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## ORIGINAL ARTICLE

# Photocatalytic Inactivation of Diarrheal Viruses by Visible-Light-Catalytic Titanium Dioxide

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## SUMMARY

**Titanium dioxide ($TiO_2$) that had been irradiated with visible light (VL) was demonstrated to inactivate rotavirus, astrovirus, and feline calicivirus (FCV). The virus titers were dramatically reduced after exposure for 24 hrs to the VL-catalytic $TiO_2$. The addition of bovine serum albumin could protect the virus against inactivation by VL-catalytic $TiO_2$ in a dose-dependent manner. This finding implied that the VL-catalytic $TiO_2$ products might somehow interact initially with the viral proteins in the process of virus inactivation. Moreover, we showed partial degradation of the rotaviral dsRNA genome. This was more prominent when the virus was exposed to the VL-catalytic $TiO_2$ treatment for at least 2 days. An attempt was made to elucidate the mechanism underlying the inactivation of the viruses. It was found that upon activation of $TiO_2$ with VL by using a white fluorescent lamp, the reactive oxygen species such as superoxide anions ($O_2^-$) and hydroxyl radicals (·OH) were generated in a significant amount after stimulation for 8, 16, and 24 hrs. We therefore assume that virus inactivation by VL-catalytic $TiO_2$ might occur through the generation of $O_2^-$ and OH followed by damage to the viral protein and genome. This is the first report, to the best of our knowledge, demonstrating the inactivation of rotavirus, astrovirus and FCV by the presence of $TiO_2$ film under VL as well as describing its mechanism. (Clin. Lab 2007;53:413-421)**

## KEY WORDS

diarrheal virus; photocatalysis; reactive oxygen; Ti $O_2$; visible light

## INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide, especially in infants and young children. Acute gastroenteritis is consistently ranked as one of the top causes of death in children [1-3]. The mortality among children due to acute gastroenteritis is greater in developing rather than in developed countries [3, 4]. Every year, approximately 3 to 6 million children die from diarrhea in Asia, Africa, and Latin America [5].

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Many different viruses can cause gastroenteritis, including rotavirus, adenovirus, sapovirus, astrovirus, and norovirus [1]. Among enteropathogenic viruses, rotavirus is recognized as the major etiologic agent of acute gastroenteritis in infants and children [6-8]. Rotavirus is a genus of the *Reoviridae* family with a particle size of 60 to 80 nm in diameter. Its genome consists of 11 double stranded RNA (dsRNA) segments, which encode six structural and six non-structural proteins. The non-structural proteins are involved in genome replication, the assortment of genome sets and the regulation of gene expression. The structural proteins form a triple-layered capsid around the dsRNA genome [6]. Despite much progress in understanding the pathogenesis and management of diarrheal illness with the widespread use of oral rehydration therapies, it remains one of the most important causes of global childhood mortality and morbidity [2-4].

Astrovirus is named so to describe the distinctive five- or six-pointed star visible on particles when viewed under an electron microscope. It is a small non-enveloped virus with a particle size of 28 nm. The virus particle contains a positive sense single stranded RNA genome with a single layered capsid protein. This virus has three open reading frames (ORFs), ORF1a, ORF1b, and ORF2. Moreover, ORF1a and ORF1b encode the viral protease and polymerase, respectively, whereas ORF2 encodes the capsid protein precursor [9]. Human astrovirus is classified into 8 serotypes (1 to 8), of which serotype 1 is the most common [10, 11].

Norovirus, a member of the human calicivirus genus, is another principal cause of nonbacterial acute gastroenteritis in all age groups and has been identified as an etiologic agent of waterborne outbreaks worldwide [12-14]. The virus particle ranges from 27 to 40 nm in diameter and has a single-stranded RNA genome with an icosahedral capsid structure [15]. There is no known animal or mammalian cell culture system that can be used for the cultivation of human norovirus. Because feline calicivirus (FCV) has a genome organization and capsid architecture similar to the human norovirus and can be easily grown in a cell culture, it has been used as a surrogate for the study of norovirus inactivation [16-18].

Since photoelectrochemical disinfection with platinum-doped titanium dioxide ($TiO_2$) was first introduced almost 20 years ago [19], many studies have utilized the strong oxidizing power of $TiO_2$ photocatalysts to purify water and air of environmentally toxic substances [20-22]. Furthermore, $TiO_2$ photocatalysts have also been applied to inactivate bacteria, phages, and cancer cells [23-25]. Hydroxyl radicals generated from the photocatalyst reactions were considered to play a significant role in microbial inactivation [26]. When water containing toxic substances or bacteria were treated by photocatalysis, a fine $TiO_2$ powder and strong light such as a mercury lamp were utilized. However, this system requires the recovery of $TiO_2$ power and a high cost of light. In most studies with the application of $TiO_2$ to inactivate microorganisms, the light source was an ultraviolet (UV) lamp or a black light lamp [27, 28]. In this study, we used a visible-light (VL) responsive $TiO_2$ instead of a conventional UV-catalytic $TiO_2$, whose mechanism of chemical photocatalytic degradation of viruses has been well documented. The VL-catalytic $TiO_2$ can be applied indoors as well as under sunlight. Therefore, the VL irradiation is more convenient, economical and safe for the inactivation of diarrheal viruses than UV catalysis. In addition, there is no available report on photocatalytic inactivation of enteropathogenic viruses.

