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RESEARCH ARTICLE

In vitro Cytoprotective Effect of Infant Milk Formula Fortified with Human Rotavirus-specific Hyperimmune Yolk Immunoglobulins (IgY)

Shofiqur Rahman, Kouji Umeda, Faustino C. Icatlo, Jr., Ki Woong Lee, Sung Han Kim, Hyuk Joon Choi, Bok-Kyung Han, Young-Seo Park, and Nguyen Van Sa

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Abstract Infant formula supplemented with hyperimmune immunoglobulin Y (IgY) against human rotavirus (HRV) was evaluated *in vitro* against HRV reassortant clinical strains ATCC VR 2273 and ATCC VR 2274. Specific anti-human rotavirus antibody powder (Rotamix IgY) was prepared. The effectiveness of Rotamix IgY alone and as a pre-mixed solution with infant formula was evaluated for neutralizing rotavirus infectivity in MA104 cells. The test infant formula cross-reacted strongly against different human rotavirus strains with titers of 80-320 using a 50% fluorescent focus (FF) inhibition test. Both rotamix IgY alone and in a pre-mixed solution with infant formula showed multi-serotypic cross neutralization activities against the major rotavirus global serotypes G1, G2, G3, and G4 alone and with other human and animal-strains *in vitro*. Cell-rotavirus adhesion and cell damage arising from rotavirus infection were significantly inhibited in a dose dependent manner, compared to control IgY supplemented infant formula.

Keywords: rotavirus, immunoglobulin Y, infant formula

Shofiqur Rahman (✉), Kouji Umeda, Faustino C. Icatlo, Jr., Nguyen Van Sa
Immunology Research Institute in Gifu, EW Nutrition Japan K.K., Gifu 501-1101, Japan
Tel: +81-58-235-7303; Fax: +81-58-235-7505
E-mail: rahman@ew-nutrition.co.jp

Ki Woong Lee, Sung Han Kim
Namyang Dairy, Sejong 339-914, Korea

Hyuk Joon Choi, Bok-Kyung Han
BK bio Co., Ltd., Seongnam 462-819, Korea

Young-Seo Park
Department of Food Science and Biotechnology, Gachon University, Seongnam 461-701, Korea

Introduction

Among viral, bacterial, and parasitic agents causing acute infectious gastroenteritis, rotavirus is the leading single etiologic agent of severe diarrhea among infants and young children worldwide during the first 5 years of life (1). It is estimated to be responsible for over 500,000 deaths in infants and young children, mostly in developing countries, each year. This represents approximately 5% of all deaths (2). From different studies, serotype G1 of rotavirus has emerged as the most common global serotype in circulation (3). Epidemics of serotypes G2, G3, and G4 have also been detected from time to time in many countries. Therefore, any proposed control measure must take into account adequate protection against the above four epidemiologically significant HRV serotypes.

Conventional treatment is largely symptomatic and involves fluid and electrolyte replacement and maintenance of nutrition. No specific, effective, and affordable therapy is currently available. The use of current vaccine regimens has been in question due to variable degrees of efficacy (4) and limitations on use for children aged less than 3 months (5). Passive immunotherapy could have advantages over vaccines due to: 1) quicker immune response, 2) broader application areas (at any age level ranging from mature to a deficient or immature immune system, like infants), and 3) lower production costs.

Passive oral immunotherapy using specific chicken antibodies (IgY) has been applied with mixed success against infectious diseases of viral, bacterial, and fungal origin in both humans and animals (6-8). Peroral administration with IgY is an attractive approach because IgY does not activate the mammalian complement or interact with mammalian Fc receptors that could mediate

