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EFFECTS OF LIPOLYSIS OR HEAT TREATMENT  
ON HIV-1 PROVIRUS IN BREASTMILK

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LIPOLYSIS OR HEAT FOR HIV IN BREASTMILK

Key Words: HIV; breastmilk; DNA; provirus; heat treatment; developing countries;  
prevention; lipolysis; antiretroviral therapy; vertical transmission

ABSTRACT

Background: Transmission of HIV-1 infection through breastfeeding is associated  
with integrated DNA (provirus) in milk cells. Reduction of HIV-1 DNA in milk  
may lessen infectivity.

Purpose: To investigate efficacy of two methods available in developing  
countries to reduce HIV-1 proviral DNA in breastmilk.

Methods: Methods simulated field conditions; milk was heated by bringing to a  
boil, as over a cooking fire, and lipolysis was at room temperature. Four HIV+  
pregnant women were recruited for this pilot study, instructed to exclusively  
formula feed and stimulate milk production via pumping. Milk was collected twice  
weekly for three weeks and analyzed qualitatively for HIV-1 proviral DNA by  
polymerase chain reaction at three stages: 1) fresh, 2) after standing for six  
hours, and 3) after bringing to the boiling point.

Results: Seventeen samples from four mothers were analyzed. Fifteen of seventeen  
fresh samples (88%) had measurable HIV-1 proviral DNA despite all mothers having  
low or undetectable plasma viral loads. Lipolysis (standing at room  
temperature) for six hours did not destroy proviral DNA: 6/7 samples (83%) were  
positive for DNA after lipolysis. No samples of milk (0%, n=8) brought to a boil  
were positive for HIV-1 proviral DNA (p<.0001).

Conclusions: This preliminary evidence suggests: a) inherent lipolytic activity  
of fresh breastmilk is inadequate for destruction of HIV-1; b) bringing  
breastmilk to a boil may result in decreased HIV-1 infectivity; and c)

breastmilk cell-associated HIV-1 may not reflect plasma viral load. Nutritional value or possible bacterial contamination of milk treated in this manner was not assessed.

#### INTRODUCTION

HIV-1 can be vertically transmitted postpartum through breastfeeding (1). Risk of this transmission is variably reported; earlier reports estimate an additional 13% to 16% of infants not infected prenatally or during delivery will be infected if breastfed (2,3), and more recent reports reveal late (after 2.5 months) post-natal transmission by breastfeeding is approximately 3.2 per 100 child-years of breastfeeding (4). An interesting report indicates exclusively breastfeeding infants may not carry as high a risk for HIV-1 transmission as mixed feeding (5). The risk of postnatal transmission may increase with higher breastmilk viral loads or mastitis (6). The effect of antiretroviral therapy on HIV-1 in breastmilk or on postnatal transmission in breastfeeding dyads is unknown.

Current recommendations in developed countries for HIV-infected mothers are to exclusively formula-feed. Recommendations for developing countries were drafted in 1998 jointly by UNICEF, WHO and UNAIDS. These recommendations support use of alternatives to breastfeeding and emphasize the HIV-infected mother's right to be informed of the risks of breastfeeding and to choose an appropriate infant feeding method. They also support the mother's right to have access to breastmilk substitutes, and resources and information for their safe preparation (7). These recommendations have sparked controversy about the benefit-to-risk ratio of artificial breastmilk substitutes for both the uninfected and infected children in the developing world.

HIV-1 transmission has been associated with the presence of integrated viral DNA (provirus) in the mother's milk cells (8), although animal studies do support that orally ingested cell-free virus may be sufficient for viral transmission (9). The relative significance of cell-associated vs. cell-free virus in post-natal transmission via breastfeeding is unknown. Given the described associations of transmission with both integrated cellular HIV-1 proviral DNA and higher breastmilk viral loads, reduction of HIV-1 proviral DNA in breastmilk may lessen infectivity. In fact, because of technical challenges in culturing HIV-1 from breastmilk in the presence of inhibitors, recovery of HIV-1 provirus in breastmilk has been considered a reasonable surrogate for infectivity (8, 10). Pasteurization at 56.0 oC to 62.5oC for 30 minutes (Holder pasteurization) has been shown to effectively inactivate infectivity of both cell-free and cell-associated HIV-1 in liquid media (11) including breastmilk (12, 13), but has been technologically unavailable in the developing world. Boiling expressed breastmilk (time unspecified) is listed in the international draft guidelines as a viable option for HIV-infected mothers (7), although the effect on HIV-1 proviral DNA or infectivity is unknown. Other treatments for making breastmilk safer which are available to mothers in the developing world need further study.