Thus, the objective of this study was to investigate the role of complex photooxidants such as superoxide anions ($O_2^-$) and hydroxyl radicals (·OH) in the inactivation of rotavirus, astrovirus, and FCV under VL irradiation. In addition, the mechanism of this inactivation process is described.

## MATERIALS AND METHODS

### Cell culture and viruses

The cell lines used in this study were: African green monkey kidney (MA104) cells, human colon carcinoma (Caco-2) cells and Crandell's feline kidney (CrFK) cells. MA104 cells and Caco-2 cells were cultured in Eagle's minimal essential medium (Eagle's MEM) supplemented with 10% fetal calf serum (FCS), 0.03% glutamine, and 0.12% $NaHCO_3$. The CrFK cells were cultured in MEM supplemented with 8% FCS, 0.03% glutamine, and 0.12% $NaHCO_3$. All cell cultures were maintained at 37°C in a humidified 5% $CO_2$ atmosphere incubator.

The following viruses were used: human rotavirus strain Odelia, a simian rotavirus strain SA11, human astrovirus serotype 1 (HAstV-1), and FCV strain F4. The Odelia and SA11 strains were propagated in MA104 cells in the presence of 2 μg/ml acetyltrypsin, while the HAstV-1 was propagated in Caco-2 cells with 5 μg/ml acetyltrypsin, and FCV was propagated in CrFK cells. All viruses were harvested from the cultured supernatants after two freeze-thaw cycles. The virus titers of Odelia, HAstV-1 and FCV were estimated by the neutralization test and expressed as 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) by the Reed-Muench method as described [29]. The SA11 virus titer was determined by a plaque assay and the titer was expressed as plaque forming units per ml (pfu/ml) [30].

### $TiO_2$ film and chemicals

$TiO_2$ thin film was prepared from $TiO_2$ particles (EX-101, 5 g/liter; ECODEVICE Co., Saitama, Japan) by dip-coating on a nitoflon sheet (66 μg/cm$^2$). $TiO_2$ can absorb visible light of between 400 and 600 nm and show high activity [31]. Bovine serum albumin (BSA) globulin free grade and the other chemicals used in this study were all purchased from Wako, Ltd. (Osaka, Japan).

### Light source and apparatus

Virus suspensions on $TiO_2$ film were irradiated with a 27-watt white fluorescent lamp (WL; model FPL27EX-N, Sanyo Electric Co. LTD., Osaka, Japan), which emitted light with wavelengths in the range of 400 to 720 nm. The cultured plate was placed 27 cm under the light source. The light intensity used in this study was 2 900 ± 100 lx, as measured by an illuminometer (Model 3423, Hioki E E Co. LTD., Nagano, Japan). The reaction temperature was controlled at 30 °C.

**Table 1: Inactivation of human rotavirus (Odelia), simian rotavirus (SA11), human astrovirus (HAstV-1), and feline calicivirus (FCV) by VL-catalytic $TiO_2$ film**

| Treatment | Odelia | | SA11 | | HAstV-1 | | FCV | |
|---|---|---|---|---|---|---|---|---|
| | $Log_{10}$ $TCID_{50}$ | $Log_{10}$ reduction | $Log_{10}$ PFU | $Log_{10}$ reduction | $Log_{10}$ $TCID_{50}$ | $Log_{10}$ reduction | $Log_{10}$ $TCID_{50}$ | $Log_{10}$ reduction |
| No | 3.40 ± 0.05 | 0 | 5.15 ± 0.09 | 0 | 4.54 ± 0.08 | 0 | 3.75 ± 0.05 | 0 |
| WL | 3.10 ± 0.03 | 0.30 | 4.27 ± 0.03 | 0.88 | 4.26 ± 0.09 | 0.28 | 3.25 ± 0.09 | 0.50 |
| $TiO_2$ | 3.10 ± 0.03 | 0.30 | 4.27 ± 0.04 | 0.88 | 4.24 ± 0.09 | 0.30 | 3.57 ± 0.08 | 0.18 |
| $TiO_2$ + WL | 1.90 ± 0.03 | 1.50 | 2.37 ± 0.03 | 2.78 | 2.12 ± 0.08 | 2.42 | 1.80 ± 0.09 | 1.95 |

Odelia ($10^{3.4}$ $TCID_{50}$/ml), SA11 ($10^{5.19}$ pfu/ml), HAstV-1 ($10^{4.61}$ $TCID_{50}$/ml) and FCV ($10^{4.80}$$TCID_{50}$/ml) suspended in MEM were treated with either WL-irradiation or $TiO_2$ film alone, VL-catalytic $TiO_2$ film with WL-irradiation, or non-treated at 30°C for 24 hrs. After treatment, the virus suspensions were assayed for the surviving virus titers.

