statistically analyzed and are not shown.

Results

Cell survival rate

The cell survival rate in the cells after virus infection was lower than that in the control group

(data not shown). As the ATRA concentration increased, an increase in the cell survival rate was observed at 24 h. However, there was no significant difference between treatments ($P > 0.05$). Cell survival rates for all treatments decreased after an additional 24 h of infection, and ATRA increased the cell survival rate in a dose-dependent manner (Figure 1). The cell survival rate was highest when the ATRA concentration was 10 μmol/L.

The lipid peroxidation and hydroxyl radical level

ATRA treatment had no significant effect on MDA content in cell culture supernatants (Figure 2). MDA content without ATRA supplementation was 152% of 1 μmol/L of ATRA. Possible reasons may have been lower MDA content in the supernatant and an insensitive analysis method.

As shown in Figure 2, ATRA had an important effect on the scavenging of hydroxyl radicals in the cells infected with PRRSV. The hydroxyl radical level in cells decreased significantly ($P < 0.05$) as the ATRA concentration increased, and was lowest when the ATRA concentration was 10 μmol/L. However, there were no significant differences in the hydroxyl radical level in cell culture supernatants between the treatment groups. The hydroxyl radical level in the group that did not receive ATRA was higher than in the other groups.

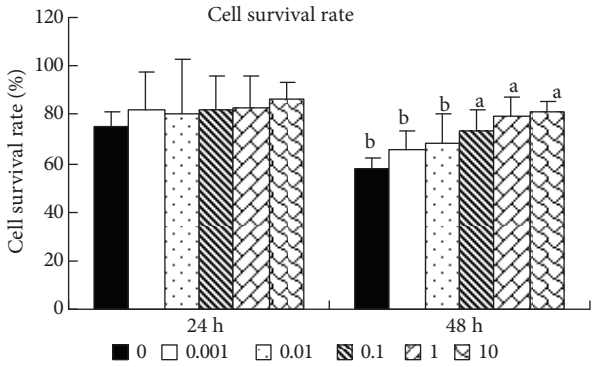

Figure 1. Cell survival rate of porcine reproductive and respiratory syndrome virus (PRRSV) infected Marc-145 cells after ATRA treatment.
Marc-145 cells were infected with PRRSV for 90 min, and then treated with 0.001, 0.01, 0.1, 1, 10 μmol/L all-trans retinoic acid (ATRA) for 20 h and 44 h. To each well was added 10 μL of the MTT labeling reagent and incubated at 37 °C for 4 h. Then 100 μL of solubilization solution was added per well and incubated at 37 °C overnight. The results were analyzed with the enzyme-linked immunosorbent assay (ELISA). Results are expressed as means ± SEM for 12 independent experiments. Different small letters denote significant differences ($P < 0.05$).
Column 1, infected cells without ATRA (control group); column 2, infected cells with 0.001 μmol/L ATRA; column 3, infected cells with 0.01 μmol/L ATRA; column 4, infected with 0.1 μmol/L ATRA; column 5, infected cells with 1 μmol/L ATRA; 6, infected cells with 10 μmol/L ATRA.

