DEACTIVATION OF HUMAN IMMUNODIFICIENCY VIRUS IN BREAST MILK USING COPPER OXIDE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY

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BY

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DECLARATION

I hereby declare that the work submitted is my original work and has not been presented for any examinations.

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RESEARCH APPROVAL

This thesis has been submitted for examination with our approval as the University supervisors.

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DEDICATION

I dedicate this work to the many HIV positive women from recourse poor suburbs of Nairobi who have to bear the burden of poverty and disease to look for better ways and means of bringing up their children.

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I express my sincere gratitude to **Professor OMU ANZALA** my first supervisor, departmental head and teacher for his support throughout my trainings in the medical microbiology world and facilitating all the arrangements that made it possible for me to be involved in the Breast Milk Study.

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LIST OF ABBREVIATIONS:

AIDS	Acquired Immunodeficiency Syndrome
CD	Cluster of differentiation, which indicates a defined subset of cellular surface that identify cell type
CD4	Cell surface glycoproteins involved in viral entry
cMAGI	Multinucleated activation of galactosidase indicator cells
DMEM	Dulbecco's modified eagle medium
ELISA	Enzyme Linked Immuno Sorbent Assay
HIV	Human Immuno-deficiency Virus
MTCT	Mother-to-child transmission
MT2	Lyphocytes cell line permissive to T-trophic HIV-1 isolates
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RPMI	Roswell Park Memorial Institute media
UNFPA	United Nations Population Fund
UNICEF	United Nations Children Education Fund
WHO	World Health Organization

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ABSTRACT

Background: HIV transmission via breastfeeding has emerged as a serious lobal challenge lacking a straightforward solution. Furthermore, evidence on the safety and cultural feasibility of methods recommended, preventing mother child transmission (PMTCT) approaches are limited. A durable platform technology introduces copper-oxide into polymeric material has recently been developed. Using the same principle, copper-oxide powder was used to treat breast milk to evaluate the deactivating properties of copper on HIV virus. This project provides a contribution for what we hope become a possible solution for reducing the transmission of HIV from mother to child via breast milk.

Methods: The focus of this study was to establish a 'proof of principle' that copper oxide can be used to deactivate HIV-1 in breast milk. A total of twenty milk samples were obtained from HIV-1 infected mothers. The milk was divided into two portions, the first portion was passed through Sartopure (Sartorius Stedim Biotech GmbH) 300 filters and filtrates collected. The second portion was treated with copper xide powder for 5 minutes and passed through Sartopure (Sartorius Stedim Biotech GmbH) 300 filters to remove copper oxide. A cell line was then exposed to the filtrate from both portions for 3-4 weeks.

Results: The exposure of the milk samples to the copper-oxide during treatment reduced by 1.8, 1.8, 2.4 and 3 (2.25±0.57; mean ± SD) log reductions (>99% reduction) of infectious viral titers as compared to the infectious titers foun in the same samples without copper oxide treatment.

Conclusions: A >99% reduction in the infectious HIV-1 in the breast milk following a short exposure to copper-oxide would significantly minimize the risk of infant infected with the virus from breast milk containing HIV. Thus a successful outcome of this project, providing a basis for further studies to confirm that copper oxide does not degrade nutritional and anti-infective components in breast milk during treatment

1.1 BACKGROUND

The safety of breast milk, in the face of mother-to-child transmission (MTCT) of human immunodeficiency virus (HIV), is a challenging problem lacking straightforward solutions. Breast milk transmission of HIV-1 can occur at any time during the entire duration of breastfeeding. The overall risk of mother to child HIV transmission in non-breastfeeding population is 15-25 % (without intervention) and 20-45% in breast feeding population [1]. Subsequent infections through breast feeding can increase overall risk to higher levels in 18-24 months. Transmission occurs typically early in the irst six months of pregnancy (infant life) [2]. However, the transmission risk is greater after 6 months of breastfeeding due to the additive effect of continuous viral exposure [3] and decreasing presence of antibody protection in the breast milk [4].

Human milk is the ideal infant food in the first 4 to months of age. Human milk contains microorganism protection, developmental-stimulating growth factors and hormones, immune function modulators, and anti-inflammatory agents that confer protection against disease and insure growth and development in spite of harsh environments [5]. While several infectious agents have been detected in breast milk, only viruses (namely HIV, human T-cell lymphotropic, West Nile, hepatitis C, herpes simplex), especially when present as co-infections of HIV, challenge the benefits of breastfeeding [6, 7].

Copper ions, either alone or in complexes, have been used for centuries to disinfect fluids, solids and tissues [8, 9]. Copper or copper-containing compounds have potent anti-viral properties [9]. The deactivation by copper of infectious bronchitis vi poliovirus, herpes simplex virus and other enveloped or non-enveloped, single- or double-stranded DNA or RNA viruses, has been reported [10]. A study by Sagripanti documented that cupric iron inactivates HIV-1 free virus and cells after 3 hours of treatment [11]. Stoichiometric concentrations of copper ions inactivate HIV-1 protease [12], an essential protein for assembly and replication of the virus. Copper ions may cause non-specific damage to HIV by damaging the envelope phospholipids and denaturizing the virus nucleic acids [10].

The capacity of copper oxide-containing filters to reduce infectious titres of a panel of viruses spiked into culture media, including HIV-1, has been demonstrated [13, 14]. These filters very efficiently reduced the infectious viral iters of all HIV-1 viral isolates tested, including wild type and drug resistant isolates, laboratory and clinical isolates, and isolates from different clades. Just a few minutes of exposure the virions to the copper oxide powder rendered them non-infectious. This exposure affected free viruses, virions being formed within the cytoplasm of cells during the cell exposure to copper, and virions prior to their budding from the cells. Cell-associated HIV-1 transmission was also attenuated in a dose dependent manner by copper oxide. The multi-site, antiviral mechanism of copper oxide [10] explains the high susceptibility of all HIV-1 viral isolates tested, with no clade or other specificity.

Copper is an essential element for humans; its biochemistry has long been studied and is well-understood [15]. Excess copper in humans at the level which is expected to be leached out into the milk, as it passes through the copper-filters (less than 1 µg/dl) is not toxic [16] on the contrary, copper oxide is widely used in vitamin supplements as the source of copper. A National Academy of Sciences Committee noted that daily intakes up to 3 mg/d in children and 8-10 mg/d for adults are considered tolerable and non-toxic [16].

The study will use Sartopure PP2 cartridges filter, optimized for wide range of prefiltration, Retention of particles and reduction of bio burden from liquids as well as gases which is ensured through fractionated defined depth filtration. Sartopure filter combine multiple layers of progressively finer pleated polypropylene depth filter material. They are ideally suited for clarification and prefilteration prior to membrane filtration. Typical application Sartopure filters is in particle removal from various media including vaccines, Biological fluids and protein solutions

This study aimed at determining, as a proof-of-principle, that copper oxide can be used to deactivate HIV-1 in human breast milk, and serves as a basis for future development of strategies tailored to the uniqueness of the maternal-child dyad feeding relationship within cultural sanctions of resource-poor societies.

The diverse approaches to breastfeeding found between nd within societies are largely based on cultural differences. The societies are socialized to breastfeed or not to breastfeed babies. Breastfeeding knowledge, when it exists, is passed down from mother to daughter in the form of consistent patterns of practices. This is internalized in a childhood experiences regarding the feeding and nurturing of infants through observation, modeling, and play (e.g., feeding of dolls with bottles or breastfeeding them). The need to change this approach to breastfeeding to avoid or solve breastfeeding difficulties can result in confusion as the society struggles to reconcile new behaviors with old beliefs. Breastfeeding patterns vary with geographical region, language, and era. Breastfeeding beliefs and behaviors can be organized around the themes of cultural dimensions. Though biology imposes constraints on successful approaches to breastfeeding, breastfeeding itudes and techniques vary betwe en cultures.

CHAPTER ONE

1.2 LITERATURE REVIEW

Out of almost 25 Million people living with HIV in Sub-Saharan Africa, about 60% are women, leading to roughly 15 million HIV-infected potential mothers living in the region, leading to 590,000 children born each year to HIV-positive mothers [17].