**Table 2: Protection of human rotavirus (Odelia), simian rotavirus (SA11) and feline calicivirus (FCV) from inactivation by VL-catalytic $TiO_2$ film**

| BSA (mg/ml) | $Log_{10}$ $TCID_{50}$ (Odelia) | $Log_{10}$ PFU (SA11) | $Log_{10}$ $TCID_{50}$ (FCV) |
|---|---|---|---|
| 0 | 1.90 ± 0.02 | 2.37 ± 0.03 | 1.80 ± 0.08 |
| 0.1 | 2.20 ± 0.02 | 2.90 ± 0.02 | 2.40 ± 0.09 |
| 1 | 2.80 ± 0.04 | 3.95 ± 0.04 | 2.83 ± 0.09 |

Odelia ($10^{3.4}$ $TCID_{50}$/ml), SA11 ($10^{5.19}$ pfu/ml) and FCV ($10^{4.80}$$TCID_{50}$/ml) suspended in MEM were treated with VL-catalytic $TiO_2$ and WL-irradiation in the absence or presence of various amounts of BSA (0.1 to 1 mg/ml) at 30°C for 24 hr. After treatment, the virus suspensions were assayed for the surviving virus titers

## Inactivation of the viruses

The virus suspensions (SA11 with $10^{5.19}$ pfu/ml, Odelia with $10^{3.40}$ $TCID_{50}$/ml, HAstV-1 with $10^{4.61}$ $TCID_{50}$/ml, and FCV with $10^{4.80}$ $TCID_{50}$/ml) of 200 µl each, were added to a dish (15 mm in diameter) in which a sheet of $TiO_2$-coated film (whole $TiO_2$ film, 176 $mm^2$) had been placed on the bottom. The dishes were then exposed to WL at 30 °C for 24 hrs and the virus suspensions were assayed for the surviving virus titer. All experiments were conducted in triplicate.

## Effect of BSA on virus inactivation by VL-catalytic $TiO_2$

In order to investigate whether the inactivation of the viruses by VL-catalytic $TiO_2$ could be reversed by BSA, 200 µl of Odelia ($10^{3.40}$ $TCID_{50}$/ml), SA11 ($10^{5.19}$ pfu/ml), and FCV ($10^{4.80}$ $TCID_{50}$/ml) suspensions were treated with VL-catalytic $TiO_2$ in the absence or presence of two concentrations (0.1 and 1 mg/ml) of BSA and WL irradiation at 30 °C. After incubation for 24hrs, the titer of the surviving viruses was determined. All experiments were conducted in triplicate.

## Analysis of rotavirus RNA by SDS-PAGE

The genomic dsRNA of rotavirus extracted by using phenol/chloroform extraction techniques was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as previously described [32]. Briefly, 20 µl of dsRNA were mixed with 5 µl of bromophenol blue and run in 10% polyacrylamide gel with a 4% polyacrylamide stacking gel in Tris-glycine buffer (25 mM Tris and 192 mM glycine, pH 8.3) containing 3.5 mM SDS. After electrophoresis at 30 mA for 2 hrs, rotavirus RNA was visualized by staining with silver nitrate. The polyacrylamide gel was scanned directly and the bands were analyzed using the Scion Image for Windows (Scion Image version 4.0.2, Scion Co., MD, USA).

## Determination of superoxide anions ($O_2^-$) and hydroxyl radicals (·OH)

The amount of $O_2^-$ was determined according to the method described [33]. Briefly, $TiO_2$-film was submerged in 5 ml of MEM phenol red free PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, 10 µM nitroblue-tetrazolium hydrochloride (NBT), and 0.1 mM EDTA. It was

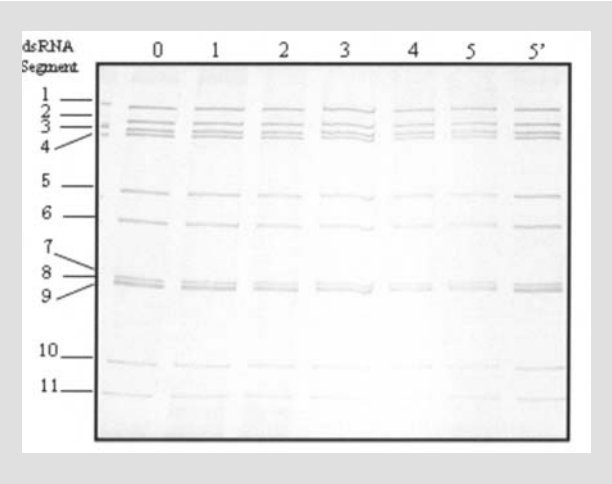


**Figure 1: The SDS-PAGE of genomic dsRNA of simian rotavirus strain SA11 with or without VL-catalytic $TiO_2$ and WL-irradiated treatment. Lane 0, electrophoretic pattern of genomic dsRNA extracted from intact virus particles. Lanes 1-5, electrophoretic pattern of genomic dsRNA extracted from rotavirus particles after exposure to the treatment for 1, 2, 3, 4, and 5 days, respectively. Lane 5, electrophoretic pattern of genomic dsRNA extracted from rotavirus particles that were kept for 5 days in the same condition without treatment.**

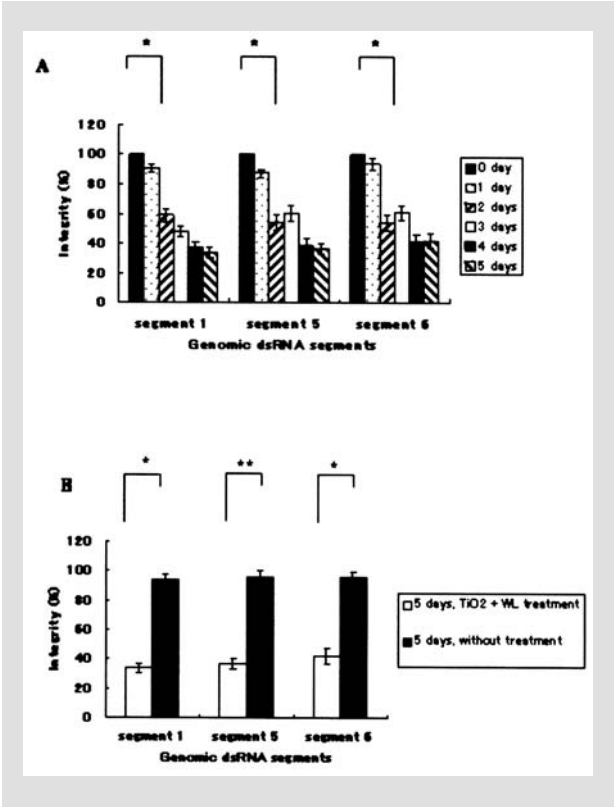


**Figure 2: The integrity of segments 1, 5, and 6 of the genomic dsRNA of simian rotavirus strain SA11 after treatment with VL-catalytic $TiO_2$ and WL-irradiation. *$p$< 0.01 (A) Percentage integrity of segments 1, 5, and 6 of the genomic dsRNA with or without exposure to the treatment for 1, 2, 3, 4 and 5 days. (B) Percentage integrity of segments 1, 5, and 6 of the genomic dsRNA on day 5 with or without treatment. *: $p$< 0.001, **: $p$=0.0001**

then irradiated with WL at room temperature for 0, 8, 16, and 24 hrs, respectively. The density of blue formazane on the film was determined by Scion Image analyzer software. The amount of hydroxyl radicals (·OH) was determined as previously described [34]. Briefly, $TiO_2$-film was submerged in 5 ml of MEM PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, 0.05 mM p-nitrosodimethylaniline, and then irradiated at room temperature for 0, 8, 16, and 24 hrs, respectively. The bleaching level of p-nitrosodimethylaniline was determined by measuring the OD (optical density) of the reaction mixture at 440 nm.

## Statistics

Student's t-tests (independent t-test or paired t-test) were performed. If the $p$ value was less than 0.05, the groups were considered to be significantly different.

## RESULTS

### Inactivation of Odelia and SA11 rotaviruses, HAstV-1, and FCV by VL-catalytic $TiO_2$

The viruses Odelia, SA11, HAstV-1, and FCV were treated with WL-irradiation alone, $TiO_2$ alone, VL-catalytic with WL-irradiation, or non-treated at 30 °C for 24 hrs. After treatment, the virus suspensions were assayed for the virus titers. The results shown in Table 1 indicate that the titers of the viruses in the suspensions remained almost the same (not significantly different) regardless of whether no treatment was given, or whether they were treated with WL-irradiation alone, or $TiO_2$ alone. The data showed that treatment of the viruses with WL-irradiation alone or $TiO_2$ alone had no effect on the virus. However, when the viruses were treated with $TiO_2$ and WL-irradiation, the titers of the viruses were dramatically reduced. The titer of Odelia was reduced by about 1.50 log from $10^{3.4}$ to $10^{1.9}$ $TCID_{50}$/ml, while the titer of SA11 was reduced by about 2.78 log from $10^{5.15}$ to $10^{2.37}$ pfu/ml. In addition, the titer of HastV-1 was reduced about by 2.42 log from $10^{4.61}$ to $10^{2.12}$ $TCID_{50}$/ml, whereas the titer of FCV was reduced by about 1.95 log from $10^{3.75}$ to $10^{1.80}$ $TCID_{50}$/ml. Taken together, these data clearly indicated that the VL-catalytic $TiO_2$ with WL-irradiation could dramatically inactivate the Odelia, SA11, HastV-1, and FCV.

