Flash-Heat Inactivation of HIV-1 in Human Milk

A Potential Method to Reduce Postnatal Transmission in Developing Countries

Kiersten Israel-Ballard, MPH, Richard Donovan, PhD,† Caroline Chantry, MD,‡ Anna Coutsoudis, PhD,§ Haynes Sheppard, PhD,† Lindiwe Sibeko, MSc,‖ and Barbara Abrams, DrPH**

Background: Up to 40% of all mother-to-child transmission of HIV occurs by means of breast-feeding; yet, in developing countries, infant formula may not be a safe option. The World Health Organization recommends heat-treated breast milk as an infant-feeding alternative. We investigated the ability of a simple method, flash-heat, to inactivate HIV in breast milk from HIV-positive mothers.

Methods: Ninety-eight breast milk samples, collected from 84 HIV-positive mothers in a periurban settlement in South Africa, were aliquoted to unheated control and flash-heating. Reverse transcriptase (RT) assays (lower detection limit of 400 HIV copies/mL) were performed to differentiate active versus inactivated cell-free HIV in unheated and flash-heated samples.

Results: We found detectable HIV in breast milk samples from 31% (26 of 84) of mothers. After adjusting for covariates, multivariate logistic regression showed a statistically significant negative association between detectable virus in breast milk and maternal $CD4^+$ T-lymphocyte count ($P = 0.045$) and volume of breast milk expressed ($P = 0.01$) and a positive association with use of multivitamins ($P = 0.03$). All flash-heated samples showed undetectable levels of cell-free HIV-1 as detected by the RT assay ($P < 0.00001$).

Conclusions: Flash-heat can inactivate HIV in naturally infected breast milk from HIV-positive women. Field studies are urgently needed to determine the feasibility of in-home flash-heating breast milk to improve infant health while reducing postnatal transmission of HIV in developing countries.

Key Words: breast milk, flash-heat, heat-treat, HIV, mother-to-child transmission, pasteurization

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It is estimated that approximately 40% of the 700,000 children who become infected with HIV each year contract the virus by means of prolonged breast-feeding.[1,2] Completely avoiding breast-feeding is not an option for many HIV-positive mothers in resource-poor areas, however, because of the cost of infant formula, unsafe water, unsanitary conditions, and sociocultural factors. In addition, the bioactive immune properties of breast milk confer protection to the infant, resulting in decreased rates of morbidity and mortality attributable to diarrheal, respiratory, and other infections compared with formula-fed infants.[3–6]

In light of this, current World Health Organization (WHO) recommendations state that when replacement-feeding options are acceptable, feasible, affordable, safe, and sustainable (AFASS), avoidance of all breast-feeding is recommended; otherwise, exclusive breast-feeding is recommended for the first 6 months of life, followed by weaning only if a nutritionally adequate and safe diet is maintained.[5,7,8] This recommendation is based on several studies that suggest the risk of HIV transmission among infants exclusively breast-fed was lower than that of those fed a mixture of breast milk and other liquids or solids.[9,10]

Modifications to breast-feeding are recommended by the WHO to reduce the risk of HIV transmission while providing breast milk's immune properties to protect the infant against other common childhood infections. One recommended alternative is manually expressed heat-treated breast milk.[7] The WHO lists 2 heating methods: (1) direct boiling, shown to cause significant nutritional damage,[11] and (2) pasteurization. The pasteurization method most commonly used in breast milk banks is Holder pasteurization (62.5°C for 30 minutes), which has been reported to inactivate HIV while retaining most of breast milk's protective elements;[12–15] however, it requires temperature gauges and timing devices that are unavailable in most at-risk communities.[16]

Flash-heat is a recently developed, simple pasteurization method that a mother in a developing country could implement over an outdoor fire or in her kitchen. Our pilot data suggest that the flash-heat method is capable of inactivating cell-free clade C HIV-1 in "spiked" breast milk samples from healthy

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From the *Division of Epidemiology, School of Public Health, University of California, Berkeley, CA; †Viral and Rickettsial Disease Laboratory, California Department of Health Services, Richmond, CA; ‡Department of Pediatrics, University of California Davis Medical Center, Sacramento, CA; §Department of Paediatrics and Child Health, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, Durban, South Africa; and ‖McGill University School of Dietetics and Human Nutrition, Montreal, Quebec, Canada.