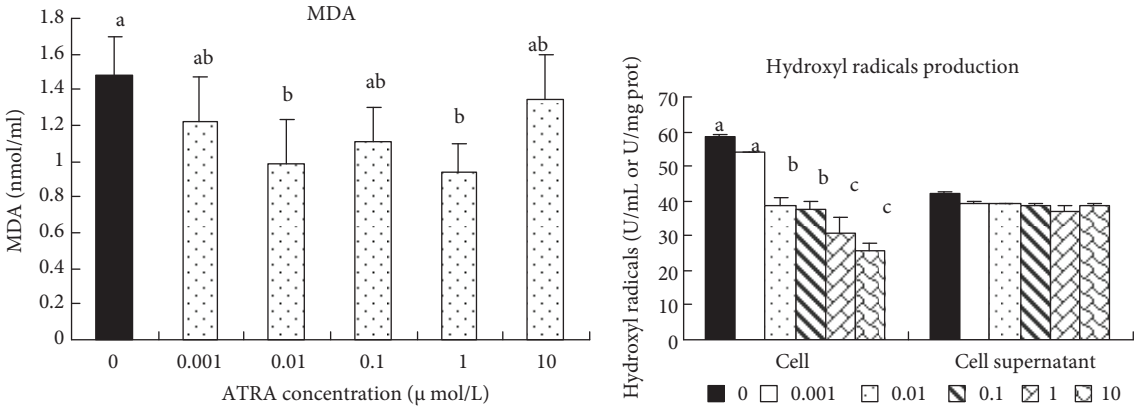

Figure 2. Effect of ATRA treatment on MDA and hydroxyl radical production.
Cells were treated with 0.001, 0.01, 0.1, 1, 10 μmol/L all-trans retinoic acid (ATRA) for 48 h after PRRSV infection. Cells and cell-free supernatants were collected from the treated cultures and the levels of MDA in supernatants and hydroxyl radical were measured. Results are expressed as means ± SEM for 4 independent experiments. Different small letters denote significant differences ($P < 0.05$).
Column 1, infected cells without ATRA; column 2, infected cells with 0.001 μmol/L ATRA; column 3, infected cells with 0.01 μmol/L ATRA; column 4, infected with 0.1 μmol/L ATRA; column 5, infected cells with 1 μmol/L ATRA; column 6, infected cells with 10 μmol/L ATRA.

The antioxidant response

The results show that ATRA supplementation at different doses increased T-AOC in the cells and culture supernatants (Figure 3). Intracellular T-AOC was highest when the ATRA concentration was 1 μmol/L, while T-AOC in the culture supernatants was highest at the 10-μmol/L concentration.

The results indicate that ATRA increased SOD activity in a dose-dependent manner 24 h after ATRA treatment (Table). Higher concentrations of ATRA continued to improve intracellular SOD activity over time. After 48 h of continuous ATRA exposure cells had a 0.99-1.26-fold increase in SOD activity, as compared to 24 h.

As shown in the Table, cellular GSH-P_X activity increased after ATRA supplementation ($P < 0.05$). Intracellular GSH-Px activity at 24 h and 48 h was highest when the ATRA concentration was 1 μmol/L. However, intracellular GSH-Px activity decreased gradually with time: at 48 h GHS-Px activity in all the treatment groups was 64%-72% of that at 24 h, while the GSH concentration significantly increased after ATRA supplementation (Figure 3). In particular, the GSH level in the 1 μmol/L of ATRA group was 2.37-fold higher than that in the non-ATRA group (75.32 ± 8.35 nmol/mg of protein vs. 31.82 ±7.03 nmol/mg of protein) ($P < 0.05$). At 48 h, the GSH level in cells dropped by 20%~40%. This could have been due to aggravation of the disease with the development of opportunistic infections, thus increasing ROS production.

Cell membrane integrity

Compared with vehicle-treated cells, ATRA-treated cell supernatant exhibited decreased LDH activity (Figure 4). The experimental data suggest that LDH activity in the culture solution greatly increased with time and that LDH activity at 48 h was 2.1-2.9-fold higher than that at 24 h, indicating cell membrane integrity was damaged and LDH was released to the cell supernatant. On the other hand, ATRA protected cell membrane integrity from virus attack.

Discussion

In the present study we observed that the cell survival rate increased significantly after ATRA supplementation. The increase in the cell survival rate might have been due to rapid proliferation and differentiation of cells. Additionally, the slower death rate and apoptosis velocity of the cells may have affected the cell survival rate, which might have inhibited virus replication. Research shows that ATRA inhibits cell growth (14). The increase in the cell survival rate after PRRSV infection may have been due to retarded cell apoptosis or cell death due to the action of ATRA. Generally, in PRRSV infected cells or animals a major pathological change is apoptosis (15). Recent investigations have shown that retinoic

Table. SOD and GSH-P_X activity.