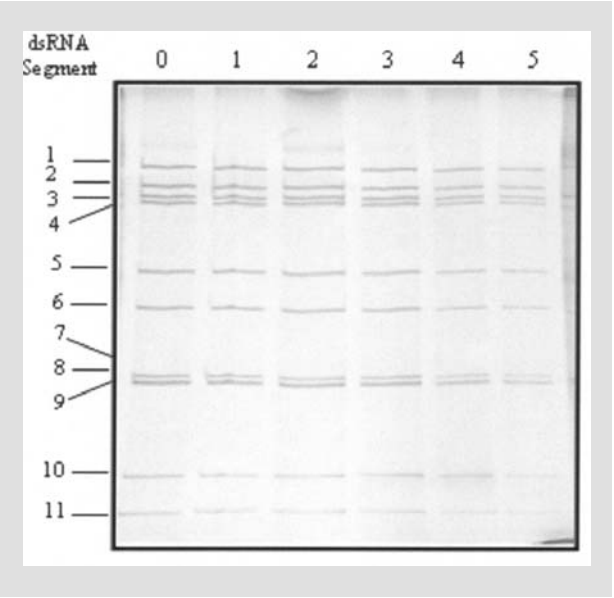


**Figure 3: SDS-PAGE of the genomic dsRNA of simian rotavirus strain SA11 treated with VL-catalytic $TiO_2$ and WL- irradiation in the presence or absence of BSA. Lane 0, electrophoretic pattern of genomic dsRNA extracted from intact rotavirus particles. Lanes 1-5, electrophoretic pattern of genomic dsRNA extracted from rotavirus particles after exposure to the treatment for 1, 2, 3, 4 and 5 days, respectively.**

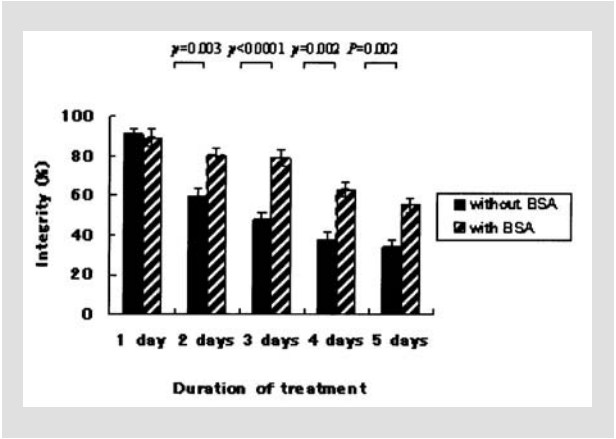


**Figure 4: The integrity of the genomic dsRNA (segment 1) of simian rotavirus strain SA11 after treatment with VL-catalytic $TiO_2$ and WL-irradiation in the presence or absence of BSA (1 mg/ml) for 1, 2, 3, 4, and 5 days.**

## Protection of viruses by BSA against inactivation by VL-catalytic $TiO_2$ with WL-irradiation

In order to elucidate the underlying mechanisms of how the VL-catalytic $TiO_2$ inactivated the viruses, we hypothesized that the effect might take place on the capsid protein of the viruses. To test this hypothesis, the viruses Odelia, SA11, and FCV were treated with VL-catalytic $TiO_2$ and WL-irradiation in the absence or presence of different concentrations of BSA (0.1 and 1 mg/ml) at 30 °C for 24 hrs. After treatment, the titers of the surviving viruses were determined. The results shown in Table 2 revealed that in the absence of BSA the virus titer of Odelia was reduced from $10^{3.40}$ to $10^{1.90}$ $TCID_{50}$/ml, while in the presence of BSA at 0.1 and 1 mg/ml the titer of the surviving virus was increased from $10^{1.90}$ to $10^{2.20}$ and $10^{2.80}$ $TCID_{50}$/ml, respectively. This protective effect of BSA was also observed in the SA11 and FCV viruses. In the absence of BSA, the titer of the virus was reduced from $10^{5.15}$ to $10^{2.37}$ pfu/ml, while in the presence of BSA at 0.1 and 1 mg/ml the titer of surviving viruses was increased from $10^{2.37}$ to $10^{2.90}$ and $10^{3.95}$ pfu/ml, respectively. Furthermore, for FCV in the absence of BSA, the virus titer was reduced from $10^{4.80}$ to $10^{1.80}$ $TCID_{50}$/ml, whereas in the presence of 0.1 and 1 mg/ml of BSA the virus titer was increased from $10^{1.80}$ to $10^{2.40}$ and $10^{2.83}$ $TCID_{50}$/ml, respectively. Altogether, the data demonstrated that virus inactivation by VL-catalytic $TiO_2$ could be abolished by BSA in a dose dependent manner.