Table 1. Rotavirus strains used in this study

Strain	Serotype
Human origin:	
HRV 408	Natural reassortant
HRV 248	Natural reassortant
Wa	G1 P[8]
KU	G1 P[8]
M37	G1 P[6]
S2	G2 P[4]
1076	G2 P[6]
YO	G3 P[8]
HK	G4 P[8]
Equine origin: HO-5	G3 P[12]
Bovine origin: Shimane	G6 P[5]
Porcine origin: S-80	G1 P[7]

inflammatory responses in the gastrointestinal tract (9). Previously, it was reported that anti-diarrheal IgY had preventive effects against diarrhea in cow (10-11), pig (12), dog (8), and cat (13). Recently, Rahman and colleagues evaluated the therapeutic efficacy of specific anti-HRV IgY egg yolk powder in a clinical trial against severe diarrhea among pediatric patients due to rotavirus (14). Beneficial effects of treatment were observed in terms of reducing the main clinical outcomes of disease, including duration of diarrhea, and achieved faster clearance of the virus based on stool evaluation (14). In this study, the *in vitro* efficacy of infant formula supplemented with anti-HRV IgY against different human rotaviruses was evaluated.

Materials and Methods

Viruses and cell lines The viruses used in this study included 1) reassortant type II subgroup human rotavirus (HRV) strains HRV 408 (ATCC 2273), HRV 248 (ATCC 2274), Wa (G1 P[8]), S2 (G2 P[4]), YO (G3 P[8]), and HK (G4 P[8]), 2) equine strain HO-5 (G3 P[12]), 3) bovine strain Shimane (G6 P[5]), and porcine strain S-80 (G1 P[7]) (Table 1). The rhesus monkey kidney cell line MA-104 (ATCC CRL-2378) was used to propagate all the above rotaviruses. MA-104 cells were maintained in Eagle's minimal essential medium (EMEM; Nissui, Tyoko, Japan) with Earle's salts, supplemented with 10% fetal bovine serum (FBS), and incubated at 37C with 5% carbon dioxide.

Preparation of Rotamix IgY and control IgY Reassortant strains HRV 408 and HRV 248 were isolated from stools of 9 and 17 month old children from Bangladesh (15). These two reassortant human rotaviruses were used as antigens for the production of anti-HRV IgY, according to methods described previously (7). Briefly, eighteen week old Hy-Line hens were immunized using intramuscular injection with an emulsified mixture of inactivated human rotavirus as single-strain or mixed-strain emulsions. Eggs laid by the immunized hens between 3 and 10 weeks after immunization were harvested and the egg yolk was isolated, pooled, and processed into a powder form in accordance with a method described previously (6). The egg yolk powder from the eggs of hens who received a mixed vaccination with the two rotavirus strains HRV 408 and 248 was designated as "Rotamix IgY". Control IgY powder was prepared using the same method as for the eggs of hens immunized using the tissue culture medium of a mock-infected MA-104 cell monolayer as an antigen. For *in vitro* studies, Rotamix IgY and control IgY were partially purified from egg yolk using chloroform extraction and ammonium sulfate precipitation (16). The antigen and antibody protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The protein contents of the Rotamix IgY and control IgY preparations were 7.5 and 7.8%, respectively, with an IgY content of 20%(w/w). The fat, carbohydrate, ash, and moisture contents of both IgY preparations were 12, 5, 0.8, and 5%, respectively.

Preparation of test and control infant formulas A powder form of commercially available infant formula "Imperial XO" (Namyang Dairy, Sejong, Korea) was fortified with Rotamix IgY for use as a test infant formula. Approximately 14 g of this infant formula was dissolved in 100 mL of sterile distilled water and mixed with 34 μg of either Rotamix IgY (referred to hereafter as test infant formula) or 34 μg of control IgY (control infant formula). The end result was 2.428 μg of test infant formula or control infant formula for every gram of powder infant milk formula. Additionally, for every 100 mL of test infant formula, 280 mg each of α-lactalbumin and β-casein were incorporated as additional protein sources. For *in vitro* studies, Rotamix IgY and control IgY were partially purified from test and control infant formulas using chloroform extraction (16).