Without any preventive measure, 30-35% of mother-to-child transmission (MTCT) cases are due to breastfeeding. The remaining portion of MTCT cases occur in utero and at childbirth. This is called perinatal transmission. As the total percentage of MTCT ranges from between 15-45% by region, it's expected that a child born to an HIV-positive mother has a 5-10% chance of acquiring the virus via breastfeeding [18]. Therefore, 50,000 babies each year will acquire HIV via breastfeeding in Sub-Saharan Africa.

Several studies suggest that treatment with ARV's can effective at reducing the risk of perinatal transmission. For example, a 2004 study showed that taking antiretroviral therapy and delivering by cesarean section reduced the HIV transmission rate to only 1% [19]. Unfortunately, the implementation of programs to prevent perinatal transmission has been slow. So even though the percentage of HIV-positive pregnant women in low- and middle-income countries that have access to ARV's has increased from 10% in 2004 to 23% in 2006 [20]. The coverage in these countries varies significantly with only 7% coverage in West and Central Africa in 2006 [20]. In 2006 the United Nation Population Found (UNFPA) reproductive Health Branch reported that, programmes to prevent perinatal transmission may have been a failure [20]. Despite the fact that the global HIV response is now awash with funding, pregnant women in developing countries still don't have adequate access to the drugs that will prevent them from passing the virus on to their children. So although progress has been made since 2006, programs in most countries still have to work to increase coverage. In developing countries the use of antiretroviral therapies to reduce mother to child transmission of HIV (MTCT) has made important advancement in preventing HIV infections in children. In well-resourced settings, treatment has evolved from initial monotherapy with zidovudine (ZDV) to the use of combination antiretrovi l therapy in pregnancy. ARV

strategies in developed countries are expanded and effective in all areas including resource limited settings, which have achieved MTCT reduction rates as low as 2- 4% [21]. However in breast feeding populations there is still a sizable postnatal transmission, such hat overall infection rate remains considerable [21].

There is significant interest in approaches that can reduce the risk of transmission during breast feeding. In contrast, in many developing countries many pregnant, HIV-infected women cannot access even basic PMTCT intervention, such as coun and testing ARV prophylaxis. As of the beginning of 2007, that only 20% of HIV-positive, pregnant women in low- and middle –income countries would access ARVs to reduce the risk MTCT of HIV [22]. However, programs to prevent post-natal transmission of HIV through breast feeding have not been evaluated as widely as the programs to prevent perinatal transmission, and the latest WHO recommendations leave the majority women in low-resource settings with difficult choices and some uncertainty as to what is best to do, since formula feeding is not a realistic option for most women.

The WHO policy states that, "when replacement feeding acceptable, feasible, affordable, sustainable and safe, avoidance of all breastfeeding by HIV-infected mothers is recommended. Otherwise, exclusive breastfeeding is recommended during the first months of life [23]. This is because using formula in low-resource settings has been shown to decrease infant survival due to increased numbers of deaths from diarrhea and malnutrition [24]. In terms of specific breast-feeding recommendations, the situation is also ambiguous. Some of the most recent published data from a study of exclusive breast feeding followed by rapid weaning in Zambia [25] has shown no benefit for the intervention group compared to a control group that followed traditional breast feeding practices. About 7% of infants in both groups became HIV infected via breast milk between four and 24 months of age.

1.2.1 Methods of Breast Milk Deactivation

Several methods have been in use in for deactivating breast milk including Flash heating and Sodium Dodecyl Sulfate (SDS).

1.2.1.1 Flash Heating

Flash heating and pretoria pasteurization a method by which milk is heat treated to inactivate the HIV virus while maintaining the nutritional value and protective properties, has been shown to effectively remove HIV. Procedurally, using the flash-heating method, a mother first expresses 75-150 mL of breast milk into a glass jar. The jar is then placed in a pot of water, which is brought to a boil. At this point the milk can be removed, and fed to the by once it cools to an acceptable temperature [26].

Flash heating utilizes materials that are already found around the home, which could put the mother's upfront cost of the device at virtually zero. Furthermore, daily cooking is done by most families, so that the boiling of the milk might be incorporated into everyday life. Again, both the flash heating and pretoria pasteurization methods have been proven to effectively remove HIV from milk [27]. Additional research has been conducted into the degree to which these methods damage the vitamin content of breast milk. Broadly speaking, no significant damage to the nutritional content of the milk was detected, though flash heating seemed slightly more nutritionally preservative [28].

However, the flash heating method could require more boiling than is typical for a mother's routine, which might require increased time, effort, and money to acquire the extra fuel. Even if the heating were somehow incorporated entirely into the daily cooking, performing the acts of manually expressing enough milk (often 600mL/day), storing the milk, and finally delivering the milk to the child are simply not practical for many women. Furthermore, by blatantly not breastfeeding a child, a woman is often ubject to the stigma of being HIV+, a reality that many women and their families are reluctant to admit. Acceptability studies looking into the heat treatment of breast milk in Zimbabwean society have been performed [29]. The results indicate that it would be acceptable if rectly introduced and understood, however this acknowledgment required a resource intensive small-group discussion even in

the study. Many people were hesitant of the methods effectiveness and the inconveniences of having to boil water on a frequent basis.

1.2.1.2 Sodium Dodecyl Sulfate (SDS)

Sodium dodecyl sulfate (SDS), also known as Sodium lauryl sulfate (SLS), is a surfactant commonly found in toothpaste, shampoo, etc. Recent in-vitro work has shown that SDS, in concentrations as low as .1%, is highly effective at eliminating HIV-1 [30]. SDS is also extremely inexpensive.

However nutritional side effects of infant SDS intake via breast milk have not been extensively researched. In this case though, nutritional in-vitro studies have been carried out, and in-vivo research is further along. SDS can also cause skin irritation, depending on the concentration and duration of contact. SDS induces aphthous ulcers by wearing away the mucin containing mucus membrane, the protective layer safeguard the gums, the tongue and mouth lining. There are also concerns of SDS affecting taste. Similar to drinking orange juice after brushing your teeth, SDS has been shown to temporarily decrease the reception of sweet tastes.

1.2.1.3 Copper

Research has shown that impregnating fibers and/or polypropylene filters with a copper-oxide mixture (70% Cu2O and 30% CuO, >99% purity [31] can be used as a biocidal method of removing HIV-1 in medium culture.

Copper, along with copper impregnated fibers, is very cheap [31]. We suspect that copper-based filters might be more easily marketed ("all-natural") than chemical microbicide based ones. However, possible effects of copper on breast milk constituents have not been well researched. Indeed, neither in-vivo nor even comprehensive in-vitro studies have been carried out. However, copper filtration does look promising, and if proven effective and appropriate, our final design could easily incorporate a copper-based filter.

This study comes up with deactivating prove for copper oxide in breast milk, then putting this idea into a user-oriented device application for a breastfeeding will be the next challenge

CHAPTER TWO

2.1 STATEMENT OF THE PROBLEM

HIV-1 transmission through breast milk is one of the main modes through which HIV-1 is transmitted from HIV-1 infected mothers to their babies. Breastfeeding is estimated to have contributed 33-50% of the HIV-1 mother-to-child transmission cases worldwide [32].

The greatest burden of HIV infection in women and their children is disproportionately borne by the poorest countries, especially in sub-Saharan Africa. Breastfeeding is a major health-promoting factor for infants and children in developing countries but the risk of mother-to-child transmission (MTCT) of HIV by this route is challenging traditional practices and health policies. Maternal and infant factors contributing to the risk of MTCT through breastfeeding are still poorly understood and well researched. Factors identified include: advanced clinical stages of infection in the mother high maternal plasma HIV-1 load, presence of mastitis and infant oral thrush. In many developing countries, ional agencies are providing support and recommendations for preventing MTCT of HIV-1 by breastfeeding. Preventive strategies supported by WHO/UNICEF and charitable agencies in some sentinel centres in sub-Saharan Africa include routine antenatal voluntary counseling and testing (VCT), PCR testing of infants of seropositive mothers at 6 weeks of age, various combinations of a shortened period (3-6 months) of exclusive breastfeeding, perinatal administration of antiretroviral (ARV) such as nevirapine and provision of affordable and safe infant replacement feeds (presently given free by UNICEF in some centres). Many problems, however, have hindered effective implementation of these interventions.