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Reprints: Kiersten A. Israel-Ballard, MPH, School of Public Health, University of California, 140 Earl Warren Hall #7360, Berkeley, CA 94720-7360 (e-mail: ballardk@berkeley.edu).

mothers in the United States, while retaining most of the milk's nutritional and antimicrobial value.[17,18] The objective of this study was to confirm that flash-heat can inactivate HIV in naturally infected breast milk from HIV-positive mothers.

METHODS

HIV-positive breast-feeding mothers not currently receiving antiretrovirals or antibiotics were recruited during postnatal clinic visits at an informal periurban settlement in Durban, South Africa, between October 2004 and July 2005. Eighty-four women agreed to participate in this study; after giving written informed consent, they provided a blood sample and gave a total of 98 breast milk samples (some women donated additional breast milk samples on subsequent visits). The women were instructed to wash their hands with soap and water and then manually express 75 to 150 mL of breast milk into sterile, locally obtained glass jars that were provided. Lactation consultants were available if mothers needed assistance with manual expression. Breast milk samples were immediately placed in an ice-water bath and transported to the laboratory. Fifty milliliters of each expressed breast milk sample was aliquoted to be flash-heated in the same glass jar, and the remaining volume was aliquoted to be used as an unheated control. Laboratory analyses of plasma and breast milk samples were performed at the Nelson Mandela School of Medicine, University of KwaZulu-Natal in Durban, South Africa, and the Viral and Rickettsial Disease Laboratory at the California Department of Health Services in Richmond, California, respectively.

The flash-heat method has been described elsewhere.[17] Briefly, 50 mL of expressed breast milk in an uncovered sterile 16-oz (455-mL) commercial glass food jar was placed in 450 mL of water in a Hart brand 1-qt aluminum pan (Hendler and Hart, Cape Town, South Africa) purchased locally. Water and milk were heated together over a single-burner butane stove, used to imitate the intense heat of a fire, until the water reached 100°C and was at a rolling boil. The breast milk was immediately removed from the water and allowed to cool to 37°C. Temperature data were collected at 15-second intervals using thermometer probes (Cole-Palmer Digi-Sense DuaLogR Thermocouple Thermometers, Vernon Hills, IL). Flash-heating typically reached temperatures greater than 56°C for 6 minutes 15 seconds and peaked at 72.9°C. Flash-heated and unheated breast milk samples were then divided into 1.5-mL aliquots and immediately stored at −80°C. Samples were shipped frozen on dry ice to the California Department of Health Services Viral and Rickettsial Disease Laboratory for viral analysis.

To determine which samples initially had detectable HIV-1, we performed TaqMan Real Time-RNA-PCR (TaqMan RT-PCR, Applied Biosystems, Foster City, CA), which has a sensitivity of 50 RNA copies/mL, on the 98 unheated breast milk samples. RNA was extracted using the Viral RNA Kit (Qiagen, Valencia, CA). The TaqMan RT-PCR assay methods have been described elsewhere.[19] Because polymerase chain reaction (PCR) assays detect only viral nucleic acid and do not distinguish between viable and nonviable HIV, we used an alternate assay to assess the inactivation of HIV remaining after heat treatment.[17] Because previous studies have shown the reverse transcriptase (RT) enzymatic activity assay to correlate well with RNA quantification[20,21] and because functional RT is required for cellular infection, we chose quantitative measurement of RT activity as a marker for the presence of viable HIV in heated versus unheated breast milk (ExaVir Quantitative HIV-Reverse Transcriptase Load Kit; Cavidi, Uppsala, Sweden). The sensitivity of this RT assay was 400 copies/mL. To apply this methodology to breast milk, the manufacturer's instructions for plasma were followed, with the exception of additional readings at 30-minute intervals starting from time 0 through 4 hours and then overnight. Plasma $CD4^+$ T-lymphocyte assays were performed.

