Antiviral Research 93 (2012) 225–233

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Inactivation of high and low pathogenic avian influenza virus H5 subtypes by copper ions incorporated in zeolite-textile materials

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ARTICLE INFO

Article history:
Received 17 May 2011
Revised 28 November 2011
Accepted 29 November 2011
Available online 9 December 2011

Keywords:
Avian influenza virus
H5 subtype
Copper ion
Zeolite
Antiviral effect

ABSTRACT

The effect of cotton textiles containing Cu^{2+} held by zeolites (CuZeo-textile) on the inactivation of H5 subtype viruses was examined. Allantoic fluid (AF) containing a virus (AF virus) (0.1 ml) was applied to the textile (3 × 3-cm), and incubated for a specific period at ambient temperature. After each incubation, 0.9 ml of culture medium was added followed by squeezing to recover the virus into the medium. The recovered virus was titrated using Madin–Darby canine kidney (MDCK) cells or 10-day-old embryonated chicken eggs.

The highly pathogenic H5N1 and the low pathogenic H5N3 viruses were inactivated on the CuZeo-textile, even after short incubation. The titer of A/chicken/Yamaguchi/7/04 (H5N1) in MDCK cells and in eggs declined by >5.0 $\log_{10}$ and 5.0 $\log_{10}$, respectively, in 30 s. The titer of A/whooper swan/Hokkaido/1/08 (H5N1) in MDCK cells declined by 2.3 and 3.5 in 1 and 5 min, respectively. When A/whistling swan/Shimane/499/83 (H5N3) was treated on the CuZeo-textile for 10 min, the titer declined by >5.0 $\log_{10}$ in MDCK cells and by >3.5 $\log_{10}$ in eggs. In contrast, no decrease in the titers was observed on cotton textiles containing zeolites alone (Zeo-textile). Neither cytopathic effects nor NP antigens were detected in MDCK cells inoculated with the H5N1 virus treated on the CuZeo-textile. The viral genes (H5, N1, M, and NP) were amplified from the virus treated on the CuZeo-textile by RT-PCR. The hemagglutinating activity of the CuZeo-textile treated virus was unaffected, indicating that virus–receptor interactions were maintained. Electron microscopic analysis revealed a small number of particles with morphological abnormalities in the H5N3 virus samples recovered immediately from the CuZeo-textile, while no particles were detectable in the 10-min treated sample, suggesting the rapid destruction of virions by the Cu^{2+} in the CuZeo-textile. The loss of infectivity of H5 viruses could, therefore, be due to the destruction of virions by Cu^{2+}. Interestingly, $CuCl_2$ treatment (500 and 5000 μM) did not have an antiviral effect on the AF viruses (H5N1 and H5N3) even after 48 h of incubation, although the titer of the purified H5N3 virus treated with $CuCl_2$ declined greatly. The antiviral effect was inhibited by adding the AF to the purified H5N3 virus prior to the $CuCl_2$ treatment. The known antibacterial/antifungal activities of copper suggest that the CuZeo-textile can be applied at a high level of hygiene in both animals and humans.

1. Introduction

The spread of the highly pathogenic (HP) H5N1 subtype avian influenza viruses (AIVs) has caused severe poultry diseases in many countries. HP H5N1 viruses have also caused a high incidence of lethal infections in humans, which has raised global concern about their potential to induce a pandemic (Webster et al., 2006). Therefore, early elimination of the virus from affected farms is important to eliminate the chances of transmission of HP H5N1 viruses from the infected poultry to humans or from humans to humans.

Because a significant amount of AIVs is shed through respiratory secretions and feces, transmission may easily occur by direct contact between the infected and susceptible birds or by indirect contact including aerosols or exposure to virus-contaminated fomites (Swayne and Halvorson, 2008). AIVs can be introduced into farms by several routes, including infected birds, contaminated

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doi:10.1016/j.antiviral.2011.11.017

mammals, water, clothes, equipment, and delivery vehicles (Swayne and Halvorson, 2008). Biosecurity measures are, therefore, critical for preventing AIV infection and its spread among the poultry; their implementation could eliminate the chances of transmission of the virus from the infected birds. Infection with AIVs such as the H5, H7, or H9 subtypes in humans is thought to occur through the accidental aspiration of contaminated materials such as droplets or dried feces because of close contact with infected birds (Swayne and Halvorson, 2008). Therefore, the development of highly effective viricidal materials or products to prevent the transmission of viruses or to eliminate the source of infection in the environment is important.

Various kinds of materials or products have been developed with the objective of removing the source of viral pathogens; among them, copper is a promising candidate for virus inactivation. Several studies have shown that copper reduced the infectivity of the enveloped or non-enveloped DNA or RNA viruses, including influenza virus, with different intensities (Borkow and Gabbay, 2004; Borkow et al., 2007, 2008, 2010; Horie et al., 2008; Jordan and Nassar, 1971; Karlström and Levine, 1991; Noyce et al., 2007; Sagripanti, 1992; Sagripanti and Lightfoote, 1996; Sagripanti et al., 1993, 1997).

Reports describing the effect of copper or copper ions on the inactivation of influenza viruses have been limited in number. Noyce et al. (2007) reported that human H1N1 virus gets inactivated on copper surfaces, but an exposure period of 6 h was required to obtain a full effect, which was possibly due to the limited amount of Cu^{2+} released from the copper surface. Faúndez et al. (2004) demonstrated the antibacterial activity of copper surfaces against *Salmonella enterica* and *Campylobacter jejuni*, and showed that the amount of Cu^{2+} released from the surface was correlated with the time of exposure. The Cu^{2+} in $CuCl_2$ and $CuSO_4$ solutions at concentrations of 2.5–250 μM was reported to inactivate AIV (H9N2 subtype) in a time-dependent manner (within 3–6 h) (Horie et al., 2008). Borkow et al. (2007) reported that the passage of the human H3N2 virus through polypropylene filters impregnated with copper oxide particles did not result in a large reduction in the viral infectivity titers (0.8–2.5 $\log_{10}$ reduction). Recently, the same group showed that human H1N1 and avian H9N2 virus titers were not recovered from the facemasks containing polypropylene layers with copper oxide particles 30 min after the aerosolized viruses were introduced into the mask (Borkow et al., 2010).