There is evidence that allowing breastmilk to simply stand may enable the lipase to release free fatty acids which may break down some viral envelopes, as previously demonstrated with vesicular stomatitis virus, herpes simplex virus type-1, and visna virus (14, 15). Medium-chain saturated and long-chain unsaturated fatty acids are all highly active against enveloped viruses. The fatty acids affect the envelope causing leakage; at higher fatty acid concentrations, complete disintegration of the envelope and viral particles occurs (15). Lipolytic activity increases with breastmilk storage, and may not be significant with fresh milk (15, 16). Controversial results have been reported with HIV-1. One study demonstrates that human milk could inactivate 200-700 units/ml of HIV-1, and that milk concentrations as low as 10% had some anti-viral activity with titer reduction occurring rapidly and more efficiently at 22 oC or 37 oC than 0 oC (12). Alternatively, another study reports that room

temperature incubation of HIV-1 in breastmilk for 16 hours did not lead to quantitative loss of RNA extraction of cell-free virus (17).

The primary purpose of this pilot study was to investigate the efficacy of two methods available in the developing world to reduce HIV-1 proviral DNA in breastmilk: heat-treating maternal milk or allowing breastmilk to undergo lipolysis by standing for six hours. A secondary purpose was to measure HIV-1 proviral DNA in breastmilk (colostrum and mature milk) from mothers on combination antiretroviral therapy.

#### METHODS

HIV+ pregnant women receiving prenatal care between 8/98 and 7/99 at the University of Puerto Rico in San Juan, Puerto Rico were recruited. Institutional Review Board approval and informed consent were obtained. Four mothers agreed to study entry. Maternal laboratory data available included CD4+ cell count and plasma viral load between 0-18 weeks gestation, 19-31 weeks gestation, after 32 weeks gestation, at delivery and at 2 months postpartum. Information on antiretroviral therapy was collected.

After delivery, mothers were instructed to exclusively formula feed and to stimulate milk production via pumping several times daily. Milk was collected on site twice weekly for three weeks postpartum in order to obtain samples of both colostrum and mature milk. Colostrum was defined as milk obtained from day 1 to day 5 postpartum, transitional milk from day 6 to day 14 postpartum and mature milk thereafter. Not all mothers participated in every prenatal and postpartum visit; one mother donated only one milk sample after delivery. Milk samples were divided into three aliquots and analyzed qualitatively for HIV-1 proviral DNA and quantitatively for HIV-1 RNA by polymerase chain reaction (PCR) (Roche-Amplicor) after three treatments: 1) fresh, 2) after standing for six hours at room temperature, and 3) after bringing to the boiling point. Methods simulated field conditions. Milk was brought to a boil in a small water bath, as could be performed over a cooking fire. The milk and water were placed over the heat source simultaneously. Milk was removed from the heat and water upon reaching the boiling point. Lipolysis was at room temperature for six hours, as this was the maximal time it would be considered bacteriologically safe without refrigeration.

After the above treatments, milk was centrifuged at 710g for 20 minutes to yield three layers: lipids, aqueous supernatant and cellular fractions. The lipid portion was discarded, the supernatant was assayed for HIV-1 RNA by PCR and the cell pellet was resuspended in RPMI 1640 with glutamine and then recentrifuged at 300g for 10 minutes three times. Dry cell pellets at  $1 \times 10^6$  cells/ml were made for proviral DNA PCR testing. PCR testing for HIV-1 RNA and proviral DNA was performed according to manufacturer's instructions for standard commercial kits (Roche). All laboratory analyses were performed at the certified AIDS Clinical Trials Group laboratory at the University of Puerto Rico.

#### RESULTS

Seventeen samples from four mothers were analyzed. All four mothers were receiving combination antiretroviral therapy with nucleoside reverse transcriptase inhibitors zidovudine and lamivudine ("Combivir") throughout the study period. All four mothers had low or undetectable plasma viral loads; 12 of 17 plasma viral load analyses done prenatally, at delivery and postpartum were undetectable. One mother had undetectable viral load at delivery, but low viral loads of 2,158 and 12,877 copies/ml 1 month before and 2 months after delivery respectively. Two other mothers had lower levels of detectable virus at some visits. Similarly, maternal mean CD4+ cell count (s.d.) was 544 (112) cells/uL at delivery. Information from the 4 mothers is summarized in Table 1.