Developing techniques that will inactivate the HIV-1 virions ingested by an infant on a daily basis during the first few months of life through breast milk is thus of critical importance.

2.2 RESEARCH QUESTION

Can copper oxide be used to deactivate HIV-1 in human breast milk?

2.3 HYPOTHESIS

Use of copper oxide in treatment of breast milk can inactivate HIV thus significantly reducing transmission of HIV from an infected mother to child through breast feeding.

2.4 JUSTIFICATION

Stoichiometric concentrations of copper ions inactivate the HIV-1 protease, essential for viral replication. Cell-free and cell-associated HIV-1 infectivity is inhibited when exposed to copper-oxide in a dose dependent manner. Passage of high titers of a wide range of HIV-1 isolates, spiked in culture media, through filters containing copper-oxide powder, resulted in their deactivation [33]

This study was set to demonstrate the effect of copper oxide in treatment of HIV-1 isolates found in breast milk obtained from HIV-1 sero-positive donors, the virus load was drastically reduced (>99%) when exposed to copper oxide. And the study was a proof-of-concept that copper oxide is effective against HIV-1 found in breast milk and serves as the basis for further research aimed at determining the po effects copper oxide may have on the nutritional and anti-infective properties of breast milk.

2.5 MAIN OBJECTIVES

To evaluate the ability of copper oxide to inactivate infect icles in breast milk of HIV positive mothers.

2.6 SPECIFIC OBJECTIVES

1. To determine the efficiency of Sarptopure 300 (retention rate filter) in reducing the infectious HIV-1 particles in breast milk samples
2. To demonstrate the decline in infectious HIV viral titers in whole breast milk after treatment with copper oxide and filtration with a 5 µm retention rate (Sarptopure 300).
3. To demonstrate the decline in infectious HIV viral titers in cell free breast milk after treatment with copper oxide and filtration with a 5 µm retention rate (Sarptopure 300).

CHAPTER THREE

3.1 METHODOLOGY

3.1.1 STUDY AREA

The site of study was Langata City Council Clinic, in constituency. Langata Constituency is an electoral Constituency in Kenya. It is one of the eight constituencies of Nairobi Province. The constituency has an area of 223 km². Kibera, Kenya's largest slum is located here, as are Karen and Langata, some of the mo affluent suburbs in Nairobi. The clinic serves residents of Kibera slums and its' environs and is by the Nairobi city council.

The laboratory work was done in the Department of Medical Microbiology within the University of Nairobi's School of Medicine at Kenyatta National Hospital.

3.1.2 STUDY POPULATION

The study participants were asymptomatic HIV positive women attending Langata city council clinics, aged between 18 and 45 years.

Only mothers, who willingly consented, were enrolled to participate in the study. Socio-demographic and medical information was obtained from the study participants using a standard questionnaire approved by Kenyatta National Hospital-University of Nairobi Ethics Research Committee.

3.1.3 STUDY DESIGN:

This was a cross sectional study on a small group of 20 HIV positive women to look at the deactivation of HIV viral particles in breast milk aft atment with copper oxide.

3.1.4 SAMPLING METHOD

The study used convenience sampling, a non-probability sampling technique, and participants were recruited as they visited the clinic. Subjects were selected because of their accessibility to the researcher and only those who gave consent where enrolled for the study.

3.1.5 SAMPLE SIZE

Studies show that the biocidial properties of copper oxide impregnated fibrix against HIV-1 viruses achieve a percentage > 99.9 % reduction in 20 minutes [10]. A fate achieved in water treatment against other viruses [10]. At 90 % confidence and confidence interval of 1.645 the sample size was derived using the fisher's formulae below.

$$n = \frac{z^2_{1-\alpha/2} P(1-P)}{D^2}$$

Where: **n =** study sample size

p = expected level of purification 98% after treatment.

D= degree of precision or a tolerance error margin or width of the confidence interval

Z= Standard Normal deviation at 90% C.I .For a 90% CI, z=1.645

For example in 99% level of purification

Baseline of 1,000.00 infectious particles when reduced to 10.00 infectious particles

Will give 1,000.00 – 10.00 = 990.00 reduction

990/1,000x 100 = 99%

10^3-10^1= 10^2 ($\log_{10}10^3/10^1 = \log_{10}10^2 = 2$ log reduction)

A 2 log reduction will significant deactivate HIV in breast milk

$$n = \frac{z^2_{1-\alpha/2} P(1-P)}{D^2}$$

Using D = 5%

$$n = \frac{(1.645)^2\ (0.02)(0.98)}{(0.05)^2}$$

n = 21.215

The calculated study sample size was 21.215 ~ **21**

3.1.6 ENROLLMENT/INCLUSION CRITERIA

Included in the study were:

1. HIV positive women attending Langata City Council clinics between the age of 18 and 45 years.

2. Women currently breast feeding.

3. Women willing to give informed consent and able to com with the study protocol, including clinical examination.

Excluded from the study were women on antiretroviral therapy.

3.2 MATERIALS

3.2.1 SARTOPURE FILTER MAUFACTURE

Sartopure PP2 Cartridges are optimized for the wide range of pre-filtration. Retention of particles and reduction of bio-burden from liquids as well as gases is ensured throug fractioned defined depth filtration. They are ideally for clarification and pre-filtration prior to membrane filtration.

Sartopure® PP2 300 is a self-contained ready to use filter is designed for use in small scale production of high valuable products. Featuring the sa materials and type of construction as any other Sartopure® PP2 filter element, is ideally suited for R&D Labs. Retention of hard, non deformable particles and reduction of bio-burden from liquids as well as gases is ensured through fractionated defined depth filtration. Sartopure® PP2 300 combines multiple layers of progressively finer pleated polypropylene depth filter materials. They are ideally suited for clarification and pre-filtration prior to membrane filtration. Applications: Typical applications for Sartopure® PP2 300 are partic removal and bio-burden reduction from various process media like: Plasma Fractions, vaccines, diagnostics, purified protein solutions, biological fluids, ophthalmic and solutions containing Preservatives.

3.2.2 EQUIPMENTS

Specimen collection system:

1. Breast Pump Kit; Medala electric pump (Symphony, 2-Phase Expression and Medela, 1907764 A 1206 model, McHenry)
2. Fifty pieces of 100ml sterile calibrated wide mouth containers for collecting m lk

A Complete working Virology laboratory with:

1. Biosafety cabinet Level 2 (BSL 2)-LABCARE
2. Inverted microscope using x10, x40 and x100 Objectives
3. Talboys standard multi-tube Vortexer alexred
4. Centrifuge that can accommodate 50ml centrifuge tubes
5. A CO_2 incubator set at 37° C
6. A Refrigerator 2-8° C
7. Freezer -20° C
8. Chemical Balance 0.1 mg accuracy
9. Source of Carbon dioxide – CO_2 Cylinder

A Complete working HIV Laboratory with:

1. Pippete aid Electric HS rota filler 3000R
2. Two Multi-channel automatic pipettes:
    i. Multi-channel automatic pipette 5-50 ul
    ii. Multi-channel automatic pipette 50-300 ul
3. Three Single channel automatic pipette:
    i. Automatic pipette 5-40 ul
    ii. Automatic pipette 20-200 ul
    iii. Automatic pipette 200-1000 ul
4. Data entry facilities, i.e. Record books or Computer and printer

3.2.3 SUPPLIES

1. 50 grams of Copper (11) oxide powder. Copper oxide powder with a D_{50} of 18 microns and no particles below 6 microns