Zeolite is a microporous aluminosilicate mineral consisting of three-dimensionally constructed tetrahedrons of SiO_4 and AlO_4 with ion exchange and adsorption capabilities. Metal ions such as Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and others can be held in the microspores of zeolites, because of its negative surface charge and ion exchange capability. A novel functional textile material (100% cotton) was developed in which zeolite A ($Na_{12}((AlO_2)_{12}(SiO_2)_{12}) \cdot 27H_2O$) was chemically synthesized on the cotton textile using the structural components (Na^+, Al^{3+} and Si^{4+}) of zeolite and then Na^+ was replaced with copper ion (Cu^{2+}) (Fig. 1a and b). The incorporated zeolite tolerates at least 1 dozen washings (as reported by the manufacturer).

The purpose of the present study was to examine the effect of Cu^{2+} held by zeolites on the inactivation of H5 subtype AIVs, and to evaluate this compound as a potent influenza control measure in the environment. In addition, the mechanisms underlying the antiviral effect of Cu^{2+} were investigated.

2. Materials and methods

2.1. Viruses

A/chicken/Yamaguchi/7/04 (Ck/Yamaguchi/7/04) (Mase et al., 2005) and A/whooper swan/Hokkaido/1/08 (Who.s/Hokkaido/1/08) H5N1 viruses were kindly provided by the National Institute of Animal Health, Japan and Dr. H. Kida, Hokkaido University, Japan, respectively. A/whistling swan/Shimane/499/83 (Whi. s/Shimane/499/83) H5N3 virus was kindly provided by Dr. T. Itho, Tottori University, Japan. The viruses was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs, and the allantoic fluid (AF) containing the virus (AF virus) was stored at −80 °C until use. In most experiments, the AF viruses were used.

The H5N3 virus was purified by ultracentrifugation at 40,000 rpm for 2 h with 30% and 60% sucrose solutions in a Himac CS-GXII micro ultracentrifuge (Hitachi High-Technologies Corporation, Tokyo, Japan). The purified virus was re-suspended in phosphate-buffered saline (PBS, pH 7.4) and stored at −80 °C until use.

2.2. Cell cultures

Madin–Darby canine kidney (MDCK) cells, which were kindly provided by Dr. H. Nagano, Hokkaido Institute of Health, Japan, were cultivated in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

2.3. Virus titration

Virus titers in the MDCK cells were quantified as the $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) by using the Behrens–Kärber method (Kärber, 1931).

For the titration of H5N3 virus in the MDCK cells, the cell growth medium was replaced with virus growth medium (VGM), which consisted of DMEM, including 25 mM HEPES, 0.01% glucose, 0.2% bovine serum albumin and trypsin (6.25 μg/ml. The inocula were removed and replaced with VGM after 1-h-long absorption at 37 °C. For the titration of H5N1 virus, VGM without trypsin (VGM(−)) was used.

In some experiments, virus titers were also measured using 10-day-old embryonated chicken eggs and were quantified as the log 50% egg infective dose (EID_{50}) as described above.

For titration, all the samples were diluted 10-fold starting at a dilution of 10.

2.4. Hemagglutination (HA) and neuraminidase (NA) tests

HA tests were conducted according to the WHO Manual on Animal Influenza Diagnosis and Surveillance (WHO Manual) by using 0.5% chicken blood cells.

NA activity was measured in microtubes by incubating 20 μl of the viral suspension with 20 μl of fetuin (Sigma, St. Louis, MO) at 37 °C for 18 h. The tubes were cooled, and then, 20 μl of periodate reagent were added to each tube, followed by shaking and incubation at room temperature for 20 min. The reaction was stopped by the addition of 200 μl of arsenite reagent. Then, 500 μl of thiobarbituric acid reagent were added to each tube followed by 15-min incubation at 100 °C. After cooling the tubes, 600 μl of butanol were added to each tube. The amount of liberated sialic acid was chemically determined by measuring the color developed in a spectrophotometer at 549 nm.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR)

Viral RNA was extracted using a QIAamp Viral RNA kit (QIAGEN, Tokyo, Japan), according to the manufacturer's instructions. The OneStep RT-PCR kit (QIAGEN) was used to detect viral H5, N1, M, and NP genes. H5, N1, and M gene specific primer sequences are described in the document recommendations and laboratory procedures for detection of avian influenza A (H5N1) virus in