Virus quantitation Rotavirus titers were determined using the 50% tissue culture infectious dose, or $TCID_{50}$ method (13). Briefly, tissue culture supernatants were assayed for virus infectivity in MA-104 cells cultivated in microtiter plates or tubes based on endpoint dilution of samples with a cytopathic effect in a 10-fold sample dilution series.

Fluorescent focus (FF) inhibition assay of IgY To determine the cross-reactivity and neutralization titer of IgY samples, the FF reduction method was used (17). In

this assay, MA 104 cells were plated onto 96-well tissue culture plates with EMEM containing Earle's salts and non-essential amino acids, and supplemented with L-glutamine, sodium pyruvate, and 5% fetal bovine serum. Cells were incubated at 37°C with a 5% CO_2 tension for 24 h, or until a confluent monolayer was formed. Different infant formula dilutions of Rotamix IgY and control IgY were prepared in phosphate buffered saline with final concentrations of 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 mg/mL. Each dilution was mixed with an equal volume of 12 rotavirus strains (248, 408, Wa, KU, M37, S2, 1076, YO, HK, HO-5, Shimane, and S-80) at a dilution that yielded 150 to 200 FF units per 0.025 mL per well, and the mixture was allowed to react at 37°C for 1 h. Approximately 50 μL aliquots of the IgY-virus mixtures were dispensed onto MA104 monolayers in a 96-well microplate and incubated for 1 h at 37°C. An amount of 100 microliters of fresh EMEM was added, followed by 16 to 18 h of cultivation at 37°C with a 5% CO_2 tension. Fixation in cold (−80°C) methanol and reactions with primary antibodies and with secondary fluorescent-labeled antibodies were performed as described previously (18). The neutralizing antibody titer was expressed as the reciprocal of the highest IgY dilution that inhibited the FF count by >50%. The mean FF inhibitory titer of IgY was calculated from 3 independent assays.

Rotavirus neutralizing capacity of Rotamix IgY-fortified infant formula The neutralization effect of infant formulas was determined using the 50% tissue culture infectious dose, or $TCID_{50}$ method (13). Briefly, MA-104 cells were cultured overnight in minimum essential medium containing 10% FBS in cell culture tubes. An amount of 1 g of test or control infant formula was dissolved in 7 mL of PBS (8 times dilution, or 125 mg/mL) and IgYs were partially purified using chloroform extraction (16). The tubes were treated with either 100 μL of phosphate buffered saline (PBS) as a control, or with 100 μL of 10-fold diluted suspensions (10, 10^1, up to 10^{10}) of HRV 408 and HRV 248 that were pre-incubated at 37°C for 1 h with 100 μL of either the control or test infant formula. Approximately 100 μL of this rotavirus-infant formula mixture was added to cell culture tubes and incubated at 37°C for 1 h. Finally, 1 mL of EMEM was added and the tubes were incubated at 37°C for 7 days. The cytopathic effect was checked every day and the virus dilution inactivated by the infant formula extract was determined.

The $TCID_{50}$ method was used to determine the minimum effective dose of infant formula. MA-104 cells were cultured overnight in EMEM using 96-well microtiter plates. Approximately 100 μL/well of 200 $TCID_{50}$ HRV 408 cells was mixed with 100 μL of serially diluted control IgY, Rotamix IgY, and test or control infant formula at final concentrations of 1,000, 50, 25, 12.5, 6.25, 3.13, and 1.56 mg/mL.

The microtiter plates were incubated at 37°C for 1 h. The HRV-IgY or HRV-infant formula mixtures (100 μL/well) were dispensed onto MA104 monolayer cells in wells and incubated at 37°C for 7 days. The cytopathic effect was checked everyday.