## Analysis of rotavirus RNA by SDS-PAGE

In order to determine whether the RNA genome inside the rotavirus particles could be damaged by VL-catalytic $TiO_2$, the viral genome was extracted from rotavirus particles after inactivation with VL-catalytic $TiO_2$ and WL irradiation and then analyzed by SDS-PAGE. It was found that all 11 segments of the rotavirus genome were partially degraded, which was indicated by the intensity of the RNA bands, compared to those of the intact rotavirus (Figure 1). Then, the Scion Image Analyzer Software was used to analyze the intensity of dsRNA segment bands. Segments 1, 5, and 6 of SA11 genomic dsRNA were chosen for this analysis. The results shown in Figure 2A revealed that after treatment with VL-catalytic $TiO_2$ and WL-irradiation for 2 days, the intensity of segments 1, 5, and 6 of SA11 genomic dsRNA was decreased dramatically to 59.1%, 54.7% and 54.1%, respectively ($p< 0.01$). The decrease of the intensity between days 2 and 3 of treatment was not significantly different. However, on day 4 of treatment the percentage intensity of segments 1, 5, and 6 declined further to 37.5%, 39%, and 41.6%, respectively.

It might be possible that the decrease of the intensity of genomic dsRNA bands observed might have been due to auto-degradation of the genomic RNA molecule rather

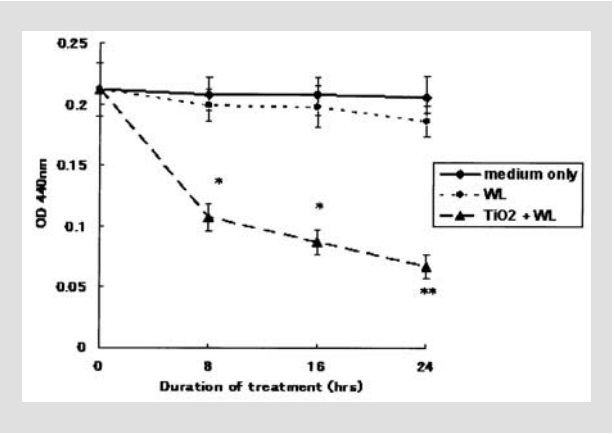


**Figure 5: Generation of ·OH from VL-catalytic $TiO_2$ film after irradiation with WL in an aqueous medium. The $TiO_2$ film was submerged in 5 ml of MEM PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, and 0.05 mM p-nitrosodimethylaniline and then irradiated with WL at 30 °C. The O.D. was measured at 440 nm after exposure for 0, 8, 16, and 24 hr. *: $p$=0.001, **: $p$<0.001 (against WL)**

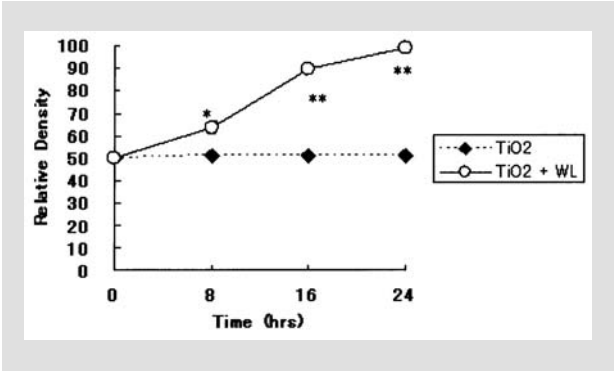


**Figure 6: Generation of $O_2^-$ on the VL-catalytic $TiO_2$ film after irradiation with WL.**
**The $TiO_2$-film was submerged in 5 ml of MEM PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, 10 µM NBT, and 0.1 mM EDTA and then irradiated with WL at 30 °C. The film was then analyzed by the Scion Image Analyzer Software to calculate the relative density of $O_2^-$. *: $p$=0.001, **: $p$< 0.001**

than the effect of VL-catalytic $TiO_2$ and WL-irradiated treatment. To solve this problem, the intensities of SA11 genomic dsRNA bands of segments 1, 5, and 6 treated with VL-catalytic $TiO_2$ and WL-irradiation were compared to those of the corresponding segments which were kept in the same condition without treatment. The results shown in Figure 2B demonstrated that the intensities of dsRNA bands of segments 1, 5, and 6 remained the same at more than 95%, while those treated with VL-catalytic $TiO_2$ were markedly decreased to 33.7%, 36.7%, and 42.4%, respectively. The data clearly indicated that the intensity decrease of genomic dsRNA bands of rotavirus was the result of VL-catalytic- $TiO_2$ and WL-irradiated treatment, and not due to auto-degradation of genomic RNA.