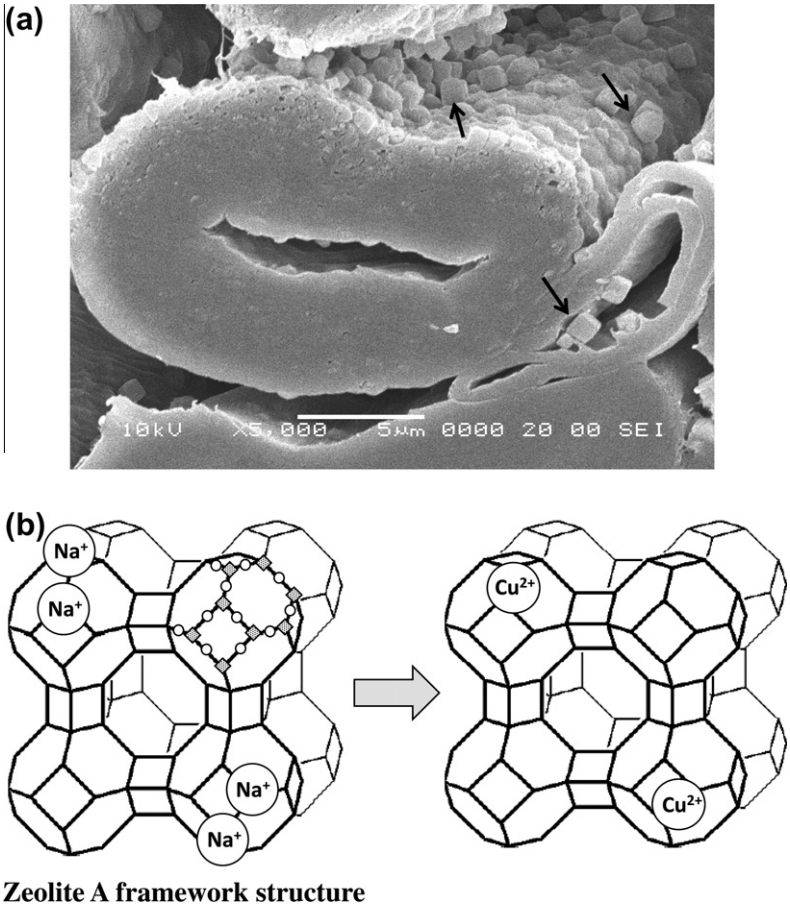

Fig. 1. (a) A scanning electron micrograph of zeolite-incorporated cotton fibers. The arrows indicate zeolite. Bar: 5 μm. Zeolite crystals chemically synthesized on the cotton textile are seen inside and outside the cotton fibers. The picture was kindly provided by Nisshinbo Holding Inc., Tokyo, Japan. (b) Zeolite A framework structure showing the position of the Si or Al atom (dotted diamond) bridged by an O atom (circle). Zeolite A is a microporous aluminosilicate ($Na_{12}((AlO_2)_{12}(SiO_2)_{12}) \cdot 27H_2O$) consisting of three-dimensionally constructed tetrahedrons of SiO_4 and AlO_4. Zeolites possess a negative charge due to the AlO_4 and Na^+ is held in the microspores. Two Na^+ are replaced with one Cu^{2+} through an ion exchange reaction. The positions of Na^+ and Cu^{2+} are shown in Figure b. The zeolite A framework structure was obtained from http://www.sist.ac.jp/~yamazaki/yamazeo.htm (accessed 10.10.11).

specimens from suspected human cases (WHO Manual). The NP gene was amplified using the primers described by Lee et al. (2001).

PCR products were electrophoresed on 1.8% agarose gels and stained with ethidium bromide.

2.6. Indirect fluorescent antibody (IFA) test

An IFA test was conducted as described previously (Bülow and Biggs, 1975). A commercially available monoclonal antibody (MAb) against the nucleoprotein (NP) of human influenza A virus (Oxford Biotechnology Ltd., UK) was used as primary antibody. Samples were observed by BIOREVO fluorescent microscope (KEYENCE, Osaka, Japan). FITC-conjugated rabbit anti mouse IgG (Rockland Immunochemicals Inc., PA) was used as a secondary antibody.

2.7. Source of copper ion (Cu^{2+})

Cotton textiles (100% cotton) incorporating synthesized zeolites with Cu^{2+} (CuZeo-textile) and zeolite alone (Zeo-textile) were used (Nisshinbo Holdings Inc., Tokyo, Japan). Cu^{2+} was present at a concentration of 0.5 g/m^2 in the CuZeo-textile.

$CuCl_2 \cdot 2H_2O$ (Wako Chemicals, Osaka, Japan) dissolved in ultrapure water at a concentration of 500 and 5000 μM was used to examine the effect of Cu^{2+} on the virus.

2.8. Treatment of the virus by CuZeo-textile and Zeo-textile

The AF virus (0.1 ml) was directly added to a piece of the textile (3 × 3-cm), and incubated at ambient temperature. Immediately or after 30 s or 1, 5, or 10 min, 0.9 ml of VGM(−) was added, followed by squeezing with a pair of forceps to recover the virus into the medium. A period of 15 s was required to squeeze and recover the medium from 1 treated textile. The treatment was performed 1 sample at a time, and the recovered virus sample from the textile was immediately diluted for titration.

To examine whether the virus is further inactivated in the medium squeezed from the CuZeo-textile during the squeezing and recovering time, a residual copper control experiment was performed. After incubating 0.1 ml VGM on the CuZeo-textile or Zeo-textile for 1 and 10 min, 0.9 ml VGM was added, followed by squeezing to recover the medium. The AF virus was then added to the recovered medium and incubated for 15 s. Samples were diluted 10-fold and inoculated onto MDCK cells for titration as described above.

The effect of Cu^{2+} (CuZeo-textile) on virus inactivation was also examined in the presence of FBS and chicken feces. The AF virus was mixed with an equal volume of 80% FBS in PBS and added to the textile, and the virus was recovered, as described above. A 20% fecal suspension in PBS was mixed with an equal volume of the virus and added to the textile, and then, the virus was

recovered in the medium, as described above. After centrifugation at 15000*g* to remove the feces, the virus was immediately titrated using MDCK cells in a 96-well microplate, as described above. The inocula in the MDCK cells were replaced with VGM 1 h after inoculation.

2.9. Cytotoxicity test

The Cytotoxicity of VGM squeezed from the CuZeo- and Zeo-textile to the MDCK cells was measured by the LDH assay using a Cytotoxicity Detection kit (Roche Applied Science, Mannheim, Germany). Briefly, the medium squeezed from the textile after a 1-min incubation was diluted 10-fold and inoculated onto the cells according to the virus titration procedure described above. The culture supernatants of the inoculated cells were examined for cytotoxicity 2 days later. The percentage of cytotoxicity was calculated by substracting the control background absorbance values from the absorbance of the experimental sample using the following equation:

$$[(\text{Experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100\%.$$

2.10. Treatment of the virus with $CuCl_2$

The AF virus (H5N1 or H5N3) was added to an equal volume of 500 or 5000 μM $CuCl_2$ and maintained for 24 and 48 h at ambient temperature. The treated virus in the mixture was titrated on the MDCK cells, as described above.

In some experiments, the purified H5N3 virus was used to examine the effect of AF on $CuCl_2$-induced inactivation of virus.