We used the Student t test, χ^2 test of independence, and bivariate and multivariate logistic regression to analyze and compare demographic data from the 84 recruited women. We identified variables a priori that were potential predictors of breast milk viral load and volume, based on previous evidence[22] and theoretic assumptions. Mothers were categorized as having detectable or undetectable breast milk HIV. For mothers who gave multiple samples, this was based on the sample from their initial visit only, because all maternal characteristics, including $CD4^+$ T-lymphocyte count and sociodemographic data, were collected at this visit. For logistic regression, all variables were analyzed as continuous, with the exception of maternal $CD4^+$ T-lymphocyte counts, which were assigned binary categoric values of $<$ or >500 cells/mm^3. For analysis of HIV inactivation and to compare concentrations of HIV in unheated versus flash-heated breast milk, we used the Sign test. RT levels less than the assay limit of quantification (400 copies/mL) were replaced by an imputed value of 400 copies/mL to quantify the undetectable levels of HIV conservatively. All statistical analyses were performed using STATA, version 8.0 (Stata Corporation, College Station, TX).

Our study was approved by the Committees for the Protection of Human Subjects at the Universities of California at Berkeley and Davis and the University of KwaZulu-Natal, Durban, South Africa.

RESULTS

Table 1 shows the maternal characteristics and sociodemographic data from the 84 HIV-positive mothers who were enrolled in this study and compares the unadjusted associations between mothers with detectable HIV in their breast milk versus those with undetectable HIV. If women provided more than 1 breast milk sample, only the first sample provided was included for this analysis. We found detectable HIV, as determined by the RT assay, in breast milk samples from 31% (26 of 84) of mothers.

We investigated the association between mothers with detectable HIV in their breast milk (>400 HIV copies/mL) and maternal characteristics that were selected a priori. Bivariate analyses showed statistically significant associations between detectable breast milk viral load and maternal age (odds ratio [OR] = 1.10, 95% confidence interval [CI]: 1.01 to 1.21), $CD4^+$ T-lymphocyte count (OR = 0.21, 95% CI: 0.07 to 0.63), use of multivitamins (OR = 4.70, 95% CI: 1.23 to

TABLE 1. Characteristics of Mothers With and Without Detectable Breast Milk HIV in Unheated Controls

Characteristic	All Enrolled Mothers (n = 84)	Mothers With No Detectable HIV in Unheated EBM (n = 58)	Mothers With Detectable HIV in Unheated EBM (n = 26)	*P**
Maternal age (y), mean (SD)	25.8 (5.4)	24.9 (5.7)	27.8 (4.5)	0.02
Maternal $CD4^+$ count (cells/mm^3), mean (SD)	502.1 (234.8)	551.1 (230.6)	398.4 (212.3)	0.01
Maternal HgB (g/dL), mean (SD)	11.6 (1.5)	11.7 (1.6)	11.2 (1.2)	0.17
Maternal weight (kg), mean (SD)	66.7 (10.8)	66.2 (9.8)	68.0 (13.0)	0.49
Maternal height (m), mean (SD)	1.6 (0.1)	1.6 (0.1)	1.6 (0.1)	0.51
Maternal BMI (kg/m^2), mean (SD)	26.8 (4.0)	26.7 (3.8)	27.1 (4.4)	0.65
Maternal triceps skinfold (mm), mean (SD)	14.8 (5.5)	14.8 (5.9)	14.8 (4.4)	0.96
Currently taking antibiotics, n (%)				0.81
Yes	4 (4.8)	3 (5.2)	1 (3.9)	
No	75 (89.3)	52 (89.7)	23 (88.5)	
Missing	5 (6.0)	3 (5.2)	2 (7.7)	
Currently taking multivitamins, n (%)				0.02
Yes	11 (13.1)	4 (6.9)	7 (26.9)	
No	70 (83.3)	51 (87.9)	19 (73.1)	
Missing	3 (3.6)	3 (5.2)	0 (0.0)	
Amount expressed (mL), mean (SD)	87.1 (17.0)	91.0 (18.4)	78.2 (7.9)	0.001
Time for manual expression (min), mean (SD)	74 (102.9)	68 (91.2)	66 (52.2)	0.92
Mode of current infant feeding, n (%)				0.55
EBF	65 (77.4)	45 (77.6)	20 (76.9)	
PBF	2 (2.4)	2 (3.5)	0 (0.0)	
MF	16 (19.1)	10 (17.2)	6 (23.1)	
Missing	1 (1.2)	1 (1.7)	0 (0.0)	
Mode of infant feeding from previous child, n				0.27
EBF	17 (20.2)	11 (19.0)	6 (23.1)	
PBF	0 (0.0)	0 (0.0)	0 (0.0)	
MF	33 (39.3)	25 (43.1)	8 (30.8)	
Formula	3 (3.6)	1 (1.7)	2 (7.7)	
Missing	31 (36.9)	21 (36.2)	10 (38.5)	
History of manual expression, n (%)				0.31
Yes	29 (34.5)	18 (31.0)	11 (42.3)	
No	45 (53.6)	33 (56.9)	12 (46.2)	
Missing	10 (11.9)	7 (12.1)	3 (11.5)	
Electricity, n (%)				0.31
Yes	16 (19.1)	12 (20.7)	4 (15.4)	
No	30 (35.7)	18 (31.0)	12 (46.2)	
Missing	38 (45.2)	28 (48.3)	10 (38.5)	
Access to a refrigerator, n (%)				0.77
Yes	15 (17.9)	11 (19.0)	4 (15.4)	
No	59 (70.2)	41 (70.7)	18 (69.2)	
Missing	10 (11.9)	6 (10.3)	4 (15.4)	
Piped water source, n (%)				0.59
Piped inside	26 (31.0)	16 (27.6)	10 (38.5)	
Public tap	24 (28.6)	16 (27.6)	8 (30.8)	
Piped in yard	24 (28.6)	18 (31.0)	6 (23.1)	
Missing	10 (11.9)	8 (13.8)	2 (7.7)	
Fuel for cooking, n (%)				0.74
Paraffin	56 (66.7)	37 (63.8)	19 (78.1)	
Electricity	17 (20.2)	12 (20.7)	5 (19.2)	
Gas	1 (1.2)	1 (1.7)	0 (0.0)	
Missing	10 (11.9)	8 (13.8)	2 (7.7)	