The quantitative RNA PCR assay performed on the breastmilk was invalid with the aqueous supernatant; an inhibitor was present in the milk.

Fifteen of seventeen fresh breastmilk samples (88%) had measurable HIV-1 proviral DNA in the cellular fraction. Lipolysis (standing at room temperature) for six hours did not destroy proviral DNA in the cellular sample: six of seven samples (83%) were positive for proviral DNA after lipolysis. No sample of milk (0%, n=8) brought to a boil had a cellular fraction which was positive for HIV-1 proviral DNA ( $p < .0001$ ). All eight of these samples were among the fifteen samples originally positive. These results are summarized in Table 2.

#### DISCUSSION

There are three important findings in this small study: 1) proviral HIV-1 DNA was destroyed by bringing the breastmilk to a boil; 2) proviral HIV-1 DNA was not destroyed by lipolysis for 6 hours at room temperature; 3) a high prevalence of cell-associated HIV-1 proviral DNA was detected in breastmilk despite combination antiretroviral therapy and low or undetectable plasma viral loads.

These findings are the first to document that HIV-1 proviral DNA is destroyed by bringing the expressed milk to a boil. Heretofore, it has been assumed that boiling breastmilk would destroy the HIV-1 virus, as HIV-1 is known to be heat sensitive, documented by destruction of infectivity with Holder pasteurization (12-14). In fact, boiling (duration unspecified) the breastmilk of HIV-infected mothers is listed as an alternative by the United Nations in the 1998 recommendations "HIV and Infant Feeding" (7). Nonetheless, healthcare providers in the developing world have urged studies to document the safety of this approach (18, 19). This method could be replicated in relatively impoverished and remote areas over cooking fires or stoves. The potentially negative nutritional impact of this treatment on the breastmilk was not assessed. Heat sensitive elements including immunoglobulins, lipase and other anti-infective properties may be altered. The effect of bringing breastmilk to a boil on its nutritional content needs to be studied. Bacteriologic activity in expressed milk which is batched and heated also needs to be studied. If refrigeration were unavailable, milk would need to be expressed and heated several times daily.

Lipolysis for 6 hours at room temperature was inadequate for destruction of HIV-1 proviral DNA. This is the maximum time a sample would be considered bacteriologically safe at room temperature and failure of adequate lipolysis within this time frame precludes this method in areas without refrigeration. This finding is not surprising given previous descriptions that anti-viral activity develops after milk storage allows lipase to release free fatty acids which then destroy the viral envelope (15); storage for 24 hours or more may be necessary (15, 16). This may account for previous discrepancies on the effect on HIV-1. It is important to note that an effect on cell-associated DNA is plausible, as the viral envelope is derived from host cell membranes and the fatty acids can be shown to also affect the cell membrane, causing cell lysis (15). Further investigation of the effect on HIV-1 proviral DNA of 24-48 hours storage at refrigerated temperatures is warranted for areas where this option is available.

Proviral DNA was documented in 88% of our 17 samples including 83% of the mature milk samples. Previous studies showed lower rates of HIV-1 in breastmilk: 70% of Haitian women were found to have HIV-1 proviral DNA in colostrum (20), 58% of breastmilk samples were described to have cell-associated HIV-1 in another report (10). The prevalence of cellular HIV-1 in milk from infected mothers in other reports has ranged from 20 to 47% and cell-free HIV-1 was found in 39% of samples (17).

The prevalence of positive samples in our study is particularly surprising given that the mothers

were on antiretroviral therapy and relatively immunocompetent as Nduati, et al. described a higher prevalence of HIV-1 infected cells in colostrum when maternal CD4+ cell counts were <400 cells/mm<sup>3</sup>, with an odds ratio, 3.1(10). There are several possible explanations. One possibility is that HIV-1 in breastmilk may not reflect the plasma viral load. Conflicting results regarding the relation between breastmilk and plasma HIV-1 viral levels have been previously published. Semba described a Spearman correlation between breastmilk HIV-1 load (performed on the aqueous portion) and plasma HIV-1 load of 0.47 in 134 women (6). Conversely, no correlation between plasma and extra-cellular breastmilk HIV-1 RNA viral levels was found recently in 42 women by You, et al. (21). Our observation would support the possibility that cell-associated virus in breastmilk may not reflect plasma viral levels, although our breastmilk assays were strictly qualitative. Of note, the study by You, et al. did not find a difference in extracellular breastmilk viral loads between transmitters and non-transmitters (21), even for 3 cases of suspected late transmission, in contrast to Semba's group which noted increased levels of virus in the aqueous breastmilk fraction in transmitting mothers (6). Unfortunately, neither of these groups examined the cellular fraction of the milk, where integrated provirus has previously been described to correlate with transmission in multiple studies (9, 22); although one study did not confirm this association (23). Another possible explanation of the high prevalence of HIV-1 provirus in the samples in our study is that HIV-1 proviral DNA is increased by some mechanism that results from exclusive pumping, perhaps increased cellularity. Finally, the ability to generalize from our very small sample size is limited.

Zidovudine and lamivudine have both been demonstrated to be excreted in breastmilk, a finding postulated to potentially reduce the viral load in breastmilk (24, 25). Whereas our assays of the cellular fraction were not quantitative, nearly all of the samples were positive despite the combined antiretroviral therapy of these mothers. This finding discredits the notion that antiretroviral therapy will significantly decrease the prevalence of cellular breastmilk infection. However, the presence of antiretroviral agents in breastmilk may potentially reduce postnatal transmission through postexposure prophylaxis of the infant, an effect which may occur independently of reducing breastmilk virus.

Presence of detectable HIV-1 provirus in the breastmilk despite low/undetectable plasma viremia may reflect productive infection of mammary epithelial cells, previously demonstrated to occur in vitro (26, 27). Macrophages and T cells in colostrum and early milk also demonstrate productive infection (27). Breast epithelial or macrophage cells may represent a reservoir for HIV-1 not easily amenable to eradication, similar to that described for other sites, e.g. B-lymphocytes or "resting" CD4 lymphocytes (28, 29). Similarly, plasma viral load does not predict lymph node virus, which is related to cell-associated DNA or RNA in the blood (30).

In conclusion, this study confirms the hypothesis that bringing expressed breastmilk to a boil does destroy HIV-1 proviral DNA and demonstrates that inherent lipolytic activity of fresh milk over a period of 6 hours is inadequate for HIV-1 proviral DNA destruction. Furthermore, it documents the high prevalence of HIV-1 infected cells in the breastmilk of women on combination antiretroviral therapy with low plasma viral loads and minimal immunosuppression. This suggests that the prevalence of cellular breastmilk HIV-1 infection may be independent of plasma viral load as well as antiretroviral therapy. These findings support current UNAIDS recommendations regarding home heat treatment of expressed breastmilk and further delineate that bringing the milk to a boil, rather than maintaining a boil, is adequate. Investigation on the nutritional impact of this method is needed.

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Table 1

Clinical and Breastmilk Sample Information from Four HIV-Infected Mothers in Study

Mother

1234Age (Years)23372131TreatmentZDV/3TCZDV/3TCZDV/3TCZDV/3TC  
 CD4+ Count (Cells/ml) >32 Weeks Gestation626609472626At  
 Delivery4445324977042 Months Postpartum887923545N/APlasma Viral Load (HIV RNA  
 Copies/ml)>32 Weeks Gestation155ND2158NDat DeliveryNDNDND1402 Months  
 Postpartum846ND12877NDSpecimens Positive for HIV-1 DNA/Total  
 TestedUntreated1/16/64/54/5LipolysisN/A3/42/21/1Heat-Treated\*0/10/40/10/2\*  
 Untreated Samples Positive ND= undetectable N/A = not available

Table 2

Qualitative HIV Proviral DNA Results in Expressed Breastmilk from Four HIV-Infected

Mothers, by Milk Treatment Group

Treatment Group

Number of

Samples

Positive for HIV

Proviral DNA (%)

None (Fresh)

17

88\*

Colostrum

5

100

Transitional

6

83

Mature

6

83

Lipolyzed

7

86

Heated

8

0\*

\*p < .0001

•PAGE •1•

•PAGE •4•