2.11. Negative staining electron microscopy (EM)

The purified H5N3 virus treated on the CuZeo-textile or Zeo-textile was recovered immediately or 10 min after its addition. The recovered virus was centrifuged at 40,000 rpm for 2 h and re-suspended in a small amount of PBS. An EM sample was prepared on a 400-mesh-carbon-coated collodion grid (NISSHIN EM Co., Ltd., Tokyo, Japan), according to the two-step method described by Chrystie (1996). The treated viruses on the grids were negatively stained for 2 min with 2% phosphotungstic acid (PTA, pH 6.5).

The grids were examined using a H7500 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.12. Histopathology and immunohistochemistry

Three embryonated chicken eggs were inoculated with a 10^2-fold dilution of the Ck/Yamaguchi/7/04 virus treated on the CuZeo-textile or Zeo-textile for 10 min. The embryos dying or surviving during the 2-day-long observation period were fixed, and the lung, liver, brain, kidney, and heart of the embryos were embedded in paraffin.

Immunohistochemical analysis was performed to detect influenza viral antigens with a Histofine Simple Stain MAX PO (M) kit (Nichirei Inc., Tokyo, Japan) according to the manufacturer's instructions. Briefly, Mab against influenza A virus matrix protein (AbD Serotec, Kidlington, UK) was used as the primary antibody. The sections were pretreated with 10 mM citrate buffer (pH 6.0) in a microwave oven at 500 W for 15 min for antigen retrieval. After quenching the endogenous peroxidase with 3% hydrogen peroxidase, the sections were incubated with the primary antibody for 30 min. After rinsing in PBS, the labeled amino acid polymers with the peroxidase and secondary antibody were added, and the samples were incubated with the substrate reagent for 5 min. After staining, the sections were counterstained with hematoxylin.

3. Results

3.1. Inactivation of the H5 subtype viruses by CuZeo-textiles

The titers of the H5 subtype AF viruses treated on the CuZeo-textile with various incubation times were measured in the MDCK cells or in eggs (Tables 1–3). Examination of the cytotoxicity of the medium squeezed from CuZeo- and Zeo-textiles to MDCK cells by the LDH assay showed no cytotoxic effect (data not shown).

As shown in Table 1, the CuZeo-textile inactivated the Ck/Yamaguchi/7/04 H5N1 virus even with short incubation times. In the experiment with the MDCK cells, virus titers were reduced by >5.0, >4.8, and >5.2 $\log_{10}$ in 30 s, 1 or 10 min, respectively. Similar results were obtained using the eggs. The inactivation effect of the CuZeo-textile was observed even when the virus was recovered immediately from the CuZeo-textile; the titer was reduced by 3.4 $\log_{10}$. The virus titers were further reduced by 5.0 and 6.0 $\log_{10}$ in 1 and 10 min, respectively. In contrast, the Zeo-textile lacking Cu^{2+} did not show an antiviral effect.

The Who.s/Hokkaido/1/08 H5N1 virus was also inactivated by the CuZeo-textile after a short incubation, with a 2.3-$\log_{10}$ reduction in the titers in 1 min (Table 2). The degree of inactivation was less significant than the effect on the Ck/Yamaguchi/7/04 H5N1 virus. However, the virus was inactivated, and the titers declined by 3.5 and >4.3 $\log_{10}$ in 5 and 10 min on the CuZeo-textile, respectively.

The titers of the Whi.s/Shimane/499/83 H5N3 virus treated on the CuZeo-textile were reduced by >5.0 $\log_{10}$ in the MDCK cells and >3.5 $\log_{10}$ in the eggs in 10 min (Table 3).

In the residual copper control experiment, the virus was incubated in the virus-free medium squeezed and recovered from the CuZeo- and Zeo-textiles to examine the antiviral effect of free-Cu^{2+} that might be eluted in the medium from the CuZeo-textile. As shown in Table 4, the titer in CuZeo- and Zeo-textile samples was not significantly different, indicating that no inactivation occurred.

3.2. Effect of FBS and feces on the inactivation effect of CuZeo-textile

The effect of FBS or feces on the inactivation of Ck/Yamaguchi/7/04 H5N1 AF virus by the CuZeo-textile was examined. FBS did not inhibit the effect of the CuZeo-textile, and the virus titer was reduced by >4.5 $\log_{10}$ compared to that on the Zeo-textile in 10 min. Similarly, in the presence of feces, the virus titers decreased by >3.3 and >3.8 $\log_{10}$ in 1 and 10 min, respectively (Table 5).

3.3. Effect of the CuZeo-textile on the H5N1 virus HA and NA activity

The HA titers of the Ck/Yamaguchi/7/04 and Who.s/Hokkaido/1/08 H5N1 AF viruses treated on the CuZeo-textile for 10 min were not reduced as compared to those of the viruses treated on the Zeo-textile or the virus control (Table 6).

On the other hand, the NA activity of the Ck/Yamaguchi/7/04 H5N1 AF virus was slightly inhibited by treatment on the CuZeo-textile for 10 min (approximately 20% reduction) compared to the virus treated on the Zeo-textile (Fig. 2). The NA activity of the virus treated on the CuZeo-textile for 30 and 60 min was reduced to a greater extent than that of the virus for 10 min, although the NA activity was not completely inhibited.

Table 1
Inactivation of the Ck/Yamaguchi/7/04 H5N1 virus by CuZeo-textile.

Incubation time[a]	Infectivity					
	Log_{10} $TCID_{50}$/ml[b]			Log_{10} EID_{50}/ml		
	Zeo-textile	CuZeo-textile	(Log_{10} reduction)	Zeo-textile	CuZeo-textile	(Log_{10} reduction)
Recovered immediately	nt[c]	nt		7.5	4.1	(3.4)
30 s	nt	<1.7	(>5.0)	8.1	3.1	(5.0)
1 min	nt	<1.9	(>4.8)	nt	nt	
10 min	6.7	<1.5	(>5.2)	8.5	2.5	(6.0)

[a] The virus was incubated on CuZeo-textile or Zeo-textile for different time intervals.
[b] Log_{10} reduction in each incubation time was calculated against virus titers obtained after a 10-min incubation on Zeo-textile.
[c] nt = not tested.