2. 100pcs of Sartopure 300 PP2 559 Filters
3. Small Plastic Petri dishes 5mm in diameter for holding media
4. Staining Tray a pack
5. 100 pcs of 10 ml syringes or 100 pcs of 20 ml syringes
6. 100 pcs of 15mls centrifuge tubes
7. 100 pcs of 50 mls centrifuge tubes
8. Sterile pipettes:
    i. I pk of 2mls sterile pipettes
    ii. I pk of 5mls sterile pipettes
    iii. I pk of 10mls sterile pipettes
9. Sterile Bags
10. Autoclavable Bags for Biohazard waste materials
11. Disposable protective clothing (gown. goggles)
12. 4pks of Powder free gloves 100pcs each
13. Pippette Tips:
    i. Pipette tips 5-50ul –yellow
    ii. Pipette tips 50-200ul –Clear/white
    iii. Pipette tips 200-=2000ul -Blue
14. 100pcs of Tissue culture test plates 96 F
15. Tissue culture flasks
16. 1000 mls phosphate Buffered saline
17. 1 pk Lens cleaning tissue
18. 1% antibiotics (Pen-Strep-Nystatin suspension, Biological Industries Israel).
19. cMAGI cell line
20. MT2 cell line
21. Culture media
    i. 5 Bottles of Prepared RPMI 500 ml (GibcoBRL, Life Technologies, Paisley, UK)
    ii. 3 Bottles of Prepared DMEM 500 ml (GibcoBRL, Life Technologies, Paisley, UK)

iii. 1 Bottle of Fetal calf Serum (GibcoBRL, Life Technologies, Paisley, United Kingdom)

22. 5 liters Ethanol
23. 5 liters Sodium Hypochlorite (JIK)
24. Grease pencil and Markers
25. Labels

3.2.4 PERSONNEL

1. Clinical staff (Medical Nurse)
2. Laboratory Personnel
3. Data Entry Clerk
4. Statistician

3.3 PROCEDURE

3.3.1 DONOR REQUIREMENTS

The HIV (+) donors' were identified through health providers' and referrals at the Langata City Council of Nairobi Maternity Department. The health providers explained the study protocol in Swahili, the national language of Kenya, and the donors signed informed consent forms. All women who agreed to participate in the study were provided with a cotton baby blanket and Ksh. 1500 (approximately USD20).

3.3.2 COLLECTION OF DEMOGRAPHIC INFORMATION

The study nurse administered a structured behavioral questionnaire to assess baseline characteristics. This questionnaire collected socio-demographic and medical information of the study participants.

3.3.3 QUALITY CONTROL

Standard operation procedures were followed in the preparation and administration of the questionnaire. One in every five questionnaire was checked by independent data entry staff at the Kenya AIDS Vaccine Initiative for correct entries.

3.3.4 SAMPLE COLLECTION (Blood and Breast Milk)

3.3.4.1 Blood samples

Two milliliters of whole blood sample was collected from each of the HIV-1 infected individuals by venipuncture in K_3EDTA vacutainer tubes after pre-test counseling and informed consent; blood collected was used for HIV testing, viral load and CD4 counts. To exclude the influence of circadian variation on lymphocytes sub-populations, samples were collected between 0800 and 1200 hours. All samples were held at room temperature and processed within 2 hours of collection.

3.3.4.2 Breast milk samples

Twenty breast milk samples (~80 ml) were obtained only from HIV-1 seropositive (HIV[+]), antiretroviral-naïve women between birth and 8 weeks postpartum presenting for clinical care at Langata City Council clinic in Nairobi, following the approval of Kenyatta National Hospital Institutional Review Boards. These breast mil samples were collected using a Medala electric pump (Symphony, 2-Phase Expression and Medela, 1907764 A 1206 model, McHenry) which uses positive/negative pressure in a fashion that mimics that of the baby's nursing at the breast. The donated breast milk samples were put in sterile containers and processed fresh within 3 hours of donation.

3.3.5 LABORATORY METHODS

3.3.5.1 MT2 and cMAGI cell lines

MT2 and cMAGI cell line were obtained from Mark Wainberg, MacGill University, Canada through Dr. Gadi Borkow Chief Medical Scientist Cupron Scientific Israel.

3.3.5.2 Preparation of cell lines

MT2 and cMAGI cell line were cultured and maintained in RPMI 1640 (GibcoBRL, Life Technologies, Paisley, United Kingdom) and DMEM (GibcoBRL, Life Technologies, Paisley, United Kingdom) respectively, each containing 10% fetal calf serum (GibcoBRL, Life Technologies, Paisley, United Kingdom) as indicated in appendix III.

3.3.5.3 Breast milk processing

The milk samples received were split into 4 portions of 20mls each. One portion was kept without treatment (whole breast milk [WM]). The second portion collected was treated with 10 grams of copper Oxide (whole breast milk treated with copper oxide [WM +]), was constantly and thoroughly mixed at room temperature with a vortex –mixer to maintain the copper oxide particles in suspension for up to 5 minutes. The other two portions were centrifuged at 2000 rpm for 10 minutes to remove fat and cells. Skim and cell free breast milk [CS] was collected from the middle part of the tubes and aseptically transferred into two sterile tube. One tube was labeled [CS-] Skim and Cell free milk without treatment and the other tube was treated with 10 grams of copper Oxide (Skim and Cell free milk treated with copper oxide labeled [CS+]), after being processed as portion one [WM+] above.

3.3.5.4 Cell culture

MT2 and cMAGI cell lines were cultured in RPMI 1640 medium and DMEM respectively, each containing 10% fetal calf serum. Fifty µl of whole Breast milk without treatment [WM -] from HIV-1 positive mother were added to 100 ml of RPMI 1640 medium and DMEM supplemented with 10% fetal calf serum, 1 mM and glutamine (Difco, Detroit, MI), containing MT2 and cMAGI cells respectively. The viabil of the cells was assessed before use by using the trypan blue exclusion assay, where cells without an intact membrane take up the coloring agent to indicate cell death (Appendix III).

3.3.5.5 Detection of HIV-1 infection

Viral growth was assessed using two methods namely a) microscopic assessment of syncytium formation by two independent observers of HIV-1 infection of CD4+ MT2 cells and b) determining the number of infected cMAGI cells.

A syncytium is a large cell like structure filled with cytoplasm and containing many nuclei. Most cells in eukaryotic organisms have a single nucleus. Syncytia are specialized forms in which the cytoplasm of one cell is continuous with that of adjourning cells, resulting in a multinucleate unit or mass of protoplasm produced by t merging. Syncytia are formed

commonly when cells are infected with certain types of virus such as HIV and paramyxoviruses. During infection,Viral fusion protein used by the virus to enter the cell are transported to the cell surface where they can cause the host cell membrane to fuse with neighboring cells.

HIV causes infected CD4 cells produce viral proteins, including fusion proteins. Then, the cells begin to display surface HIV glycoproteins, which are antigenic. Normally a cytotoxic T cell will immediately come to "inject" lymphotoxins, such as perforin or granzyme, which kill the infected T helper cells. However if they are nearby T helper cells, the gp4 molecules that initiate the fusion process will help HIV receptors displayed on the surface of the T helper cells bind to other similar lymphocytes [34]. This makes the dozens of T helper cells fuse cell membranes into a giant, nonfunctional syncytium, which causes the HIV virion to kill many T helper cells by infecting only one.

The titer was calculated by using the Reed-Muench end point dilution method [35] or b) determining the number of infected cMAGI cells [36]. MT2 and cMAGI cell lines were cultured in RPMI 1640 medium (GibcoBRL, Life Technologies, Paisley, United Kingdom) and DMEM (GibcoBRL) containing 10% fetal calf serum (GibcoBRL) and 1% antibiotics (Pen-Strep-Nystatin suspension, Biological Industries). The cMAGI cell line is a lymphocyte cell line stably transfected with a plasmid containing the HIV-1 LTR fused to ß-galactosidase [35]. These cells stain blue only when infected with HIV-1.

3.3.5.6 Inhibition of HIV-1 infectivity of breast milk samples

Twenty ml aliquots of breast milk obtained from HIV (+) donors without any treatment [WM -] were put in a syringe attached to a membrane and by using a plunger the milk was pushed through the membranes. The eluates were collected and subjected to multiple sequential dilutions in RPMI for use with MT2 cells and DMEM for use with cMAGI cell. Forty micro liters (40ul) of breast milk eluate [WM -] was added to 120ul of appropriate media and multiple sequential dilutions of 1/4, 1/16, 64, 1/256 etc prepared. Hundred micro liters (100ul) of each dilution was then added to corresponding 100ul of media with $2x10^5$ MT-2 or cMAGI target cells in 96-well micro-plates using 3 replicate wells per

dilution. Viral infectivity was determined after 3-5 days of incubation at 37ºC in a 5% CO_2 moist incubator, as described using a cytopathic effect assay (MT2) or counting cells stained blue (cMAGI).

3.3.5.7 Reed-Muench end point dilution method

The end point dilution assay was used to measure virus titer before the development of the plaque assay and is still used for viruses that do not form plaques. Serial dilution of a virus stock were prepared and inoculated onto triplicate cell cultures, in multi-well formats (96 well plastic plates). The number of cell cultures that were infected was then determined for each virus dilution by looking for cytopathic effect. For example, 10 monolayer cell cultures can be infected with each virus dilution. After an incubation period, plates that displayed cytopathic effect were scored as +, ++, +++ etc. At hi dilutions none of the cell cultures are infected because there are no particles are present. At lower dilutions, every cell culture is infected. Half of cell cultures showing cytopathic effect at the 10^{-x} dilution. This is the **end point**, the dilution of the virus at which 50% of cell cultures are infected. This number can be calculated from the data and expressed as 50% infectious dose (ID_{50}) per milliliter. The virus stock in this example contains 10^{x} ID_{50} per ml.

In real life, 50% end point does not usually fall exactly on a dilution as shown in the example. Therefore statistical procedures are used to the end point of the titration. The statistical method of reed and munch was used to determine the 50% end point. In this method, the results are pooled and the mortality at each dilution is calculated. The 50% end point, which falls between the fifth and sixth dilutions, for example, is calculated to be $10^{-6.5.}$ Therefore the virus sample contains $10^{6.5}$ LD_{50} units [35].

For the second test twenty ml aliquots of breast milk from HIV (+) donors were mixed with 10 grams of copper (II) oxide powder. After being thoroughly mixed for 5 minutes at room temperature, the milk samples [WM +] were put in a syringe attached to a membrane and by using a plunger the milk was pushed through the membranes. Elute [WM +] were collected and subjected to multiple sequential dilutions in RPMI for use with MT2 cells and DMEM for use with cMAGI cell. 40 µl of breast milk elute [WM +] was added to

120 µl of appropriate media and multiple sequential dilutions of 1/4, 1/16, 1/64, 1/256 etc prepared. Hundred micro liters of each dilution was then added to corresponding 100 µl of media with cell line $2x10^5$ MT-2 or cMAGI target cells in 96-well micro-plates using 3 replicate wells per dilution. Viral infectivity was determined after 3-5 days of incubation in a humidified CO_2 incubator at 37ºC as described above.

Breast milk from HIV positive donors not treated with Copper oxide was used as a positive control. Forty micro liters of milk was added to 120 µl of appropriate media to prepare a ¼ dilutions. The dilution was then added to MT2 or cMAGI target cells in 96-well microplates using three replicate wells per specimen. Following 3 to 4 days of incubation, the infectious viral titers were determined using a cytopathic effect assay in MT2 and/or counting cells stained blue in cMAGI cell lines.

Culture medium without breast milk, passed through the filter, was used as a negative control for antiviral activity and for determination of cytotoxicity. Viral infectivity of target cells was also examined over 3 weeks of incubation.

Skim and Cell free milk without treatment, was processed in a similar manner as whole breast milk without treatment, Skim and Cell free milk treated with copper oxide, was processed in a similar manner as whole breast milk treated with copper oxide.

3.3.5.8 Quality control

Standard operating procedures were followed to ensure correct patient's identification, proper specimen collection and Proper specimen labeling and processing.

3.3.5.9 CD4 Testing

CD4 Cell count was tested at KAVI laboratories. Dual-color immuno-phenotyping was performed using standard whole blood methodology as previously described.

3.3.5.10 Plasma HIV Viral load Testing

Maternal plasma viral load was determined at KAVI laboratories, using AMPLICOR Monitor Kit, version 1.5 (Roche Diagnostic).

3.4 DATA MANAGEMENT

3.4.1 DATA COLLECTION INSTRUMENTS

Various data collection techniques were used:

1. Questionnaire**:** A set of systematically structured questions was used by the researcher to get information from respondents. The questionnaire was presented in an interview format, it included a check lists among other research methods.
2. Face-to-face interviews were performed by the nursing staff.
3. Projective technique was performed by the nursing staff.
4. A clinical examination report was as given by recruiting nurse.
5. Laboratory reports obtained from test results.

3.4.2 DATA ANALYSIS

Data was collected and analyzed using Statistical Package for Social Science (SPSS) for windows version 16.0

CHAPTER FOUR

4.1 RESULTS

During the month of February, 2010 whole blood and breast milk Samples were collected from Twenty HIV positive mothers enrolled in the Study. The youngest donor was 19 years of age while the oldest was 41 years. The CD4 T Cell counts of the participants ranged from 161 to 1, 287 cells/μl while he viral load ranged from <50 to 102, 322 copies/ml. The donor's population showed normal distribution as indicated in figure 4.1.

Figure 4.1: Distribution of breast feeding mothers according to their ages.

Fig. 4.1 above is a bar graph showing the distribution of breast feeding mothers in the study according to their ages. The figure details the distributed of the study population six age groups. The youngest age group of 18-20 years had one participant, 21- 25 years age group had five participants, 26-30 years age group had five participants, 31-35 years age group had six participants, 36-40 years age group had two participants and the more than 40 years age group had only one participant.

	Age in years	T Lymphs (CD3+ CD4+) Abs Cnt (cells/µl)	Plasma Viral Load (Copies/ml)	Culture of whole breast milk after filtration only [wm-]	Culture of whole breast milk after treatment with cuo and filtration [wm+]	Culture of fat and cells free breast milk after filtration only [cs-]	Culture of fat and cells free breast milk after treatment with cuo and filtration [cs+]
1	35	874	Undetected (<50)	NEG	NEG	NEG	NEG
2	26	502	2,592	NEG	NEG	NEG	NEG
3	**32**	**283**	**102,322**	**+ve 1/64**	**NEG**	**+ve 1/64**	**NEG**
4	30	761	1,063	NEG	NEG	NEG	NEG
5	25	503	1,063	NEG	NEG	NEG	NEG
6	23	436	523	NEG	NEG	NEG	NEG
7	**34**	**472**	**48,449**	**+ve 1/64**	**NEG**	**+ve 1/64**	**NEG**
8	26	418	1,640	NEG	NEG	NEG	NEG
9	31	742	Undetected (<50)	NEG	NEG	NEG	NEG
10	25	1,287	423	NEG	NEG	NEG	NEG
11	32	634	Undetected (<50)	NEG	NEG	NEG	NEG
12	**24**	**233**	**15,828**	**+ve 1/1024**	**NEG**	**+ve 1/1024**	**NEG**
13	23	473	4,124	NEG	NEG	NEG	NEG
14	34	622	19,229	NEG	NEG	NEG	NEG
15	36	528	Undetected (<50)	NEG	NEG	NEG	NEG
16	41	562	Undetected (<50)	NEG	NEG	NEG	NEG
17	**26**	**161**	**754**	**+ve 1/256**	**NEG**	**+ve 1/256**	**NEG**
18	39	605	Undetected (<50)	NEG	NEG	NEG	NEG
19	19	925	22,071	NEG	NEG	NEG	NEG
20	38	422	16,713	NEG	NEG	NEG	NEG

Table 4.1: Data on breast feeding mothers showing age distribution

Table 4.1 above showing data on breast feeding mothers according to age; CD4 Counts (in cells/µl.), viral load (Copies /ml) and viable viral culture in breast milk.

The data in the table 4.1 provide details of age, T Lymphocytes counts and Plasma viral loads of the twenty participants enrolled in the study. The results included test results of Breast Milk samples, four samples tested positive for viable viral culture before treatment and negative for viable viral culture after treatment with copper oxide as shown in the table.

Figure 4.2: Specimen testing positive for viable virus in culture according to CD4 counts

The figure 4.2 provided details of CD4 counts in blood specimens obtained from twenty participants. The lowest CD4 value recorded was below CD4 cells /µl while the highest recorded was above 1000 cells/ µl. Ten participants out of 20 enrolled had CD4 counts of between 401 and 600 cells / µl. Four participants one with CD4 count of less than 200 cells/ µl, two with CD4 counts between 201 and 400 cells/ µl and one with CD4 values between 401 and 600 cells/ µl produced viable viral titers in breast milk.

Table 4.2: Age group of breast milk donor in relation to culture results

Age of Donors in years	**Number of Donors**		**Donors that produced sample with viable viral titer**	
18 – 25	6	55%	2	33.3 % Positive
26 – 35	11	30%	2	18.2 % Positive
36 – 45	3	15%	0	0 % Positive
Total	**20**		**4**	

Table 4.2 above showing the Distribution of breast milk samples with positive viral culture according to the different age groups

Table 4.2 give details on the age of Donors enrolled in the study, their distribution in different age groups and the viable virus test results. 55% participants were in the age group of 18-25 years, 30% in the age group of 26-35 years and 15% in the age group of 36 to 45 years. Two samples out of six collected from participants in the 18-25 years age group tested positive for viable viral culture, resulting to a percentage positive rate of 33.3%. In the 26-35 years age group 2 samples out of 11 collected tested positive for viable viral culture, resulting to a percentage positive rate of 18.2%. While in the 36 -45 age group all the 3 samples tested negative for viable viral culture.

Figure 4.3: viable viral culture results produced for breast milk against plasma viral load of Donors

The figure gives the plasma viral load of blood specimen collected from 20 participants. Six participants had undetected levels of virus (levels below 50 copies per ml), three had between 50 and 1000 copies per ml, another three had between 1001 and 2000 copies per ml, one produced between 2001 and 3000 copies per ml, another one produced between 4001 and 5000 copies per ml and another six produced more than 5000 copies per ml. Out of 20 Breast Milk Samples collected from participants with varying v ral copies per ml, only four tested positive for viable viral cultures. Three patients with over 5000 viral copies per ml and only one with viral copies of between 50 and 1000 copies tested positive for viable viral cultures.

Figure 4.4: Culture results of breast milk samples after treatment as compared to marital status of the participants

Out of twenty participants enrolled in the study 14 were marr women and only six were single. The 4 samples that tested positive for viable re from married participants.

Figure 4.5: Culture results of breast milk samples after treatment as compared to the level of education of participants

Majority of participants enrolled in the study had sec dary level of education; three participants had primary level of education as the highest academic qualification and one participant had tertiary level of education. All participants who tested positive for viable viral culture had secondary level of education.

Figure 4.6: Culture results of breast milk samples after treatment as compared to occupation of participants

Participants enrolled in the study were either house wife or women in small scale business. Most of the participants enrolled were house wife and only four participants were involved in small scale business activities. Three participants tested positive for viable viral culture among the house wives and from the business group.

Table 4.3: Difference in dilution and log reduction of viable viral titers

Sample	Highest Dilution that showed positive viable viral growth in culture before treatment.	Highest Dilution that showed positive viable viral growth in culture after treatment with CUO	Difference in dilution	Log reduction
104	1:64	0	1:64	1.8
109	1:64	0	1:64	1.8
115	1:1024	0	1:1024	3
120	1:256	0	1:256	2.4

Table 4.3 above, showing the difference in dilution and log reduction of viable viral titers from four samples that produced growth in culture before treatment with copper oxide. Two specimens tested positive for viable viral growth in culture before treatment at 1:64 dilution, one specimens tested positive for viable viral growth culture before treatment at 1:1024 dilution and the last specimens tested positive for viable viral growth in culture before treatment at 1:256 dilution. All the specimens tested ve for viable viral growth in culture after treatment with CUO. The finding resulted to a log reduction of 1.8, 1.8, 3 and 2.4 respectively in the specimens tested

4.2 DISCUSSION

This study tested the ability of copper oxide particles to deactivate HIV-1 free virus in Breast Milk. This data demonstrated that treatment of breast k with copper oxide particles can inactivate cell free HIV-1 Virus in Naturally infected Breast Milk from HIV positive mothers. After 5 minutes of treatment of breast milk with copper oxide HIV-1 was undetected in the samples that initially had tested po ve for viable viral culture.

In this study, donors of lower age group showed increased isolation rate for viable virus in culture with 33.3% in age group 18 to 25 years as compared 18.2% in age group 26 to 35 years, samples from the donors in older age group did not show any viable virus growth in culture. Despite the increased viral isolation rate in breast milk among young mothers, Younger participants accounted for a minority of the study population's total HIV-1 infections. We found that the older donors 26 -35 years of age accounted for a greater portion (55.5 %) of the total numbers of HIV-1 positive mothers who were involved in the study. This percentage was greater than 30% ($p>0.001$) value accounted for by the 18 -25 years old. This compares well with several studies which show that there is an elevated risk of HIV among young adults [37].

The more viable viral titers was achieved from Breast ilk samples collected from participants with lower CD4 counts as compared to that from samples from participants with higher CD4 Counts as shown in table 4.2. The findings agree well with the findings of the isolation rate of viable viral titers from donors with the highest viral load as indicated in table 4.3. This directs to the inverse relationship where in general patients with low CD4 counts have higher viral loads. The findings compares well with results of a study that evaluated three cellular and five serologic markers affected by infection with the human immunodeficiency virus type 1 (HIV-1) for their ability to predict transmission and progression of acquired immunodeficiency syndrome [38].

The donors from whom the plasma viral loads were undetected produced milk samples that showed no viral activity in the cell culture as shown in table 4.1. In untreated HIV infection, HIV replication usually produces billions of new HIV copies da ly.

Primary human immunodeficiency virus type 1 infection in adults is characterized by high level of virus replication. This is manifested as a steep rise in plasma HIV -1 RNA levels that reach a peak of between 10^5 and 10^6 copies/ml approximately 2 weeks after infection. Once the host defenses are mobilized against the virus, there is a slow decline to the steady-state viral load, or set point, of between 10^4 and 10^5 copies/ml at approximately 4 months post infection [39, 40, 41]. Peak viral levels in adults are not predictive of the rate of disease progression. However, the viral set point, which is most likely, a measure of the dynamic between the virulence of the infecting virus strain and the ability of the host immune system to contain the virus, is highly predictive of disease progression. This has exhibited in studies in which high steady-state level of HIV-1 RNA in plasma at 4 to 12 months post infection translate to significantly faster progression of AIDS [40, 41]. Several studies have shown that the plasma viral load is much higher than breast milk viral load [41]. In this study four specimens with plasma viral load of 102,322 copies per ml, 48,449 copies per ml, 15,828 copies per ml and 754 copies per ml produce viable viral titers on culture while 7 specimens with plasma viral loads higher than 1000 copies per ml and one as high as 22,071 copies per ml did not produce viable titer as shown in figure 4.3. This agrees with a HIV transmission study done in Zimbabwe that reported a viral detection in breast milk sample of two women whose plasma viral load were 74,000 and 43,0000 copies per ml and non from women whose viral load were higher [41]. The above named study which seems to suggest that shedding of the virus in breast milk may depend o many yet to be established factors. However viral load test do not show that infectious HIV is present in the body cells containing HIV. Virions may be inactivated by breast milk, saliva or infant gut and the inactivated cells will still register positive test by molecular techniques.

A viral load below the level of detection ("undetectable") indicates inability of the assay to detect HIV in the plasma, but does NOT indicate absence of the virus from the body [42].

Undetectable HIV plasma viral load are values below the lower limit of detection for the particular test assay that are being used. Early viral load tests could only test down to 10,000 copies. Modern tests are able to test down to 500 copies of the virus per milliliter of plasma. Some ultra sensitive viral load assays can detect 25 to 50 copies/ml. Currently ultra-ultra

sensitive assays available in research laboratories can test down to a single copy per ml! However, even in HIV-positive patients with HIV plasma viral loads below 1 copy/ml, this does not imply the donors have no viruses in their body. HIV may still exist inside cells in the blood, lymph nodes, semen, vaginal and rectal fluids, **breast milk**, and other compartments [42].

In this study we used the AMLICOR Monitor kit, version (Roche Diagnostic) in a PCR machine whose limit of detection was 50 copies /ml.

Prior to testing the capacity of copper oxide particles to deactivate HIV-1 in breast milk, a suitable membrane which was not going to alter the composition of east milk was identified.

The membrane needed was to block passage of copper oxide particles, as these particles would kill cells in culture. In consulting with membrane manufacturers, Sartopure 300, 5 microns pores' size was selected for not clogging or affecting the milk integrity. Copper oxide particles that did not clog or pass through the ure membrane was used in the study.

After solving the above described technical difficulties, we examined the capacity of the copper oxide powder to reduce HIV-1 infectivity when incubated at room temperature for 5 minutes with fresh milk samples obtained from HIV positive women.

From twenty samples of breast milk of HIV positive mothers tested only four tested positive for viable virus in culture. Several dilutions of the four Breast Milk samples tested positive before treatment with copper oxide and the viable viral test results recorded did not depend on the level of Donor plasma viral counts as shown in fig.4.3. All the four positive samples tested negative for viable viral culture after treatment with copper oxide and in all 4 samples exposure of breast milk samples to copper oxide resulted in 1.8, 1.8, 2.4 and 3 (2.25 ± 0.57; mean ± SD) log reductions (>99% reduction) in infectious viral titers as compared the infectious titers found in the same samples passed through the same filters without copper oxide treatment. There were no significant log reduction in samples that had been filtered without copper oxide treatment, equally there were no significant difference in viable viral

titers between whole milk treated with copper oxide [WM+] and Skim and Cell free milk treated with copper oxide [CS+] as shown in table 4.1.

Studies have shown in previous research that free-flow passage of medium containing HIV-1 or other viruses through filters containing copper oxide results in significant reductions of the viral infectious titers [13, 14]. In this study it was possible to demonstrate that HIV-1 infectious titers, when present in breast milk of HIV positive women, could be significantly reduced when exposed to copper oxide for 5 minutes. These results are a proof-of-principle that copper oxide can inactivate HIV-1 free virus in human breast milk and serve as the impetus to further explore the possibility of using copper oxide in the context of reduction of Mother to Child to Transmission of HIV.

Obviously further development is necessary. A filtering device can be developed in which copper oxide particles with a significant smaller particle size should be used. In this study very large copper oxide particles were used, reducing significantly the surface area of the copper oxide in contact with the milk. Ideally the copper oxide powder should be in the nano-micron particle size range. Such particle size may allow reducing significantly the exposure time needed to achieve >99% reduction of HIV-1 infectivity. We believe that a reduction of >99% in the infectious HIV-1 units present in the breast milk following the milk exposure to the copper oxide particles would enormously reduce the risk of a baby becoming infected with the virus during breast feeding. The positive results of this study strongly encourage us to explore the development of a filter strategy tailored to the uniqueness of the maternal-child feeding inter-relationship within cultural sanctions of resource-poor societies.

4.3 CONCLUSION

Given the difficulties and lack of certainty regarding prevention methods for MTCT through breastfeeding, this study identified copper oxide in combined use with a filter definite and significant and an appropriate compound for inactivating HIV viral particles in breast milk.

4.4 RECOMMENDATION

For most applications, if copper technology is to be useful, complete neutralization of viruses in a given treatment system should be achieved. Depending on the solution composition and volume and the virus particles present, appropriate amount of copper powder will have to be calculated. Clearly, a larger volume of the active copper oxide is needed. The retention time of the solution in the powder should also be determined. This copper-based technology may, therefore, represent a novel and inexpensive means to uickly deactivate viruses in breast milk and other body fluids. A technological innovation that would come-up with filters that can be reactivated by heating or reusable filters that will bring this dream to the reach of all mothers

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APPENDIXES

APPENDIX I

CONSENT

TITLE: DEACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS IN BREAST MILK USING COPPER OXIDE

CONSENT FORM

From the Research Student: I Meshack Juma an Msc Student in Medical Microbiology at the Department of Medical Microbiology, University of Nairobi, is conducting a research in this clinic on deactivation of human immunodeficiency virus in breast milk using copper oxide. Through the university I am seeking your support to participate in the study. Participation in the study is voluntary and failure to participate will not jeopardize your chances of getting services from the family health clinic.

Physical Address: Medical Microbiology Department, School of Medicine at Kenyatta National Hospital, Hospital Rd. off Ngong Rd, Nairobi, Kenya

Introduction

This Consent form contain information about the research named above. In order to be sure you are informed about being in this research, we are asking you to read (or have it read to you) this consent form. You will also be asked to sign on it (or make your mark in front of a witness). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything that you may not understand.

Reasons for Research

You are being asked to take part in a research to help us document on the deactivation of human immunodeficiency virus in breast milk using copper oxide. This research will help us find places where future research can be done to promo ways of life that can lead to conditions that will reduce chance of transmission of HIV to breast feeding children.

General information about the Research

This research is being planned to take place at the Department of Medical Microbiology at Kenyatta National Hospital. Only HIV positive breast feeding mothers will be involved in this study.

This study only involves one visit, with an interview, physical examination of the breast, collection of about 80 mls of breast milk sample using a breast milk pump and blood sample less than one-half teaspoonful (2mls) for additional HIV tests, the additional testing will help researchers tell the difference between old HIV infection and Recent HIV infections. Your name will not be sent to the research lab.

Our part in Research

If you agree to be in the research study today, we will:

- Ask you questions to see if you are eligible to be in the study
- Get you permission to be in the study by you signing this consent form
- Ask you to give us 2 mls of blood and allow us to collect a breast milk samples from you.
- Ask you questions about your life and about your interest in being part of the HIV prevention research in future.

You do not need to join the research to continue receiving the usual health care services at the family health clinic. If you decide to participate in this study the blood will be taken from your arm with a small needle. Your part in the research will last about 30 minutes. There will be no extra cost on you for taking these tests.

Possible Risks

Some people may feel dizzy when blood is drawn from them. There will be some pain or discomfort from the needle prick. You may have a bruise or swelling where the needle goes into your arm.

Possible benefits

This study will not help you directly but it will help the researchers find some facts about the possible deactivation of human immunodeficiency virus in breast lk using copper oxide and this may lead to further HIV research.

If you decide not to be in the Research

You are free to decide if you want to be in this research. Your decision will not affect the health care you would normally receive at the clinic.

Confidentiality

We will protect information about you and your taking in this research to the best of our ability. Your name will not be on the interview form. name will not be given to the research laboratory staff and you will not be named in any report.

Compensation

Mothers who agreed to participate in the study will be provided with a cotton baby blanket and 1500 Ksh (approximately USD20) compensation for participation.

Leaving the Research

If you choose to be in the study, you can still decide not to complete the interview or not to give blood or avail yourself for breast milk collection. If you leave the study, please tell the interviewer why you are leaving.

If you have problems or other questions

If you have problems that you think might be related to taking part this research or any question about the research, please call Meshack Juma on 0722 875477.

If you get sick or have other health problems

If you have severe bruises bruising or an infection from needle stick, please phone the clinic or Meshack Juma or come back to the clinic right away, at any time during the research.

Your right as a participant

Please feel free to ask any additional question concerning the study at any point. In case of any questions arising from the conduct of this study or any information that has not been explained to your satisfaction or any questions about your rights as a research participant you may need to contact an autonomous body, please fill free to contact the ethics committee from the addresses below.

Ethical Committee: The Secretary, Kenyatta National Hospital-Ethics Review Committee (KNH-ERC) Hospital Rd. Along, Ngong Rd

P.O. Box 20723, Nairobi

Tel: +254 02 2726300-9

Fax: +254 02 2725272

Telegram : MEDSUP'', Nairobi

Email : KNHplan@Ken.Healthnet.org

VOLUNTER AGREEEMENT

This document described the procedures, benefits and risks for the research titled "Deactivation of human immunodeficiency virus in breast ilk using copper oxide" in Medical Microbiology Department has been explained to me. I have been given an opportunity to ask questions about the research and all explanations given to me are satisfactory. I agree to participate as a volunteer.

Print name of Volunteer……..

Signature of Volunteer…….. Date……..

If volunteer can not read this form themselves, a witness must sign here

I was present throughout the entire informed consent process with the volunteer. All questions from the volunteer were answered and the volunteer has to take part in the research.

Print name of Volunteer……..

Signature of Volunteer…….. Date……..

I certify that the nature and purpose, the potential benefits, and possible risk associated with participating in this research have been explained to the above individual.

Print name of Person who obtained Consent ……..

Signature of Person who obtained Consent ……
Date……..

Additional consent for other tests

We expect that there might be some little blood left a testing for this project. Any little left blood or breast milk could be useful for further Such research could help lead to better ways to manage mother to child HIV transmission. The exact research to be done may be decided at a later date. Would you be willing for us to use any left materials for other research activities.

Yes … I agree that other HIV/AIDS research may be done on ny left over blood collected from me.

Signature of Volunteer…….. Date……..

No …. I DO NOT agree for any other tests to be done on any leftover blood collected from me.

Investigator: Name: MESHACK JUMA

Mobile No: +254 722 875477

Email: mojuma@uonbi.acke or mailmolo@yahoo.com

Signature of investigator……..

APPENDIX II

QUESTION GUIDE

Name _____ Clinic No _____

Date _____ Time _____

1. Date of birth of Participant _____

2. What is your marital status

- ☐ Single
- ☐ Married
- ☐ Cohabiting/Living together
- ☐ Separated
- ☐ Divorced
- ☐ Widowed

3. Highest education level

- ☐ none
- ☐ Adult education
- ☐ Primary
- ☐ Secondary
- ☐ College/university

4. Occupation

- ☐ House wife
- ☐ Farmer
- ☐ Professional employment
- ☐ Small scale business
- ☐ Others…….

5. Do you have any medical condition known to you i.e. diabetes mellitus?

No_____, Yes_____,

6. Date of last baby delivery…….

8. Date of HIV Diagnosis…….

9. Antiretroviral (ARV) treatment prior to delivery ……

Yes…… No…… Unknown……

10. If yes which ARV…….

11. Antiretroviral HIV treatment <u>after delivery</u>……Yes……No……unknown……

12. If yes which ARV……

APPENDIX III

Preparation of cell lines

Two cell lines used are

MT2 cells wild lymphocytes, grow in RPMI 1640 with L-Glutamine media composed of

1. L-Glutaine 5ml to 500 ml
2. Antibiotics 5ml
3. 50 ml (10%) of fetal calf serum to 500ml
   MT2 Cell expresses CCR4

Cmagi cells grow in DMEM with L-Glutamine composed of

1. L-Glutaine 5ml to 500 ml
2. Antibiotics 5ml
3. 50 ml (10%) of fetal calf serum to 500ml
   Cell expressing CCR4, CCR5

Freezing Cells:

Cells should be growing well or known to be in log phase

Count, collect and pellet cells in a 15mL test tube

Resuspend in freezing media so that the concentration no more than 5x10^6 cells/mL of cold freezing media

Transfer 1mL of cells to appropriately labeled cryovials and maintain on ice for approximately 30minutes

Transfer vials to -80C freezer for 24hrs

Transfer to liquid nitrogen dewar or -140C freezer for long-term storage.

Freezing media

- 10% DMSO
- 90% FCS
- you'll need 1mL per 5x10^6 cells

Thawing Cells :

Remove vial from Liquid Nitrogen or -140C freezer and immediately transfer to 37C water bath

While holding the tip of the vial, gently agitate the ial, being careful not to allow water to penetrate the cap or seal

When completely thawed, transfer contents of vial to 15mL test tube

Slowly add 10mL warm complete media and spin at 1000g in

Decant media and resuspend pellet in a volume of complete media appropriate for flask or macro well

Transfer cells to flask or 24 well plates and incubate at 37C and 5%CO2

Cells can be checked visually or counted, beginning at approximately 1hr, for an estimate of viability. Immediate cell counts can be misleading

Stocking cells:

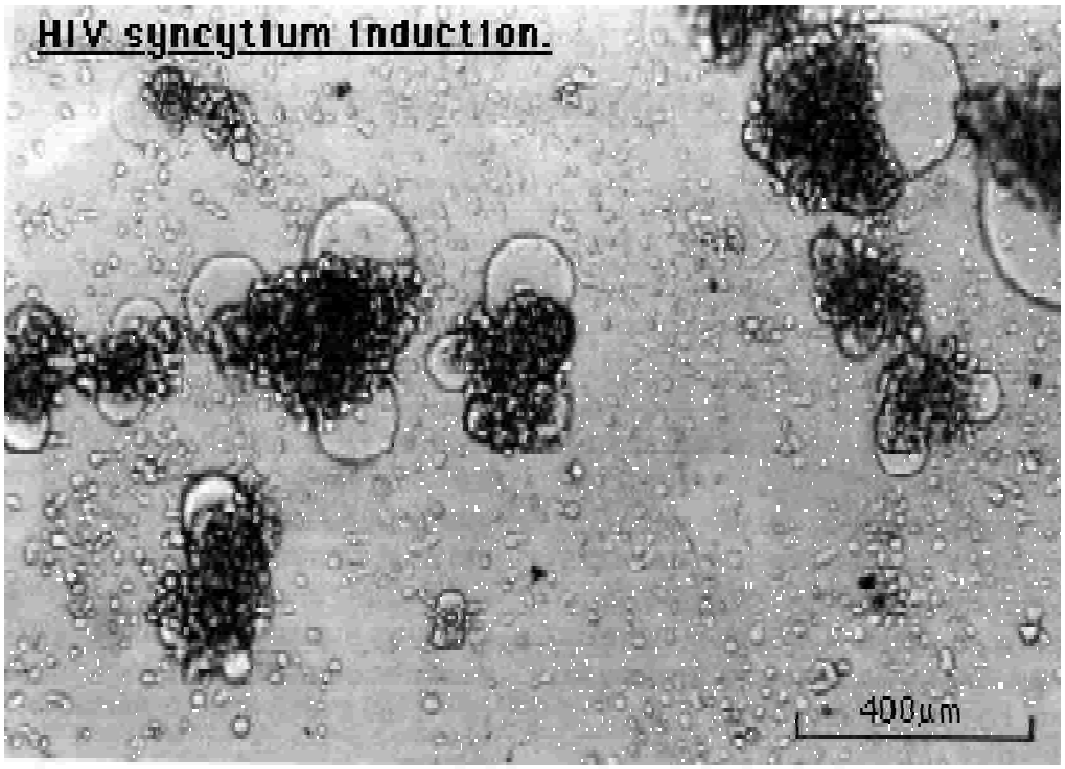
1. Filter sterilize 700ml of RPMI-1640 (2mM L-Glutamine) and 200ml fetal bovine serum (FBS) through a 0.22µm filter
2. Add 100ml sterile dimethyl sulfoxide (DMSO)
3. Aliquot into tubes as appropriate

Caution: Do not filter DMSO, it will dissolve the cellulose acetate membrane. Store the tubes at -80^{o}C for up to one year.

APPENDIX IV

Syncytium

Photograph No. 1 A Microscopic display of syncytium formation by HIV-1 infected CD4+ MT2 cells



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Photograph 1: A Microscopic display of syncytium formation by HIV-1 infected CD4+ MT2 cells

APPENDIX V

Procedure for staining cMAGi cell lines for ß-gal

REAGENTS

1. Staining solution

Reagent	1 plate	3 palates	8 plates
PBS	11.3ml	33.9ml	90.4ml
0.2M Ferric CN	250 µl	750 µl	1.84 ml
0.2M Ferro CN	250 µl	750 µl	1.84 ml
0.5M Mgcl2	12 µl	36 µl	96 µl (24 µl of 2M)
40mg/ml X-Gal	120 µl	360 µl	960 µl

X-Gal substrate (5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside)

2. Fixative
   i. 5 minutes with 1% formaldehyde and 0.2% glutaldehyde in PBS
   ii. 1% formaldehyde……10 ml
   iii. 0.2% glutaldehyde in PBS……2 ml

PROCEDURE

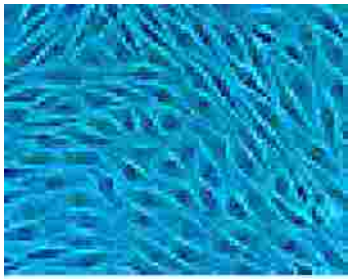
1. Remove the media from cMAGi plates remove the 200ul of media completely by setting the multichannel at 250ul and sucking to remove completely.
2. Add 100ul of fixative solution and incubate at room te 25?C-30? C for maximum of 5 minutes.
3. