

Microwave Treatment Prevents Cytomegalovirus Transmission to Preterm Infants through Human Milk

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Abstract

Background: Mother's own milk (MOM) is the best nutrition for preterm infants; however, milk from cytomegalovirus (CMV)-seropositive mothers may cause the transmission of the virus to the infants. As extremely preterm infants are at a risk of developing serious conditions due to CMV infection, even MOM is sometimes pasteurized. Previously, we demonstrated that microwave heating of 50 mL formula milk containing CMV at 500 W for 40s prevented CMV infection in living cells. However, we do not know if this is the case with reactivated CMV in human milk (HM). Therefore, we investigated if microwave heating of HM would be effective in preventing CMV infection in living cells.

Methods: Twenty-three CMV-IgG-positive and four CMV-IgG-negative mothers who donated HM to the human milk bank during 6 to 16 weeks postpartum were enrolled. The HM was treated with microwave at 500 W. HFL-III cells were seeded in culture dishes. Aliquots of HM samples subjected to microwave was inoculated onto susceptible cell monolayers. Infectious viral titer was measured by the 50% median tissue culture infectious Dose (TCID₅₀) assay.

Results: The TCID₅₀ assay was decreased in five out of eight 100-mL samples after microwave at 200W for 60 s. The CMV infectivity assay was negative in three, decreased in eight, and no change in six 100-mL samples, and negative in four and decreased in eight 50-mL samples.

Conclusions: Microwave heating of HM at low settings could decrease CMV transmission through HM, however, to obtain entire prevention is difficult when loss of bioactive properties is considered.

Keywords: Human milk; Cytomegalovirus; Microwave; Preterm infant

Introduction

Human milk (HM) is an optimal source of nutrition for infants and is even considered therapeutic for preterm infants. However, HM from mothers who are seropositive for cytomegalovirus (CMV) can be a source of postnatal infection in preterm infants. The estimated cumulative rate of transmission of CMV from seropositive mothers to preterm infants is 37% [1]. Extremely preterm infants are at a high risk of developing a sepsis-like syndrome, cholestasis, or bronchopulmonary dysplasia because of the absence of the transmission of maternal antibodies. The strategy to prevent CMV transmission via HM differs among countries. France and Austria recommend pasteurization of HM from CMV-seropositive mothers until 32 and 34 weeks post-conception, respectively [2]. However, it is unclear whether HM from CMV-seropositive mothers should be pasteurized as this procedure can cause loss of bioactive properties. There has been no clear consensus on whether HM from CMV-seropositive mothers should be treated. Ben-Shoshan et al. [3] demonstrated the relevance of microwave (MW) heating on prevention of CMV infection in HM. We have previously added CMV with a viral load of 5.0×10^3 plaque-forming units/mL to 50 mL of formula milk, resulting in 772 plaque-forming units/mL at baseline. After MW heating, the supernatants (0.1 mL) were inoculated onto susceptible cell monolayers and incubated for 90 min to allow viral attachment

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to cells [4]. There was no cell infection observed following 500 W heating for 40 s. Furthermore, MW heating of 100 mL HM at 500 W for 40 s did not result in a significant reduction in secretory immunoglobulin A (SIgA), lactoferrin, or transforming growth factor (TGF)-beta levels in HM [5]. Although MW heating of formula milk at 500 W for 40 s was effective in preventing the CMV infection in living cells, we do not know if this is the case in reactivated CMV in HM. Furthermore, the viral load of CMV in HM differs depending on the individuals and period after the delivery. As reported in the previous study, heating to 60 °C for a short time would be effective in preventing CMV infection. Formula milk (100 mL) at an initial temperature of 10 °C reaches 60 °C when exposed to 500 W MW heating for 40 s [4]. However, 100 mL of breast milk did not attain a temperature of 60 °C following treatment with 500 W MW heating for 40 s under the same conditions [6]. Therefore, we used HM derived from CMV-positive mothers to determine whether MW treatment prevents CMV infection in living cells.

Methods

Participants

We used HM derived from 27 participants aged 29-41 y (mean 32.1 y), who gave birth from March to August 2021, and were 6-16 weeks postpartum (Median, 9; Q1, 7; Q2, 9; Q3, 10; interquartile range, 3) when they donated their HM. This HM was frozen prior to use for up to 3 months. All participants were donors for the HM bank in Japan. The HM samples were tested for CMV IgG antibodies, Human Immunodeficiency Virus (HIV), Human T-cell Leukemia Virus (HTLV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and syphilis at the time of registration. The inclusion criteria for the donors were same as human milk banking association of North America (HMBANA); the babies were exclusively breastfed and growing well, and the donors had no comorbidities [7]. To maintain confidentiality, we allocated a unique number to each donor. Data were stored in a personal computer that was never connected to the internet. Samples of HM used in this experiment were rejected when excessive bacterial counts were recorded, and permission to use donor's HM for this study was given by all donors. Prior to use, frozen HM was placed in a refrigerator, thawed overnight, and subsequently, stirred well by hand. The experiment was performed in the research laboratory of the Department of Pediatrics, Showa University School of Medicine, Japan.

Data Analysis

Treatment of HM with MW heating

A MW oven (DR-D278B; Twinturbo Co. Ltd, Niigata, Japan) was used for the experiment. After MW treatment, HM samples were stored at -20 °C until further use.

Experiment 1

Milk samples (100 mL) were placed in 160-mL glass bottles and heated using a MW oven for 60 s at 200 and 500 W.

Experiment 2

Milk samples (50 and 100 mL) were placed in 160-mL glass bottles, followed by heating for 60 s at 500 W.

Determination of HCMV-DNA copies

Determination of the number of HCMV-DNA copies was performed as previously described, with minor modifications [4]. Briefly, DNA was extracted from HM using a QIAamp DNA mini kit (Qiagen, Hilden, Germany), and a real-time polymerase chain reaction (real time-PCR) was performed using LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland).

Investigation of CMV infectivity titer

Investigation of CMV infectivity titer was performed using median tissue culture infectious dose (TCID50). The HFL-III cells were propagated in 15% fetal bovine serum supplemented with DMEM/Ham's F-12 medium. Cells were maintained in 5% CO₂ and 37 °C in an incubator. The HM samples were centrifuged at 8,000 g for 5 min, and the supernatant was collected for determining the infectious viral titer. The supernatants of HM obtained after centrifugation (0.1 mL) were inoculated onto susceptible cell monolayers and incubated for 90 min to allow viral attachment to cells. After 14 days of incubation, TCID50/mL was measured to quantify the CMV infectivity titer.

Results

Experiment 1

CMV-DNA was detected in all CMV-positive mother's milk samples and was below the detection limit in CMV-negative mother's milk samples (Table 1). After 200 W or 500 W of MW treatment, the number of CMV-DNA copies did not significantly differ from that of control samples (Table 1). The TCID50/mL value was zero for CMV-negative mother's milk samples, and infectious virus was not detected. However, the infectious virus was determined in seven of eight samples of non-treated CMV-positive mother's milk after freezing at -20°C. After 200 W of MW heat treatment, TCID50/mL decreased in five out of eight samples of CMV-positive mother's milk (Table 1). Although a significant decrease was observed in TCID50/mL after 200 W MW heat treatment of milk samples for 60 s (Wilcoxon rank sum test, p=0.036) compared to that in control samples, the infectious CMV still persisted in the treated samples. A similar

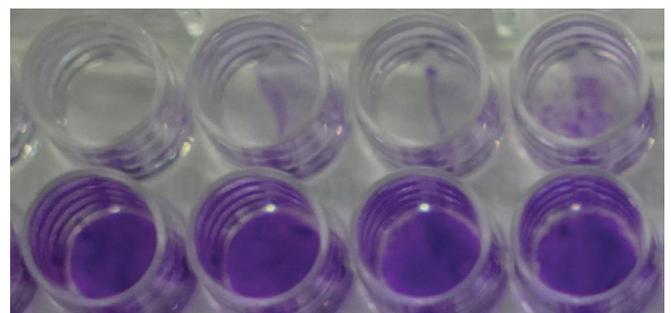


Figure 1: CMV infected cells

result was obtained after 500 W MW heat treatment. Therefore, we decreased the volume of milk to determine whether a smaller volume improved the prevention of CMV infection following treatment with the same MW heat setting (Experiment 2).

Experiment 2

The volume of HM was reduced from 100 mL to 50 mL, followed by MW heat treatment. CMV-DNA was detected in all the milk samples used in experiment 2, with viral titers ranging from 2,840 to 117,467 copies/mL (Table 2). After 500 W, 60 s of MW heat treatment, the number of CMV-DNA copies did not differ significantly among treated or control samples (Table 2). The TCID50/mL was 100 for all non-treated HM samples in experiment 2 and CMV was not inactivated by freezing at -20 °C. After 100 mL of HM was treated with MW heating at 500 W for 60 s TCID50/mL decreased in 6 of 10 CMV-positive milk samples. In contrast, TCID50/mL decreased in all 12 CMV-positive samples with 50 mL volume after exposure to 500 W for 60 s. Thus, MW heat treatment inactivated CMV with a decrease in TCID50/mL; infectivity of CMV was completely lost in 4 of 12 samples of HM with 50 mL volume following MW heat treatment for 60 s. In total, 17 HM samples (100 mL) were treated with MW heating at 500 W for 60 s, resulting in significant

decrease in TCID50/mL (Wilcoxon signed rank test, $p < 0.001$) across experiments 1 and 2. A significant decrease in TCID50/mL (Wilcoxon signed rank test, $p < 0.001$) was also demonstrated when 50 mL of HM was treated with MW heating (500 W) for 60 s. When we compared the decrease in TCID50/mL between 50 mL and 100 mL samples, a significantly higher decrease was observed in the 50 mL samples than that in the 100 mL samples (Wilcoxon signed rank test, $p = 0.016$).

Number of CMV-DNA copies in HM samples was same that we reported in our previous study [8]. The number of CMV-DNA copies at baseline did not change even if MW heat treatment was performed. This result supports our previous study [4]. In addition, the association was not recognized between DNA copy number and CMV infectivity titer.

Discussion

In this study, we demonstrated that infectious CMV persisted even if HM samples were kept frozen for more than one month. Furthermore, we demonstrated that MW heat treatment with 200 W or 500 W can prevent CMV infection to some extent. We have already reported that MW heating of formula milk, to which CMV was artificially added, at 500 W for 40 s was effective in

Table 1: The number of HCMV-DNA copies and the value of TCID50 in the human milk (100ml) after 200W/500W for 60s.

Sample	Number of CMV-DNA copies per milliliter			TCID50 per milliliter		
	No treat	200 W treat	500 W treat	No treat	200 W treat	500 W treat
A	679 ± 73	1352 ± 96	1281 ± 310	100	10	10
B	27300 ± 1229	3680 ± 1060	1056 ± 247	100	10	10
C	10760 ± 510	7087 ± 136	13030 ± 1811	100	10	10
D	1649 ± 271	1183 ± 351	1603 ± 217	10	10	10
E	1952 ± 604	1071 ± 323	1633 ± 993	0	0	0
F	2145 ± 1684	3636 ± 2323	3975 ± 2022	10	0	0
G	1336 ± 115	1300 ± 616	1083 ± 408	10	10	10
H	2329 ± 1613	2327 ± 446	2980 ± 225	10	0	0
I	< 200	ND	ND	0	ND	ND
J	< 200	ND	ND	0	ND	ND

ND: not done

Table 2: The number of CMV-DNA copies and the value of TCID50 in the human milk with 500W for 60s (50ml and 100ml)