Inhibition of *in vitro* cell damage and attachment to MA-104 cells by infant formula fortified with Rotamix IgY Cell damage inhibition by Rotamix IgY-fortified infant formula was assessed using the 50% tissue culture infectious dose, or $TCID_{50}$, method (13). Briefly, the rhesus monkey kidney cells MA-104 were cultured overnight in minimum essential medium containing 10% FBS in 96-well microtiter plates. The MA-104 monolayer cells were treated with 50 μL of PBS as a control, or 50 μL of 200 $TCID_{50}$ HRV 408 solution pre-incubated for 1 h at 37°C with 50 μL of either the control or test infant formula (50, 25, 12.5, and 6.25 mg/mL). This rotavirus-infant formula mixture that was overlaid onto MA-104 monolayer cells was allowed to incubate at 37°C for 7 days. On reading day, the plates were gently washed three times with PBS to remove dead and detached cells, and the remaining adherent cells were counted after trypan blue staining.

The FF inhibition method was used (17) to assess inhibition of rotavirus attachment to MA-104 cells by the Rotamix IgY-fortified infant formula using the human rotavirus strains HRV 408 and 248 as test virus strains. In this assay, MA 104 cells were plated in a 96-well tissue culture plate and incubated at 37°C with a 5% CO_2 tension for 24 h, or until a confluent monolayer was formed. Different dilutions of test and control infant formula in PBS were prepared with final concentrations of 0.78, 1.56, 3.13, and 6.25 mg/mL. Each dilution was mixed with an equal volume of one of the 2 different rotavirus strains (248 and 408) at a dilution that yielded 150 to 200 FF units per 0.025 mL per well, and the mixture was allowed to react at 37°C for 1 h. The rest of the method followed the same procedures as for the Rotamix IgY FF inhibition assay. The mean FF inhibitory titer of IgY was calculated from 3 independent assays.

Statistical analysis All data are presented as the mean ±SD of three independent experiments. Data for the inhibition of cell damage and attachment were analyzed using Student's *t*-test. A statistical significant was evaluated and verifies at the level of $p<0.05$ using SPSS (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Range of *in vitro* cross reactivity of infant formula against representative rotavirus serotypes Based on an FF assay, the test infant formula strongly cross-reacted serologically with different human rotavirus strains (248, 408, Wa, KU, M37, S2, 1076, YO, and HK) and 1 horse rotavirus strain (HO-5) (Table 2). The test infant formula cross-reacted weakly with porcine S-80, and not with the bovine strain Shimane. The control infant formula showed no reaction to any serological strains (neutralization titer <20). The neutralization titers of Rotamix IgY, as measured using an FF inhibition assay, against the HRV strains 408 and 248 serotypes, were 10,240 and >40,960, respectively.

Results of cross-neutralization with different host-specific serotypes revealed that the test infant formula strongly cross-neutralized all the major global HRV serotypes (G1, G2, G3, and G4) (Table 2). This *in vitro* cross-reactivity profile suggests that infant formula can provide general multi-serotypic passive protection *in vivo,* particularly among rotavirus infected infants and young children, considering the broad serotypic diversity of human rotaviruses in different regions of the world.

Neutralizing capacity of infant formula against human rotaviruses In neutralization tests, the control infant formula extract showed no inactivation of human rotaviruses, but the test infant formula extract completely inactivated $10^{8.5}$ and $10^{7.75}$ virions of the two human rotaviruses strains 408 and 248, respectively. Since the test infant formula (1 g) was diluted 8 times with PBS, 125 mg of infant formula has the power to inactivate at least $8\times10^{8.5}$ $TCID_{50}$ of HRV 408 virions, and $8\times10^{7.75}$ $TCID_{50}$ of HRV 248 virions (Table 3). The minimum effective dose of the test infant formula against HRV 408 was 12.5 mg/mL with an equivalent neutralization titer of 80, while the minimum effective dose of the control infant formula was 1,000 mg/mL with a neutralization titer of less than 20 (Table 4).