TABLE 1. (*continued*) Characteristics of Mothers With and Without Detectable Breast Milk HIV in Unheated Controls

Characteristic	All Enrolled Mothers (n = 84)	Mothers With No Detectable HIV in Unheated EBM (n = 58)	Mothers With Detectable HIV in Unheated EBM (n = 26)	*P**
Times of cooking per day, n (%)				0.66
1 time	47 (56.0)	33 (59.9)	14 (53.9)	
2 times	18 (21.4)	14 (24.1)	4 (15.4)	
3 times	2 (2.4)	1 (1.7)	1 (3.9)	
Missing	17 (20.2)	10 (17.2)	7 (26.9)	
Infant age (wk), mean (SD)	13.4 (9.5)	12.8 (9.8)	14.6 (8.6)	0.43
Birth weight (g), mean (SD)	3091.8 (475.9)	3088.3 (516.7)	3100.0 (375.8)	0.92

*Paired Student t test for continuous variables and χ^2 test of independence for categoric variables.
BMI indicates body mass index; EBF, exclusive breast-feeding; HgB, hemoglobin; MF, mixed feeding; PBF, predominantly breast-feeding.

17.88), and volume of breast milk expressed (OR = 0.93, 95% CI: 0.88 to 0.97). These variables, along with other potential predictors of breast milk viral load, were retained in our final model. In the multivariate logistic regression analysis shown in Table 2, controlling for maternal hemoglobin, age, height, weight, triceps skinfold thickness, $CD4^+$ T-lymphocyte counts, use of multivitamins, volume of breast milk expressed, and infant age, we found a statistically significant association between the odds of detectable HIV in breast milk and a decrease in maternal $CD4^+$ T-lymphocyte count ($P = 0.045$) and the volume of breast milk expressed ($P = 0.006$) and an increase in multivitamin use ($P = 0.03$). Multivariate linear regression was used to determine if infant age or mode of infant feeding was a significant predictor of the volume of breast milk expressed; a significant association was not observed, whether or not the previous covariates were retained in the model.

Of the total 98 breast milk samples tested before heating, 3 samples were positive for HIV RNA by TaqMan RT-PCR but were less than the level of RT detection. Thirty samples were positive for HIV RNA by TaqMan RT-PCR and for RT by the RT assay. Thus, the RT assay was effective in determining the viral status of 91% of the breast milk samples known to be positive by TaqMan RT-PCR.