Sample	Number of CMV-DNA copies per milliliter			TCID50 per milliliter		
	No treat	50 mL treat	100 mL treat	No treat	50 mL treat	100 mL treat
K	3367 ± 151	3373 ± 31	ND	100	10	ND
L	108667 ± 14257	117467 ± 29223	73267 ± 60802	100	10	10
M	5393 ± 1068	3120 ± 647	3793 ± 1437	100	10	ND
N	45533 ± 8778	40067 ± 4163	35067 ± 5558	100	0	0
O	28600 ± 6762	23200 ± 3650	37400 ± 4915	100	10	100
P	4500 ± 1733	4173 ± 978	3387 ± 220	100	0	10
Q	5307 ± 808	2840 ± 410	3560 ± 92	100	0	10
R	4440 ± 1193	2893 ± 527	2947 ± 613	100	0	10
S	3267 ± 1196	5927 ± 2400	2827 ± 437	100	10	100
T	3680 ± 260	4426 ± 338	4220 ± 253	100	10	100
U	23467 ± 2203	20927 ± 2024	21473 ± 8192	100	10	100
V	30400 ± 721	9873 ± 471	30733 ± 2715	100	10	10

ND: not done

preventing CMV infection. There was a discrepancy between the results of our previous study and this study. One of the reasons is that formula milk was heated to a higher temperature than HM with the same MW heat setting and for the same duration. Consequently, even if the same volume of HM is heated with 500 W for 60 s, the temperature attained is less than 60 °C. Furthermore, in our previous study, we added live HCMV to formula milk and then treated with MW heating, but in this study, we used HM expressed by CMV IgG positive donors. Reactivated CMV in HM might have better viability compared to the HCMV Towne strain. In general, viral agents in HM show lower rates of infection compared to those in buffers [9]. Our experiments demonstrated that CMV cell infection could be prevented to some extent by treatment with 500 W 60 s MW heating for 100 mL samples of HM. When we decreased the volume of milk from 100 mL to 50 mL, the effectiveness of MW irradiation in reducing viral titer was significantly larger. We have previously demonstrated that the levels of SIgA, lactoferrin, and TGF-beta in 100 mL of HM samples were not affected by irradiation with 500 W for 60 s [5]. The temperature of 50 mL HM samples increased significantly compared to that for 100 mL samples at the same MW heat settings. Therefore, the bioactive properties in HM are decreased more in 50 mL milk samples. We always must consider a balance between the benefit and risk whenever we utilize a new intervention. We have previously found that there was some decrease even with 200 W 60 s MW heat treatment that did not lead to a loss of bioactive properties in HM milk samples [5]. Furthermore, rise in temperature of HM samples after exposure to 200 W for 60 s is between 22.8 and 24.2 °C. Although the temperature itself does not rise enough to inactivate CMV, some positive effects remain, probably due to the power of MW radiation. A 60 s treatment of 100 mL HM samples using either 200 W or 500 W did not reduce the CMV titer, and therefore further examination is required to establish a means for complete prevention of CMV infection. However, a significant fraction of bioactive substances in HM would be lost following application of higher power MW. To decrease CMV infection as well as to minimize the loss of bioactive properties in HM, we suggest investigating a combination of 100 mL of milk and 500 W for 60 s using randomized control trials to establish the clinical prevention of CMV transmission through HM.

Ethics approval and consent to participate

This study was approved by Showa University Research Ethics Review Board (approval number: 2714)

Consent for publication

We obtained written consent for publication from participants.

Availability of data and materials

Competing interests

We do not have any competing interests.

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Authors' contributions

Provide individual author contribution

Conception and design of study

K Mizuno; acquisition of data: K Mizuno and C Kohda; analysis and/or interpretation of data: C Kohda, and H Den.

Drafting the manuscript

K Mizuno, H Den, and C Kohda; revising the manuscript critically for important intellectual content

Approval of the version of the manuscript to be published (the names of all authors must be listed)

K Mizuno, C Kohda, and H Den, N agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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