Table 2
Inactivation of the Who.s/Hokkaido/1/08 H5N1 virus by CuZeo-textile.

Incubation time[a]	Infectivity (Log_{10} $TCID_{50}$/ml)		
	Zeo-textile	CuZeo-textile	(Log_{10} reduction)[b]
Recovered immediately	nt[c]	4.8	(1.7)
1 min	nt	4.2	(2.3)
5 min	nt	3.0	(3.5)
10 min	6.5	<2.2	(>4.3)

[a] The virus was incubated on CuZeo-textile or Zeo-textile for different time intervals.
[b] Log_{10} reduction was calculated against virus titers obtained after a 10-min incubation on Zeo-textile.
[c] nt = not tested.

Table 3
Inactivation of the Whi.s/Shimane/499/83 H5N3 virus by CuZeo-textile.[a]

Type of textile	Infectivity	
	Log_{10} $TCID_{50}$/ml	Log_{10} EID_{50}/ml
CuZeo-textile	<2.0	6.0
Zeo-textile	7.0	>9.5
(Log_{10} reduction)	(>5.0)	(>3.5)

[a] The virus was incubated on CuZeo-textile or Zeo-textile for 10 min.

Table 4
Residual copper control experiment.

Type of textile	Infectivity (Log_{10} $TCID_{50}$/ml)[a] Incubation time of virus-free medium on the textile	
	1 min	10 min
CuZeo-textile	6.3	6.0
Zeo-textile	6.3	5.3

[a] The virus was added to the virus-free medium squeezed from the textile after incubation on it and titrated 15 s later. Simultaneously, the virus incubated on both textiles was also titrated. The titers after 1- and 10-min incubation were <2.3 $TCID_{50}$/ml and <1.8 $TCID_{50}$/ml, respectively.

3.4. Detection of viral genes by RT-PCR

Viral H5, N1, M, and NP genes were amplified from the Ck/Yamaguchi/7/04 AF virus treated on the CuZeo-textile by RT-PCR (Fig. 3).

3.5. Detection of cytopathic effect (CPE) and viral antigens

CPE and fluorescent NP antigens were not observed in the MDCK cells inoculated with the Ck/Yamaguchi/7/04 AF virus treated on the CuZeo-textile (Figs. 4 and 5). In contrast, severe CPE and abundant fluorescent antigens were found in the cells inoculated with the virus treated on the Zeo-textile.

Table 5
Effect of FBS and feces on the inactivation of Ck/Yamaguchi/7/04 H5N1 virus by CuZeo-textile.

Treatment	Type of textile	Infectivity (Log_{10} $TCID_{50}$/ml)[a]	
		1 min	10 min
FBS	CuZeo-textile	nt [b]	<1.5
	Zeo-textile	nt	6.0
	(Log_{10} reduction)		(>4.5)
Feces	CuZeo-textile	<2.8	<1.7
	Zeo-textile	6.1	5.5
	(Log_{10} reduction)	(>3.3)	(>3.8)
PBS	CuZeo-textile	nt	<1.8
	Zeo-textile	nt	5.7
	(Log_{10} reduction)		(>3.9)

[a] The virus was mixed with an equal volume of 80% FBS or 20% fecal homogenate and was incubated on CuZeo-textile or Zeo-textile for 1 and 10 min.
[b] nt = not tested.

Table 6
HA titers of the H5N1 viruses incubated on CuZeo-textile.

Sample	HA titer[a]	
	Ck/Yamaguchi/7/04	Who.s/Hokkaido/1/08
CuZeo-textile	32, 64	32, 64
Zeo-textile	16, 32	16, 32
Virus control[b]	16, 32	16, 32

[a] The viruses were incubated on the textiles for 10 min. The hemagglutination (HA) test was performed twice. The HA titer is shown as the reciprocal of the highest dilution showing HA.
[b] The virus was mixed with PBS in the ratio 1:9 to obtain the same dilution as that of the virus treated on the textile.

3.6. Histopathological and immunohistochemical examination in embryos

All the embryos inoculated with the Ck/Yamaguchi/7/04 AF virus treated on the CuZeo-textile survived during the observation period, unlike those treated on the Zeo-textile.

There were no significant histological lesions in the dead or surviving embryos. Matrix antigens were detected in the vascular endothelial cells of the lung, liver, spleen, kidney, heart, and brain of the dead embryos inoculated with the Ck/Yamaguchi/7/04 virus treated on the Zeo-textile. In contrast, no antigens were detected in surviving embryos inoculated with the virus treated on the CuZeo-textile (Fig. 6).

3.7. EM analysis

The morphology of the purified H5N3 virus treated on the CuZeo-textile was examined (Fig. 7). Typical influenza virus particles could be easily seen on the sample grid containing the virus treated

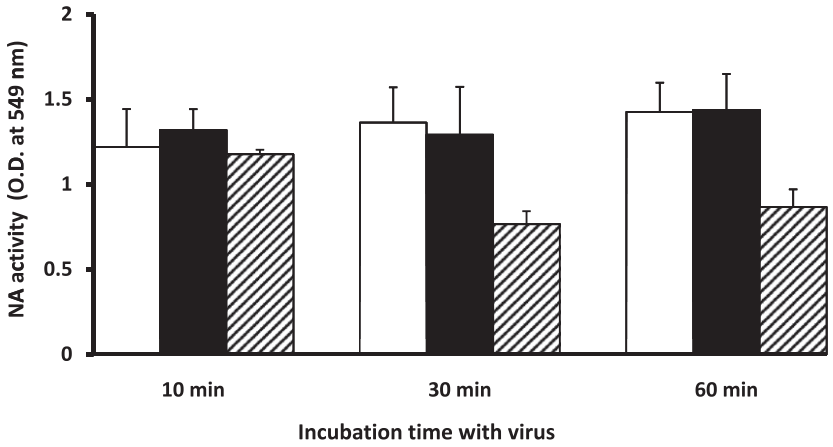

Fig. 2. Inhibition of the Ck/Yamaguchi/7/04 H5N1 virus NA after treatment with CuZeo-textile. Open bar: the virus was treated with PBS; closed bar: the virus was treated with the Zeo-textile; hatched bar: the virus was treated with the CuZeo-textile.