TABLE 2. Multivariate Logistic Regression Analysis Results Showing the Adjusted Relation Between Maternal Characteristics and Detectable HIV in Breast Milk

Breast Milk Viral Load >400 Copies/mL	OR (95% CI)
Maternal age (y)	1.03 (0.91 to 1.17)
Maternal $CD4^+$ count (>500 cells/mm^3)*	0.18 (0.03 to 0.96)
Maternal HgB (g/dL)	0.65 (0.40 to 1.05)
Maternal weight (kg)	1.07 (0.98 to 1.16)
Maternal height (cm)	1.02 (0.92 to 1.13)
Maternal triceps skin fold (mm)	0.95 (0.82 to 1.10)
Multivitamins used*	15.72 (1.26 to 195.53)
Amount of breast milk expressed (mL)*	0.90 (0.83 to 0.97)
Infant age at collection (wks)	1.04 (0.93 to 1.17)

*Statistically significant results.
HgB indicates hemoglobin.

To compare the impact of flash-heat on breast milk viral load, we assayed the unheated and flash-heated aliquots of these 30 samples using the RT assay. The 30 unheated breast milk samples had an arithmetic mean of 8266 HIV copies/mL (SD = 15,376) and a mean log of 3.45 HIV copies/mL (SD = 0.586); however, all the corresponding flash-heated samples (100%) showed undetectable levels of HIV in the RT assay (Fig. 1; see Table 2). This difference in viral loads between the 30 unheated and corresponding flash-heated breast milk samples was highly statistically significant ($P < 0.00001$).

DISCUSSION

These data demonstrate that a simple pasteurization method, flash-heat, can inactivate cell-free HIV-1 in naturally

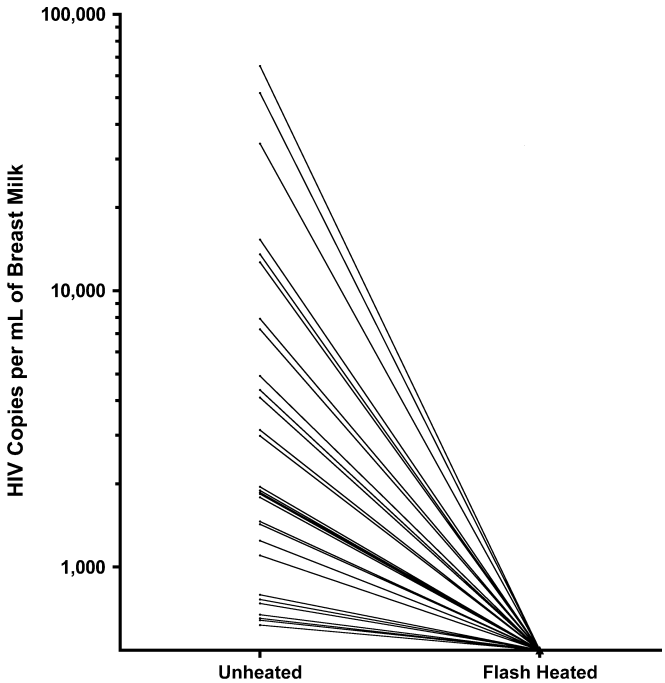

FIGURE 1. Log scale comparison of copies/mL of cell-free HIV-1 as detected by RT activity in unheated versus flash-heated naturally infected breast milk samples (n = 30).

infected breast milk from HIV-positive women. After flash-heating, HIV was undetectable by the RT assay in all 30 samples that had detectable HIV in unheated controls. This finding confirms our previous pilot data, which showed that flash-heat was capable of inactivating much greater concentrations of HIV in breast milk spiked with cell-free clade C HIV-1.[17]

We have previously shown that TaqMan RT-PCR assays are not useful in differentiating active virus versus inactive viral fragments,[17] a problem described previously in evaluating heat resistance of other viruses.[23] Similarly, studies have shown that coculture, although considered the "gold standard" for determining infectivity, is not an effective method for demonstrating inactivation of HIV in breast milk because of the assay's inherent insensitivity,[24] particularly in breast milk, because of its antiviral activity. Indeed, such data and our experience with HIV isolation suggest that only a small fraction (if any) of the 30 RT-positive samples would have been positive by HIV isolation cocultures. Thus, neither TaqMan RT-PCR nor coculture is an appropriate method for assessing HIV activity that remains after heating. The RT assay allowed us to compare a surrogate of infectious HIV in unheated and flash-heated breast milk. Although the lower limit of detection of the RT assay is 400 HIV copies/mL and, theoretically, a minimal amount of infectivity could remain in the samples showing undetectable levels of RT, the RT assay has greater sensitivity than would coculture, and was thus our method of choice. Moreover, even in samples "spiked" with high concentrations of HIV, our previous study showed undetectable levels of RT after heating.[17] The RT enzyme as a proxy for HIV infectivity could be an underestimate of viral inactivation because of the greater heat resistance of enzymes compared with surface proteins and the viral envelope, which, if disrupted, first could render the virus particle noninfectious before destruction of the RT enzyme.[25]

After controlling for other maternal characteristics, detectable breast milk viral load was significantly associated with an increased likelihood of multivitamin use and lower $CD4^+$ T-lymphocyte counts and volumes of breast milk expressed. Although we found only borderline statistical significance, previous studies have shown lower maternal $CD4^+$ T-lymphocyte counts to be predictors of increased breast milk viral load and a higher probability of transmission of HIV.[22] Likewise, previous studies have shown multivitamin intake to be protective against HIV progression;[26] yet, we found an increased risk of breast milk viral load with multivitamin use. Wide confidence intervals associated with multivitamin use, however, limit our ability to interpret these data. Factors not included in our data collection, such as fatigue and stress, may have contributed to the amount of breast milk expressed, and thus limit our understanding of the lower milk volumes expressed by some participants.[15] It is noteworthy that regardless of our findings, expressed milk volume was still substantial from mothers with a detectable breast milk viral load, with a mean of >78 mL. Moreover, there was no difference in the average time it took these mothers to express their breast milk manually, suggesting that this process was no more difficult for them than for the other mothers with less advanced disease.

Further investigation to confirm the nutritional, immunologic, and antimicrobial safety of flash-heated breast milk is needed, although preliminary data are promising.[17,27] This study investigated only 1 standardized heating protocol, using 50 mL of breast milk heated in a 450-mL water bath over a butane stove. Additional research is needed to determine if variations in milk and water volumes, jar or pan size or shape, heat source, and even altitude have an impact on the effectiveness of flash-heat. Although the WHO recommends heat treatment, if the flash-heat method is used in communities, we caution that the heating protocol described here should be strictly adhered to until additional field tests and thermal inactivation studies are completed and we better understand the margin of heating error allowable to ensure HIV destruction. Furthermore, the RT assay was limited to detection of cell-free virus only; however, recent data suggest that cell-associated HIV may play a more important role in transmission of HIV by means of breast-feeding than does cell-free virus.[28] More research is needed to address the impact of flash-heat on cell-associated HIV-1, although we hypothesize that cell-associated infectivity would be destroyed along with the cell under flash-heat conditions.

Although heat-treated breast milk may be used from birth, it may be most feasible during times of increased transmission risk, such as during mastitis[29] and, perhaps more practically, during or after the transition from exclusive breast-feeding to replacement feeds or the addition of complementary foods. Milk production would be well established at this point, and other complementary foods would then be an additional source of infant nutrition. Recent data have described several risks encountered during the weaning period. Inadequate feeding practices attributable to the lack of sufficient nutrition could result in impaired growth and malnutrition, especially without the immune protection from breast milk.[30,31] Moreover, a study in Zambia found a significant increase in breast milk viral load during the weaning period, suggesting that if a mother were to breast-feed during this time, the risk of transmission would be increased.[32] Perhaps heat-treated breast milk could be used during these high-risk times and could be viewed as a complementary food that is HIV-free, nutritious, affordable, and available.

In summary, our findings indicate that flash-heat is capable of inactivating cell-free HIV-1 in naturally infected breast milk from HIV-positive mothers. Field studies are urgently needed to determine the feasibility of flash-heating breast milk in a home setting and its ability to improve overall infant health while reducing the risk of postnatal HIV transmission in resource-poor settings.

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