ATRA concentration (μmol/L)	0	0.001	0.01	0.1	1	10
GSH-P_X (U/mg protein)						
24 h	508 ± 47^{c}	555 ± 16^{bc}	582 ± 60^{abc}	600 ± 37^{ab}	651 ± 39^{a}	621 ± 47^{ab}
48 h	365 ± 28^{b}	391 ± 15^{ab}	412 ± 35^{a}	422 ± 13^{a}	421 ± 27^{a}	418 ± 24^{a}
SOD (U/mg protein)						
24 h	16.70 ± 4.49^{c}	27.28 ± 6.21^{b}	34.39 ± 6.04^{b}	51.70 ± 5.59^{a}	62.12 ± 6.32^{a}	55.15 ± 9.19^{a}
48 h	25.16 ± 4.02^{d}	27.16 ± 4.90^{d}	50.14 ± 6.07^{c}	62.15 ± 4.06^{bc}	71.24 ± 9.83^{ab}	81.38 ± 6.12^{a}

Cells were treated with 0.001, 0.01, 0.1, 1, 10 μmol/L all-trans retinoic acid (ATRA) for 24 and 48 h after PRRSV infection. Cells were collected and SOD and GSH-P_X activity were measured. Results are expressed as means ± SEM for 4 independent experiments. Mean values within variable carrying no common letters are significantly different at $P < 0.05$.

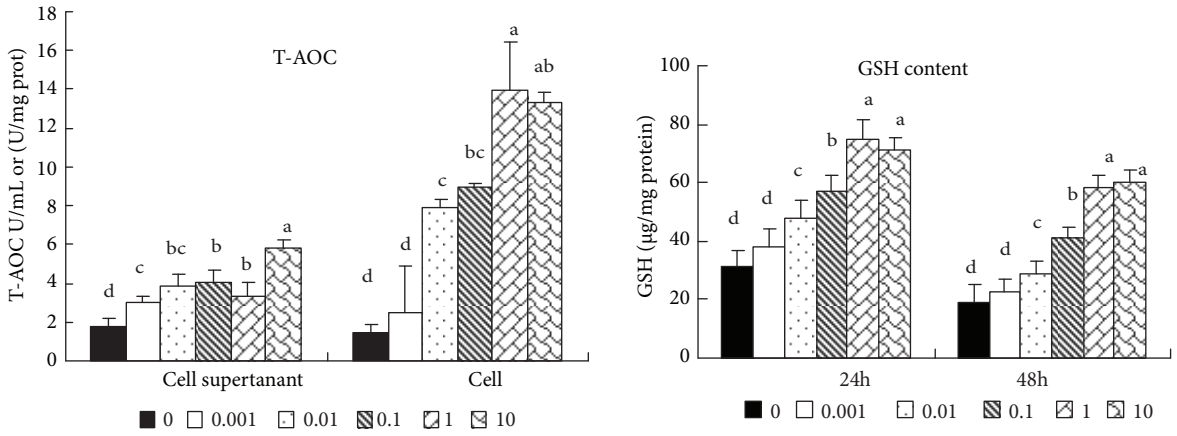

Figure 3. ATRA improved total antioxidant capability (T-AOC) and glutathione (GSH) content in cell lysates and cell supernatant.
Cells were treated with 0.001, 0.01, 0.1, 1, 10 μmol/L all-trans retinoic acid (ATRA) for 48 h after PRRSV infection. Cells and supernatants were collected from the treated cultures and T-AOC and GSH content were measured. Results are expressed as means ± SEM for 4 independent experiments. Different small letters denote significant differences ($P < 0.05$).
Column 1, infected cells without ATRA; column 2, infected cells with 0.001 μmol/L ATRA; column 3, infected cells with 0.01 μmol/L ATRA; column 4, infected with 0.1 μmol/L ATRA; column 5, infected cells with 1 μmol/L ATRA; column 6, infected cells with 10 μmol/L ATRA.

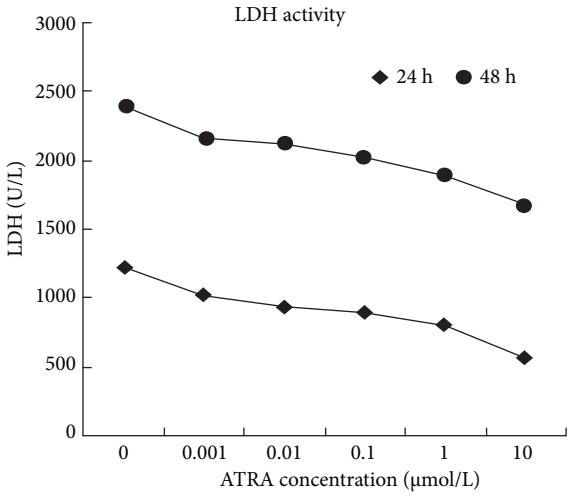

Figure 4. Effect of ATRA on lactate dehydrogenase (LDH) activity in supernatant.
Cells were treated with 0.001, 0.01, 0.1, 1, 10 μmol/L all-trans retinoic acid (ATRA) for 24 and 48 h after PRRSV infection. Cell-free supernatants were collected and the LDH activity was measured. Results are expressed as means ± SEM for 4 independent experiments.

acid inhibits H_2O_2-induced apoptosis of mesangial cells in a dose-dependent manner (16). Nakajoh et al. (17) reported that retinoic acid prevented the decrease of cell viability and reduced apoptosis in BEAS-2B cells and A549 cells. These data suggest the novel potential of RA as an inhibitor of cell apoptosis or death. This action of ATRA was ascribed, at least in part, to suppression of the cell death pathway mediated by JNK–c-Jun/AP-1 (18), which is worthy of further investigation in a PRRSV infection model.

The intracellular redox state is the result of dynamic equilibrium between oxidant and antioxidant molecules. It can affect the success of viral replication within a host cell, and then directly or indirectly influence biochemical and cytological processes (19). T-AOC exhibited similar changes to SOD in the experiment. Supplementation with vitamin C in the diet of broilers significantly increased T-AOC activity in the blood and reduced the incidence of ascites (20).

It is thought that SOD plays a major role in the metabolism of ROS. It converts superoxide into hydrogen peroxide, which is metabolized by catalase and GSH-P_X, in synergy with GSH. This enzyme is the first line of defense against superoxide anion radicals and can be induced rapidly in some conditions, such as exposure to viruses or oxidative stress in cells. Correlate analysis shows that there was a positive correlation between SOD activity and the cell survival rate (24 h: $r = 0.897$, $P < 0.05$; 48 h: $r = 0.756$, $P < 0.05$),

which indicates the protective effect of ATRA in the PRRSV infected-cells. Research has suggested that retinoic acid inhibits the decrease in SOD activity induced by high glucose, protects neurons from oxidative stress (21), and prevents a decrease in SOD-1 and SOD-2 levels in male Sprague-Dawley rats after heart overload with pressure (22).

GSH-Px is able to destroy hydrogen peroxide and has a high affinity for hydrogen peroxide. In the present study cell GSH-Px activity increased greatly as the ATRA concentration increased. GSH-P_X activity at 48 h was lower than that at 24 h. It may be considered that GSH-Px activity first increased under lipoperoxidation, via an adaptive response, and then decreased as a result of its consumption after PRRSV infection. This could have been due to aggravation of PRRSV infection with the development of opportunistic infections, thus increasing production of ROS. A decrease in GSH-Px may lead to an elevation in reactive oxygen species and thus to further impairment of cell function. Correlated analysis shows there was a positive relationship between the cell survival rate and GSH-Px (24 h: $r = 0.825$, $P < 0.05$; 48 h: $r = 0.809$, $P < 0.05$), which suggests that improvement in the cell survival rate after ATRA supplementation may be beneficial in alleviating oxidative stress. Delmas-Beauvieux et al. (23) reported GSH-P_X activity in HIV-infected patients was higher than in uninfected controls. Supplementation with selenium and vitamin E before and during parainfluenza3 virus (PI_3V) infection in lambs significantly increased blood and plasma GSH-Px activity, and the serum PIsV antibody titer (24).

GSH, a major intracellular antioxidant, is known to play a major role in regulating cell immune functions. Experiments have shown that cellular glutathione content contributes to the host cell's ability to down-regulate influenza virus replication (25). There was a positive correlation between the cell survival rate and GSH content (24 h: $r = 0.921$, $P < 0.05$; 48 h: $r = 0.885$, $P < 0.05$), which suggests that the observed improvement in the cell survival rate may have been related to GSH synthesis and cell antioxidant capacity. GSH and other antioxidants inhibited Sendai virus (6) and influenza virus (5) replication. Cells of different origin display differential permissivity for influenza A virus replication, depending on their intracellular redox power, as reflected by Bcl-2 expression and GSH content (25). Moreno-Manzano et al. (18) reported that RA could prevent H_2O_2 cytotoxicity in human renal mesangial cells by increasing GSH content. A similar result was reported for pantothenic acid, vitamin C, and vitamin E. These studies' results are similar to those of the present study.

The hydrogen radical is a very reactive ROS produced under normal conditions, but especially under pathological conditions of cell respiration. After PRRSV infection the hydroxyl radical level increased greatly, as compared to that of non-infected cells, but decreased with ATRA supplementation. Correlative analysis shows that the cell survival rate was positively correlated with hydrogen radical content at 48 h (24 h: $r = -0.573$, $P = 0.083$; 48 h: $r = -0.752$, $P < 0.05$). It is known that hydroxyl radicals initiate peroxidation of unsaturated fatty acid in cell membranes (biofilm), resulting in their rupture. Similarly, following attack by hydroxyl radicals, LDH was released into culture supernatant because of cell membrane damage or rupture. In the present study, LDH activity in cell culture supernatant was 1.42-1.76-fold higher than that in the non-uninfected group. This shows that the integrity of cell membranes was damaged after PRRSV infection. LDH is a suitable marker for following virus growth and cell permeability changes. It is suggested that the release of LDH by cells may be related to change in cell membrane integrity after ATRA treatment; however, there was no regularity in MDA content data after ATRA treatment. In mice infected with influenza virus (A/Aichi/2/68) serum MDA levels were significantly higher than those in healthy mice. Supplementation with exogenous vitamin E in mice resulted in a marked decrease in MDA levels in the blood and lungs (5).

Research has suggested that high concentrations of ROS, particularly the highly reactive hydroxyl radical, induces lipid peroxidation, DNA and RNA fragmentation, and cell membrane structure and integrity damage, and that the DNA repair capacity is related to the level of intracellular antioxidant activity (26). In the absence of a proper antioxidant system the DNA repair capacity of the cell may be altered and failure to repair DNA damage may trigger apoptosis.

Lee and Kleiboeker (27) reported that cellular apoptosis appeared after PRRSV infection and that cells treated with 9-cis-retinoic acid could protect DNA from damage by directly removing ROS (28). In the present study ATRA increased the cell survival rate after PRRSV infection. Further research is needed to assess whether there exists a relationship between the redox state and cell apoptosis after PRRSV infection and ATRA supplementation.

Conclusion

In conclusion ATRA supplementation increased the PRRSV infected-cell survival rate. This may have been due to strengthening of the endogenous antioxidant defense systems and reduced oxidative stress due to viral infection. Such studies might be useful in increasing our understanding of the influence of intracellular redox alterations on the PRRSV disease process, and the direct or indirect effects of ATRA on cell antioxidant capacity and the cell survival rate. The present study provides evidence that ATRA may be a potentially important nutrient for the prevention of PRRSV infection and/or in adjuvant therapy strategies for PRRSV infection.

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