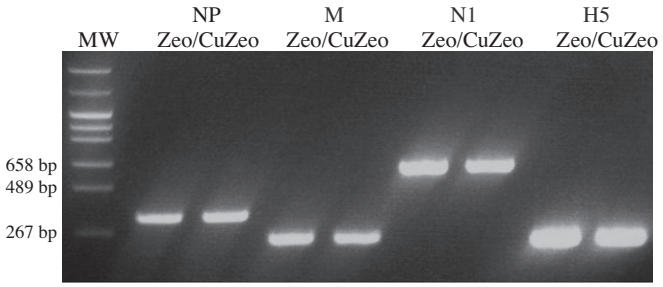

Fig. 3. Detection of H5, N1, M, and NP genes of the Ck/Yamaguchi/7/04 H5N1 virus treated on the Zeo-textile (Zeo) and CuZeo-textile (CuZeo) by RT-PCR. MW: DNA MW standard marker (pHY marker; Takara Bio Inc., Otsu, Japan). The virus was incubated on the textile for 10 min.

on the Zeo-textile, while only a small number of deformed and ambiguous particles were found on the sample grid with the virus immediately recovered from the CuZeo-textile. No particles were detectable on the sample grid containing the virus treated on the CuZeo-textile for 10 min (data not shown).

3.8. Effect of $CuCl_2$ on the H5 subtype virus

The effect of $CuCl_2$ on the inactivation of the Ck/Yamaguchi/7/04 H5N1 and Whi. s/Shimane/499/83 H5N3 viruses was examined. When the H5N1 AF virus was used as the virus source, no reduction in the viral titers was observed even at high concentrations of $CuCl_2$ (5000 μM) and 48 h of incubation (Table 7).

In contrast, when the purified H5N3 virus was similarly treated with 500 μM $CuCl_2$ for 48 h, the titer declined by >5.5 $\log_{10}$, while the titer of the AF virus did not decrease (Table 8). When AF was added to the purified virus prior to the addition of the $CuCl_2$ solution, the titer did not decline (Table 9).

4. Discussion

Reports on the efficacy of copper or copper ions (Cu^{2+}) against influenza virus infection are limited in number (Borkow et al., 2007, 2010; Noyce et al., 2007; Horie et al., 2008).

In the present study, we evaluated the antiviral ability of Cu^{2+} held by a microporous negatively charged zeolite (synthetic hydrated aluminosilicate) in comparison with Cu^{2+} in a $CuCl_2$ solution. Cu^{2+} held by zeolites rapidly and effectively inactivated the H5 subtype viruses (Tables 1–3). Surprisingly, the inactivation of the H5N1 virus by Cu^{2+} was already observed in the viruses recovered immediately from the CuZeo-textile. This is the first report showing the strong and rapid effect of Cu^{2+} on the inactivation of H5 subtype viruses. However, the Ck/Yamaguchi/7/04 H5N1 virus showed a higher susceptibility to Cu^{2+} exposure, and was inactivated with a higher $\log_{10}$ reduction of infectivity than the Who. s/Hokkaido/1/08 H5N1 virus after a very short exposure period (1 min) (>4.8 vs. 2.3 $\log_{10}$ reduction, Tables 1 and 2). This result may indicate that different viral strains have different sensitivities to Cu^{2+}.

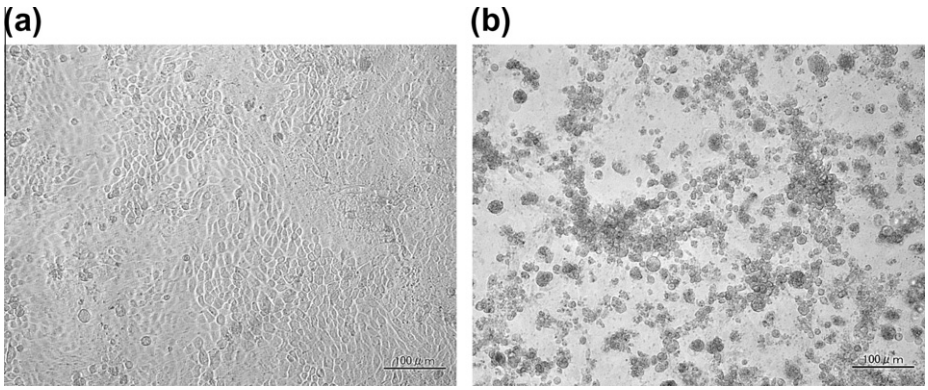

Fig. 4. Cytopathic effects in MDCK cells inoculated with the virus treated with the CuZeo-textile (a) and Zeo-textile (b). The virus was incubated on the textile for 1 min.

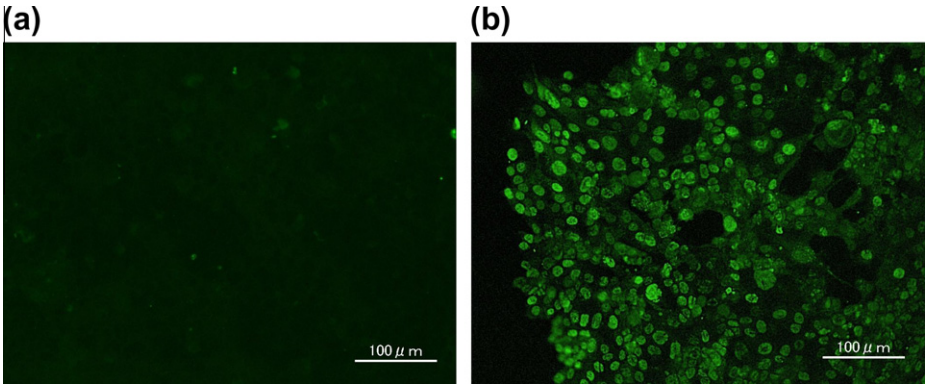

Fig. 5. IFA tests to detect NP antigens in MDCK cells inoculated with the virus treated with the CuZeo-textile (a) and Zeo-textile (b). The virus was incubated on the textile for 1 min.

