Flash-heat Inactivation of HIV-1 in Breast Milk

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Introduction

Mother-to-child transmission (MTCT) is responsible for approximately 90% of the 725,000 HIV infections that occur each year among the world's children, of which 90% are in sub-Saharan Africa[1]. In contrast to the estimated 200,000-350,000 infants who contract HIV through breastfeeding each year [2], WHO estimates that 1.5 million infants died because they were not breastfed [3]. We previously reported that Flash-heat (FH), a simple in-home pasteurization method for use in developing countries[4], is capable of inactivating cell-free HIV in "HIV-spiked" breast milk samples, while retaining the milk's nutritional value [5]. However, Becquart *et.al* has found that cell-associated HIV which includes latently infected resting CD4 T lymphocytes are a significant source of transmissible virus [6]. This is further supported by the occurrence of transmission to the infant that is seen even after controlling for low levels of cell-free virus [7].

➢ **Breast milk samples were spiked with "cell-associated virus" (infected cells) and cell free virus**

➢ **Studied Un-heat (UH) and post flash-heat (FH) breast milk infectivity by Peripheral Blood Mononuclear Cell (PBMC) and TZM-bl assays**

➢ **Examined the potential of cell-associated transmission by spiking PBMCs into breast milk and determined cell viability of UH and FH**

Methods

❑ **Sample collection**

- 5 breast milk samples were collected from healthy, volunteer mothers (not infected with HIV)
- Breast milk (EBM) was aliquoted into FH or UH controls.

❑ **Heat treatments**

- Simple method that could be implemented by mothers in kitchens or over a fire, was simulated (Figure 1)
  - ➢ 1 qt. aluminum pan from containing 450 ml of water
  - ➢ 16 oz. glass peanut butter jar for 50 ml breast milk
  - ➢ single burner butane stove to imitate intense heat of a fire

- Flash-heat protocol
  - ➢ Breast milk and water are heated together in water bath
  - ➢ When water boils, remove milk immediately from both the water and heat source
  - ➢ Typically, peak temperature 72.9ºC and temperature maintained above 56.0ºC for 6 min 15 sec

Figure 1. Flash-heat experiment using simple equipment

❑ **Virus**

- The virus used in all spiking experiments was HIV-1 clade C, strain ZA12.
- This virus was grown in PM-1 cells.

❑ **Flow Cytometry Protocol**

- UH and FH samples centrifuged at 400g, supernatant aspirated and washed twice with Hanks Balanced Salt solution.
- ~10^6 cells added to well of 96 well plate and centrifuged/washed with phosphate buffered saline (PBS)
- Stained with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit for 405 nm *excitation* from Invitrogen.
- Samples were read on 8-color BD LSRII flow cytometer
- 100,000 lymphocyte events were acquired and recorded

❑ **TZM-bl Infectivity Assay**

- UH and FH samples were serially diluted 10-fold in DMEM growth media (containing 15% FBS)
- 5ul of each dilution was added to a black, flat-bottom 96-well plate in quadruplicate
- 25ul of DMEM and 50ul of TZM-bl cells were added to each well for a total volume of 100ul.
- After an incubation at 37ºC for 48 hours, the adherent cells were washed with PBS.
- The cells were lysed using buffer Cell Culture Lysis Reagent (Promega Corp) and 50ul of Promega Luciferase Assay Substrate (TB282) was added to each well
- Luminescence was measured using a Topcount luminometer
- Wells that produced relative luminescence units (RLU) > 2.5x background were scored positive and the TCID50 was calculated by the Reed and Muench method.

❑ **Peripheral Blood Mononuclear Cell (PBMC) Infectivity Assay**

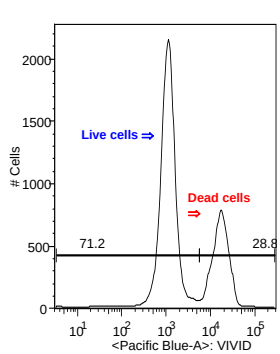
- EBM samples were spiked with up to 5.25 logs/100ul of virus. FH and UH samples were then diluted 10-fold prior to assay
- 100ul of each dilution was added in quadruplicate to a round bottom 96-well plate containing 250,000 cells/well
- The plate was incubated at 37ºC for 2 hours
- 100ul of RPMI-B growth media (containing FBS and IL-2) was added to each well.
- The assay was washed on day 3 twice with PBS and resuspended in 200ul of RPMI-B
- The assay was harvested on day 4 by adding Triton-X detergent to each well.
- Virus was quantified by using p24 antigen capture EIA (Zeptometrix)

❑ **Revised Peripheral Blood Mononuclear Cell Infectivity Assay**

- First three steps same as above
- After 2hr incubation at 37ºC the plate was washed twice with PBS and resuspended in RPMI-B and immediately harvested and quantified as described above

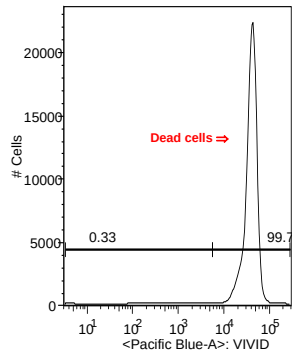
Results

❑ UH breast milk spiked with ~2 million lymphocytes/ml had a lymphocyte viability of 71% and after FH had a viability of 0.3% (Figures 2, 3)



Lymphocytes
Pre Heated Milk_PBMC_A2_A02.f
Event Count: 59480

Figure 2. UH breast milk lymphocyte viability measurement by flow cytometry indicating **71.2%** viability



Lymphocytes
Post Heated Milk_PBMC_A3_A03.
Event Count: 314094

Figure 3. FH breast milk lymphocyte measurement by flow cytometry indicating **99.7%** cell death

❑ TZM-bl infectivity assay showed an UH titer of ~10^7 TCID50/ml reduced to ~ 0 after FH (Figure 4)

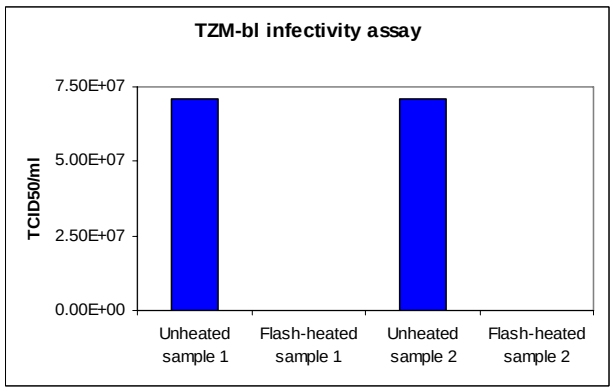


Figure 4. UH and FH milk infectivity by TZM-bl assay

❑ The PBMC infectivity assay showed decrease in virus of up to 3.75 logs/100ul and a residual of 1.5 logs/100ul of virus.

❑ The revised PBMC infectivity assay showed that there was no increase after 4 days of residual 1.5 logs/100ul virus.

Discussion

❑ The PBMC assay and detection by p24 antigen is a good way of measuring true infectivity and was able to show a decrease of up to 3.75 logs/100ul of virus after FH. However, residual p24 of approximately 1.5 logs remained.

❑ The follow-up Revised PBMC assay demonstrated that new infectious virus was not propagated in the course of the assay. Thus indicating that the remaining p24 is a residual inoculum artifact of this assay and does not indicate that there is residual infectivity after heating.

❑ The TZM-bl assay has a greater dynamic range than the PBMC/p24 assay due to not having the limitation of detecting defective virions and viral antigens in residual inoculum. This assay showed a complete loss of infectivity after FH.

❑ Flow cytometry measurements after FH demonstrated 99.7% of the cells were dead.

Conclusions

❑ **FH inactivates HIV-1 as confirmed by RT, PBMC and TZM-bl assays**

❑ **FH heat causes 99.7% cell death, thus predicting that latently infected lymphocytes will not transmit HIV after heating**

❑ **Flash-heat may be a safe infant feeding method for HIV positive mothers in developing countries who need alternatives to formula feeding, especially during times of high risk, such as during mastitis or the transition from exclusive breastfeeding to replacement foods.**

Acknowledgements

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References

1. UNAIDS/WHO. AIDS epidemic update. UNAIDS/05.19E. Geneva: UNAIDS, 2005.
2. WHO. Nutrition risk factors throughout the life course. Nutrition for Health and Development.
3. WHO. Consensus Statement: Held on behalf of the Inter-Agency Task Team (IATT) on Prevention of HIV Infections in Pregnant Women, Mothers and their Infants. WHO HIV and Infant Feeding Technical Consultation 2006, Geneva.
4. Israel-Ballard K, Donovan R, Chantry C, Coutsoudis A, Sheppard H, Sibeko L, Abrams B. Flash-heat inactivation of HIV-1 in human milk: a potential method to reduce postnatal transmission in developing countries. *J Acquir Immune Defic Syndr 2007 Jul 1;45(3):318-323.*
5. Israel-Ballard K, Coutsoudis A, Chantry CJ, Sturm AW, Karim F, Sibeko L, Abrams B. Bacterial safety of flash-heated and unheated expressed breast milk during storage. *J Trop Pediatr* 2006;52(6):399-405.
6. Becquart P, Petitjean G, Al Tabaa Y, Valea D, Huguet MF, Tuaillon E, Meda N, Vendrell JP, Van de Perre P. Detection of a large T-cell reservoir able to replicate HIV-1 actively in breast milk. AIDS 2006, 20: 1453-1454.
7. Rousseau CM, Ndauti RW, Richardson BA, John-Stewart GC, Mbori-Ngacha DA, Kreiss JK et al. Association of levels of HIV-1 infected breast milk cells and risk of mother-to-child transmission. *J Infect Dis* 2004; 190:1880-1888

Topic: C25 Interventions to prevent mother-to-child transmission.

Abstract: Flash-heat Inactivation of HIV-1 in Breast Milk

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Background: Heat-treated expressed breast milk (EBM) is recommended by WHO as an option to reduce vertical transmission of HIV. The objective of this study was to evaluate the effect of Flash-heat (FH), on cell-free and cell-associated HIV in breast milk.

Methods: Fresh EBM was collected from 5 healthy mothers in California and spiked with 6.6 $\log_{10}$ HIV copies per ml of cell-free HIV. Milk was aliquoted into unheated (UH) controls or FH (50mL EBM heated in 450mL water jacket until water boiled, then EBM removed), simulating field conditions with a glass peanut butter jar containing the heated milk in an aluminum pan over an open flame. We assayed for cell-free HIV by reverse-transcriptase (RT) and luciferase production in TZM-bl indicator cells. Cell-associated virus was studied by Peripheral Blood Mononuclear Cell (PBMC) assay. To assess HIV transmission via reactivation of latent provirus, lymphocytes were spiked into milk and cell survival after heating was evaluated by flow cytometry.

Results: Cell-free HIV in FH EBM was reduced by more than two logs as detected by RT, and 7 logs by TZM-bl assay. RT and PBMC results have limited interpretation, however, due to false positive controls and residual inoculum artifacts, respectively. Lymphocyte viability was reduced to 0.3%.

Conclusions: RT and TZM-bl results suggest inactivation of cell-free HIV in FH EBM. Cell-associated inactivation was seen in the PBMC assay and cell death seen by flow cytometry. FH may be a safe and affordable infant feeding option for HIV positive mothers in developing countries, especially during times of high risk, such as during mastitis or the transition from exclusive breastfeeding to replacement foods. Spiking studies are useful for demonstrating safety margins when treating samples with low naturally occurring levels of virus.

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