The serotypic diversity among rotaviruses is due to genetic reassortments arising from interspecies transmission and/or mixed infection. Reassortant serotypes are clinically important inasmuch as they cause severe forms of diarrhea among children worldwide. The development of any immunologic intervention against rotavirus infection must, therefore, take into account strongly cross-reactive strains against the four major HRV serotypes G1, G2, G3, and G4, and also take into account their reassortant strains, which together account for 96% of all rotavirus infections in the world (19-22). In this study, the two naturally unique human rotavirus reassortant clinical isolates ATCC VR 2273 and ATCC VR 2274 were selected and used to prepare a Rotamix IgY powder produced by mixing the two serotypes as antigens. These two viruses derive their genomic segments from parental strains of different infant formula genogroups and/or serotypes belonging to subgroup II of the prototype DS-1 human rotavirus strain (15). HRV 408 was shown to have subgroup II specificity (I1 genotype according to recent nomenclature) (16), and the RNA profile was long, which is characteristic of subgroup II rotaviruses (15). HRV 408 had G3 specificity, but could also be neutralized by polyclonal antisera to G1, G2, and G3, and may induce neutralizing antibodies to at least G1 and G2 (23). HRV 248 was shown to have G4P [4] specificity, subgroup II specificity (I1 genotype of VP6), and a long RNA pattern. Using an RNA-RNA hybridization assay, it was found that 7 of 11 RNA segments of HRV 248 are derived from the Wa genogroup, and the remaining 4 are from the DS-1 genogroup. Thus, HRV 248 is shown to be an intergenogroup reassortant (24) strain.

To determine the cross reactivity profile, several rotavirus serotypes originally derived from human, horse, cattle, and swine were reacted *in vitro* against Rotamix IgY-supplemented infant formula. Results revealed that the test infant formula cross-neutralized all the major HRV serotypes (G1, G2, G3, and G4) along with other human and animal strains (Table 2). This *in vitro* cross-reactivity

Table 3. Titer of human rotaviruses neutralizable by test of infant formula

Infant formula	Human rotavirus strain	Inactivated virions ($TCID_{50}$ /mL)
Control (125 mg/mL)	408	No neutralization
	248	No neutralization
Test (125 mg/mL)	408	$10^{8.50}$
	248	$10^{7.75}$

Table 2. *In vitro* cross neutralization activity of test infant formula with human and animal rotavirus strains as determined using an FF inhibition assay

IgY sample	Neutralization titer/0.1 mL IgY[1)] against different rotavirus strain											
	408	248	Wa	KU	M37	S2	1076	YO	HK	HO-5	Shimane	S-80
Control infant formula	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Test infant formula	160	80	160	160	160	80	320	160	160	160	<20	40

[1)]IgY titer is expressed as the dilution factor of 1 g of infant formula powder that reduced the FF count by >50% in an FF inhibition assay. Results are presented as the mean of three independent experiments.

Table 4. *In vitro* neutralization effect of infant formula against rotavirus strain HRV 408

Test sample	Dilution factor (mg/mL)						
	0 (1,000)	20 (50)	40 (25)	80 (12.5)	160 (6.25)	320 (3.13)	640 (1.56)
Control IgY	----[1)]	++++	++++	++++	++++	++++	++++
Rotamix IgY	----	----	----	----	----	----	++++
Control infant formula	----	++++[2)]	++++	++++	++++	++++	++++
Test infant formula	----	----	----	----	+++[3)]	++++	++++

[1)]+, cytopathic effect positive tube; -, cytopathic effect negative tube. Cytopathic effect score: +, <20%; ++, 20-50%; ++++, 50-100%. Neutralization titer of infant formula: [2)]Control <1, 20; [3)]test 1, 160

profile suggests that Rotamix IgY-fortified infant formula can provide a general multi-serotypic passive protection *in vivo,* particularly among rotavirus infected infants and young children, considering the broad serotypic diversity of human rotaviruses in different regions of the world.

***In vitro* cell damage inhibition by infant formula** In cell damage inhibition assays, HRV 408 added to an MA104 monolayer cell culture resulted in mammalian cell death and subsequent detachment from the plates. Pretreatment of the virus with infant formula protected the cells from damage and significantly increased cell survivability ($p<0.05$), compared to the non-treated control groups, in a dose-dependent manner. The control infant formula did not show any protection effects (Fig. 1).