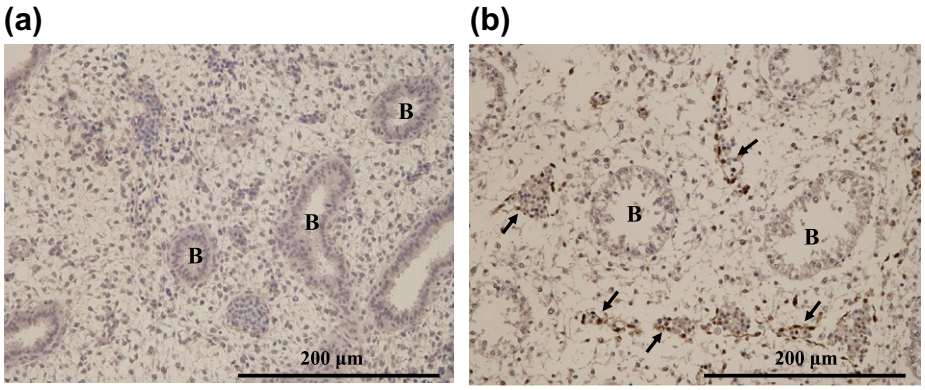

Fig. 6. Immunohistochemistry of the lung of the embryos. (a) No matrix antigens were detected in the lung of the surviving embryo inoculated with the H5N1 virus treated on the CuZeo-textile. (b) Matrix antigens (arrows) that were detected in vascular endothelial cells of the lung of the dead embryo inoculated with the virus treated on the Zeo-textile. B = bronchus.

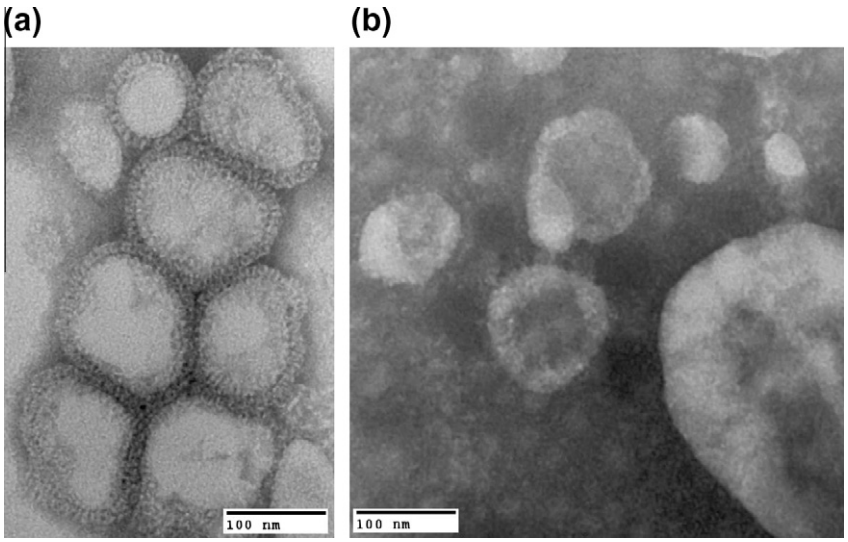

Fig. 7. EM analysis. (a) The purified Whi. s/Shimane/499/83 H5N3 virus that was treated on the Zeo-textile. (b) The purified virus that was treated on the CuZeo-textile. The viruses were recovered from both Zeo-textile and CuZeo-textile immediately after adding the viruses on the textiles and staining with 2% PTA (pH 6.5).

A previous study using purified virus reported that 25 μM $CuCl_2$ reduced H9N2 virus infectivity by nearly 4 $\log_{10}$ within 6 h (Horie et al., 2008). Unexpectedly, the present results using AF viruses indicated that the H5 viruses were not inactivated by $CuCl_2$ even at higher concentrations (500 or 5000 μM) of $CuCl_2$ and a long incubation period (48 h) (Tables 7 and 8). Although the reason why Cu^{2+} in the $CuCl_2$ solution was ineffective against the H5 AF viruses was unclear, the same concentration of $CuCl_2$ solution strongly inactivated the purified H5N3 virus (Table 8). However, the antiviral effect was inhibited by pretreatment of the purified

Table 7
Effect of $CuCl_2$ on the Ck/Yamaguchi/7/04 H5N1 virus.[a]

$CuCl_2$	Infectivity (Log_{10} $TCID_{50}$/ml) Incubation time with $CuCl_2$	
	24 h	48 h
500 μM	6.8	6.5
5000 μM	nt[c]	6.8
Virus control[b]	7.1	7.1

[a] The allantoic fluid (AF) containing the virus (AF virus) was mixed with an equal volume of $CuCl_2$ solution. The mixture was serially diluted 10-fold after incubation and titrated on the MDCK cells.
[b] Virus control: the virus was mixed with an equal volume of ultrapure water.
[c] nt = not tested.

Table 8
Effect of $CuCl_2$ on the Whi.s/Shimane/499/83 H5N3 virus obtained from a different source.[a]

Source of virus	Infectivity (Log_{10} $TCID_{50}$/ml) 500 μM $CuCl_2$		
	+	–	(Log_{10} reduction)
Purified virus	<1.5	7.0	>5.5
AF virus	7.2	7.1	

[a] The purified or AF virus was used as the virus source and was incubated with 500 μM $CuCl_2$ for 48 h.

Table 9
Adverse effects of AF on antiviral ability of $CuCl_2$ on the purified Whi.s/Shimane/499/83 H5N3 virus.[a]

500 μM $CuCl_2$	Infectivity (Log_{10} $TCID_{50}$/ml) Pretreatments of the purified virus		
	AF[b]	PBS	(Log_{10} reduction)
+	5.7	<1.7	>4.0
–	6.2	6.5	

[a] The purified virus was used as a virus source. The pretreated viruses were incubated with 500 μM $CuCl_2$ for 48 h.
[b] AF was collected from uninfected embryonated eggs.

H5N3 virus with AF (Table 9). This result suggests that some components present in the AF may have acted in opposition to the antiviral activity of Cu^{2+} in the $CuCl_2$ solution.

Cu^{2+} seems to be involved in the electronic layer called "the double layer" surrounding the surface of zeolites (Oonkhanond and Mullins, 2005). As zeolites have a microporous structure with a large surface area, it is possible that the CuZeo-textile induces a strong antiviral effect through a direct, close, and efficient contact of the viruses with the Cu^{2+} on the surface of zeolites incorporated in the dense cotton fibers. Although the reason why the zeolite-held Cu^{2+} and not the Cu^{2+} in the $CuCl_2$ solution is effective against the AF virus is unclear, it may be that the undesirable components in the AF could be absorbed by zeolites or cotton fibers, rendering them inactive.

A previous report showed that changes in the H9N2 virus titers caused by treatment with $CuCl_2$ and $CuSO_4$ were completely blocked by the concomitant presence of ethylenediaminetetraacetic acid (EDTA), which binds to Cu^{2+} (Horie et al., 2008). In this study, the inactivation effect of Cu^{2+} on the H5N1 virus was also inhibited by pretreatment of the CuZeo-textile with EDTA (data not shown).

Viral proteins including NP are synthesized from viral mRNA in the cytoplasm of the infected cells. NP is associated with replication of viral RNA and mRNA in the nucleus and is synthesized in far greater quantities than the other internal proteins in the infected cells (Bennink et al., 1987; Pallese and Shaw, 2007). In this study, no NP antigens or CPE were detectable in the MDCK cells inoculated with the H5N1 virus treated on the CuZeo-textile (Figs. 4 and 5). M antigens were not detected in the infected cells either (data not shown). Although these findings suggested that the viral proteins including NP were not synthesized from viral mRNA and virus assembly did not occur, the step in the replication cycle that was inhibited was not clear. However, viral genes were successfully amplified by RT-PCR (Fig. 3), suggesting that no profound damage was observed to the viral genes.

The influenza virus is known to first bind to the glycoprotein receptors containing sialic acid on the cell surface via HA to enter the cell by endocytosis (Pallese and Shaw, 2007). In this study, the HA titers of the H5N1 viruses recovered from the CuZeo- and Zeo-textiles were not different (Table 6). These findings suggested that the receptor binding ability of the virus was not affected by Cu^{2+}. Our previous report indicated that many morphologically abnormal particles of the Cu^{2+} treated-H9N2 virus were found, suggesting that a structural change could be associated with the antiviral effect of Cu^{2+} (Horie et al., 2008). The present study confirmed this finding (Fig. 7). Interestingly, however, no viral particles were found in the samples of viruses treated on the CuZeo-textile for 10 min, suggesting that the viruses were highly and rapidly disrupted by the zeolite-held Cu^{2+}.

Emergence of "new" pandemic influenza A viruses in humans with high mortality is of global concern (Webster et al., 2006). One of the candidate strains is the HPAI H5N1 subtype virus circulating in the bird population, although the H5N1 virus has not yet acquired the ability to transmit among humans. Since it is impossible to eradicate the virus from the bird population, prevention and control measures are critical to reduce transmission from birds to birds (Webster et al., 2006). In addition, these measures are also critical to reduce interspecies transmission of the virus to avoid a new human pandemic strain. Influenza A virus is easily transmitted to the susceptible hosts via virus-laden droplets or feces. In addition, various kinds of virus-contaminated fomites such as environmental surfaces can transmit the virus. It is, therefore, important to eliminate the source of infection from the environment. Although the high and rapid inactivation of the H5 virus by Cu^{2+} held by zeolites was shown, a powder form of Cu^{2+}-zeolite may be inadequate for application to the environment to control influenza viruses, because it will be difficult to remove it from the environment. In contrast, cotton textiles containing the Cu^{2+}-zeolite (CuZeo-textile) offers certain advantages based on easy handling and processing. It is well known that resistance of viruses to physicochemical agents is enhanced when the viruses are contained in certain materials such as feces or mucosal secretions. In this study, it was clearly demonstrated that the high and rapid inactivation effect of the CuZeo-textile on the H5N1 virus was not affected even in the presence of FBS and feces (Table 5), and such properties would be very useful in actual applications in both animal and human environments such as in farms or hospitals to eliminate virus-containing materials.

Copper has been widely used as an algaecide, fungicide, nematocide, molluscicide, or bactericide; the viricidal activity of copper has also been examined (Borkow and Gabbay, 2005). We are currently examining the inactivation effects of CuZeo-textile on various kinds of viruses. We believe that the CuZeo-textile has wide applications as a sanitary cidal agent for pathogens or in environmental healthcare goods, as other useful features of zeolites are also applicable in both medical and veterinary fields, such as deodorizing ability, removal of harmful gases or heavy metals from the environment, etc. As an example, the CuZeo-textile can be widely applied as a comprehensive healthcare item such as in protective wear (clothes, masks and gloves), sheets covering beds or pillows in hospitals, and air or water purifiers in facilities such as hospitals or farms, etc.

Acknowledgements

We would like to thank Dr. Masayuki Tani for his helpful discussion and Ms. Sachiko Matsuda for excellent technical assistance. This work was partially supported by Grants from the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, and by Grant-in Aid for Exploratory Research (19659115), MEXT Japan, and was also supported in part by a grant from Global COE Program from Japanese Ministry of Education, Science, Sports, Culture and Technology.

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