As shown in Table 2, BSA could reverse the inactivation of the viruses by VL-catalytic $TiO_2$ and WL irradiation: this raised the question of whether the BSA could also protect the viral RNA genome from degradation by VL-catalytic $TiO_2$ and WL-irradiated treatment. To answer this question, the genomic dsRNA (segment 1) of the SA11 strain treated with VL-catalytic $TiO_2$–WL irradiation for 1, 2, 3, 4, and 5 days in the presence of BSA (1 mg/ml) was analyzed by SDS-PAGE. The electrophoretic pattern of genomic dsRNA of simian rotavirus strain SA11 shown in Figure 3 revealed a relatively normal intensity of genomic dsRNA bands on days 1, 2, and 3. However, the intensity of the bands was low on days 4 and 5. In order to estimate the intensity of the bands more accurately, the Scion Image Software was used to analyze the intensity of the dsRNA band. Only the segment 1 band was used for this analysis.

The results shown in Figure 4 revealed that in the absence of BSA, the intensity of the genomic dsRNA electrophoretic band (segment 1), compared to that of the intact dsRNA was gradually reduced to 90.7% on day 1 and then strikingly reduced to 59.1%, 48.0%, 37.5% and 33.7% on days 2, 3, 4, and 5, respectively. In the presence of BSA (1mg/ml), the intensity of the genomic dsRNA band was recovered to 80.0% on day 2, 79.0% on day 3, 63.0% on day 4, and 55.0% on day 5. The data indicated that the intensity recovery of the dsRNA band ranged from approximately 21.0 to 31.0% ($p$< 0.01).

## Generation of hydroxyl radical from VL-catalytic $TiO_2$ film with WL-irradiation in an aqueous medium

Attempts were made to elucidate the mechanism of VL-catalytic $TiO_2$ film with WL irradiation in the inactivation of the enteropathogenic viruses by looking for the generation of ·OH, which is toxic to viruses. The results shown in Figure 5 demonstrated that VL-catalytic $TiO_2$ irradiated with WL generates ·OH by bleaching the p-nitrosodimethylaniline to decrease the OD significantly after exposure to the treatment for 8, 16, and 24 hrs (at 8 hrs and 16 hrs: $p$=0.001, at 24 hrs: $p$< 0.001). In contrast, in medium alone or medium irradiated with WL, the OD remained at the same level after 8, 16, and 24 hrs of treatment.

### Generation of superoxide anion on the VL-catalytic $TiO_2$ after irradiation with WL in an aqueous medium

Since $O_2^-$ is another radical that is toxic to microorganisms, it was interesting to investigate whether $O_2^-$ could also be generated from the VL-catalytic $TiO_2$ irradiated with WL. The generation of $O_2^-$ was indicated by the formation of blue formazane on the VL-catalytic $TiO_2$ film. Since the blue formazane could not be eluted into the aqueous medium, the intensity of the blue formazane formation on the film was determined by using Scion Image Analyzer Software. The results shown in Figure 6 demonstrated that the density of blue formazane was markedly increased to 63.4% at 8 hrs, 89.7% at 16 hrs, and 99% after 24 hrs of treatment. In contrast, the formation of the blue formazane in the VL-catalytic $TiO_2$ film not irradiated with WL remained at the baseline regardless of whether the duration of the treatment was 8, 16, or 24 hrs. The results indicated that the VL-catalytic $TiO_2$ irradiated with WL indeed generates $O_2^-$.

## DISCUSSION

Viral gastroenteritis is still a health burden in both developing and developed countries. Rotavirus and norovirus are the major causative agents of non-bacterial gastroenteritis and are associated with outbreaks as well as sporadic causes of this illness worldwide [1-4, 6, 35, 36]. The outbreak of diarrheal virus is sometimes associated with poor sanitation, especially in developing countries. Therefore, the discovery of a simple and effective method for inactivation of the viruses, particularly those contaminating environmental surfaces, would be a useful tool for the prevention of diarrheal virus transmission to humans.

The photocatalytic oxidation of $TiO_2$ occurred by electron transfer reactions and the generation of hydroxyl radicals [37]. The hydroxyl radical reacts with most biological molecules. The $TiO_2$ photocatalytic oxidation received wide attention and has been used for purification of water and clearing toxic substances from the environment [20-22]. Although the application of $TiO_2$ photocatalysts has been well established for inactivation of poliovirus and coliform bacteria [24, 38] as well as of cancer cells and other microorganisms [23, 25], there is no report on diarrheal viruses. The reactive oxygens (·OH [26] and $O_2^-$) generated from the photocatalytic reaction are considered to play a significant role in the microbial inactivation. The results of these studies were obtained from the use of UV-catalytic $TiO_2$.

This study is the first to apply photocatalytic $TiO_2$ under the activation by VL in the inactivation of viruses. The virus titers were dramatically reduced by up to 1.50-2.82 log10 after exposing the viruses to VL-catalytic $TiO_2$ for 24 hrs. It was also found that the inactivation of the viruses by VL-catalytic $TiO_2$ also continued after 24 hrs (data not shown). VL irradiation alone, or $TiO_2$ alone without VL irradiation, caused no harm to the virus, as indicated by the virus titers remaining at the same level as those without treatment. The data suggested that inactivation of the viruses by the VL-catalytic $TiO_2$ in this study is more likely caused by the radicals generated from the VL-catalytic $TiO_2$ reaction. In fact, we demonstrated that the reactive oxygen species, $O_2^-$ and ·OH, were produced when $TiO_2$ was irradiated with VL. The hydroxyl radical (·OH) has a high reactivity which makes it a very dangerous compound to the organism. It can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidetion) and amino acids (e.g. conversion of Phe to m-Tyrosine and o-Tyrosine). It reacts at diffusion rates with virtually any molecule found in its path including macromolecules such as DNA, membrane lipids, proteins, and carbohydrates. In terms of DNA, the hydroxyl radical can induce strand breaks as well as chemical changes in the deoxyribose and in the purine and pyrimidine bases. On the other hand, even superoxide anions ($O_2^-$) only exhibit mild oxidative properties. They have, however, a larger free diffusion path length and can undergo a series of interesting reactions. One of them might be dismutation to hydrogen peroxide, which then through Fenton chemistry, yields aggressive hydroxyl radicals [39]. Inactivation of diarrheal viruses required 24 hrs, which is much longer when compared to the 30 min required for the inactivation of poliovirus [38]. This might be due to the difference in the light source used in the inactivation of poliovirus 1, which was sunlight and black light with an emission spectrum of below 400nm. Therefore, the effect of disinfection may be enlarged by the inactivation ability of UV.

The electrophoretic analysis of genomic dsRNA rotavirus after treatment with VL-catalytic $TiO_2$ revealed a dramatic change in the intensity of the RNA bands, especially on days 2-5 of the treatment. The intensity reduction of electrophoretic RNA bands might be due to degradation of individual RNA segments rather than to random cleavage of the viral genome into smaller fragments, as observed in the phage PL-1 [28]. On the other hand, because of the triple-layered capsid protein structure of rotavirus, it could be possible to provide better protection of the viral genomic dsRNA from degradation by ·OH, generated from the VL-catalytic $TiO_2$. The degradation of genomic RNA segments of the rotavirus observed in this study is unlikely to have been due to the auto-degradation of the RNA molecule, since the genomic RNA kept in the same condition without treatment with VL-catalytic $TiO_2$ remained intact, with the intensity of the electrophoretic band being more than 95% of that extracted from the intact viral particles. This finding implies that the reactive oxygen species generated from VL-catalytic $TiO_2$ may somehow penetrate directly or indirectly into the capsid protein to cause damage to the genome of the virus.

The inactivation of rotavirus by VL-catalytic $TiO_2$ was partially recovered by the addition of BSA and the protection was in a dose-dependent manner. A similar observation was also found when other increasing doses of BSA such as 2 mg/ml, 3 mg/ml were tested (data not shown). The recovery rate was not completely restored, but rather stayed at about 21.0% - 31.0% no matter how long the genomic dsRNA of the rotavirus was treated with VL-catalytic $TiO_2$. It is possible that the inactivation effect exceeded the protecting effect of the BSA. On the other hand, it has been reported that the degradation of proteins occurred by phoctocatalytic $TiO_2$ with UV irradiation [38]. Our results suggested that the capsid protein might be the primary target of the reactive oxygen species in the initial phase of virus inactivation.

The light sources used in the inactivation of poliovirus 1, phage PL-1, and phage MS-2 were either a black light lamp or black-light blue lamp that emitted light with a spectrum of 300-400 nm, which was equivalent to that of the UV light [26, 28, 40]. The advantage of this study, regarding the light source, was the use of WL, which is simple and harmless to humans. The inactivation of enteropathogenic virus required a great deal of time, and was partially inhibited by the addition of BSA. As water may also be contaminated by other organic materials and micoorganisms in the real condition, the inactivation could be reduced. When considering safe long-time use of the reaction of TiO2 with VL, a potential application may be in places where there is no technical infrastructure, such as the walls of bathrooms and public facilities.

In conclusion, this is the first indication to demonstrate the inactivation of rotavirus, astrovirus and FCV by the presence of $TiO_2$ film under VL as well as describing its mechanism. This also underscores the potential as well as the importance of $TiO_2$ film in the future specific treatment of enteropathogenic virus infection.

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