Attachment inhibition assay using monolayer MA 104 cells In order to investigate the attachment inhibitory activity of the infant formula, an *in vitro* adhesion inhibition assay was performed to examine the adherence ability of infant formula-treated HRV 408 and 248 virions using MA104 as cell substrates. The test infant formula-treated HRV strains showed significant reductions in adherence capacities, compared with the control infant formula-treated HRV strains (Fig. 2). On the other hand, the control infant formula-treated HRV strains did not show any quantifiable

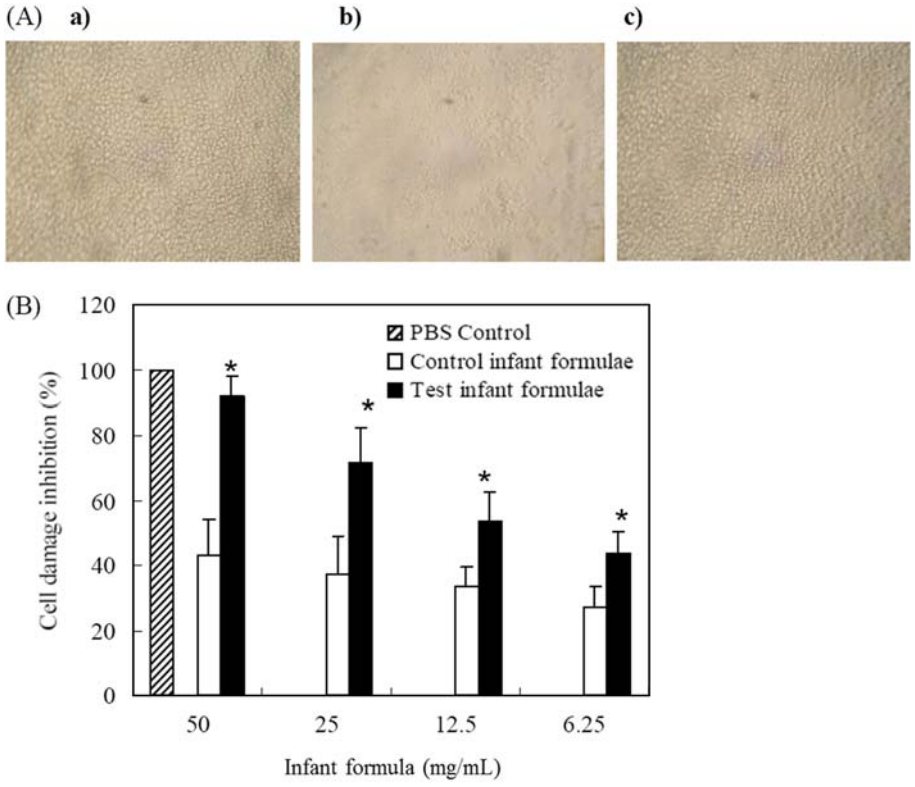

Fig. 1. Protection of MA104 cells against cytopathic effects and infection by HRV 408 using anti-HRV IgY-enriched infant formula. (A) Photomicrographs (×100 magnification) of MA104 cells treated with: a, PBS; b, control infant formula (50 mg/mL); c, test infant formula (50 mg/mL). (B) Dose dependent efficacy of infant formula. *$p<0.01$ (Student's *t*-test) indicates significant differences between the test and control infant formula groups.

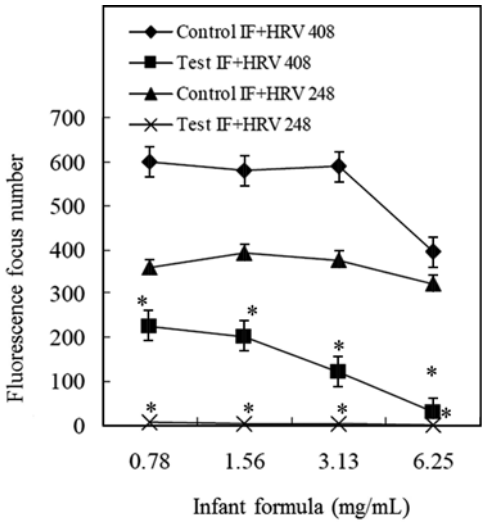

Fig. 2. Effect of anti-HRV IgY-enriched infant formula on blocking HRV infection in MA104 cells. *$p<0.01$ (Student's *t*-test) indicates significant differences between the test and control infant formula groups.

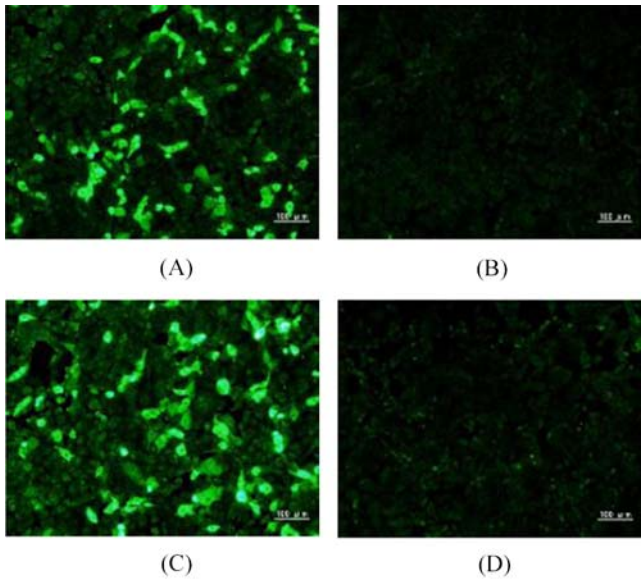

Fig. 3. Inhibitory effect of infant formula (6.25 mg/mL) against attachment of HRV to MA104 cells (FF assay). Fluorescence focus photomicrographs (×100 magnification) of MA104 cells treated with a premixture of control infant formula+HRV 248 (A), test infant formula+HRV 248 (B), control infant formula+HRV 408 (C), test infant formula+HRV 408 (D).

reduction in adhesion ability. In addition, the dose-response efficacy of the test infant formula was examined. The inhibition of HRV adhesion was directly correlated with the test infant formula concentration (Fig. 2). These results indicate that the adhesion activity of HRV was reduced after incubation of HRV with test infant formula, and the reduction of the adhesion activity was a specific and non-random event since the degree of inhibition correlated with the test infant formula concentration. In the FF assay, photomicrographs show that the test infant formula-treated HRV 248 and 408 strains inhibited viral attachment to MA 104 cells completely (100%) and partially (92%), respective to the two HRV strains (Fig. 3).

IgY has been used experimentally in mice, cat, and calves for the last 20 years for preventive and therapeutic effects. Compared to other available anti-rotaviral preventive measures, IgY powder from chickens has several advantages, including a highly specific activity, rapid onset, local site of action, distribution of products without requiring a cold chain system for storage and transport, non-toxicity (being a normal part of the human diet) (9), and a non-invasive action. IgY is also relatively safe, especially when used for a long period of time (25). Moreover, development of new IgY treatments against emerging or novel rotavirus serotypes is much easier and quicker, compared to development of new vaccines.

The cytoprotective effect of anti-HRV IgY used as a fortification for an infant formula as protection against HRV-induced diarrhea in humans was documented. Cytoprotection was evident from the anti-adhesive and anti-cell damage activities of the test infant formula. Cytoprotective activity was associated with neutralization of rotavirus strains representing the major HRV serotypes circulating worldwide. These *in vitro* findings indicate that Rotamix IgY acting within fortified infant formula is a promising tool for prevention and adjunctive treatment of children with rotavirus diarrhea.

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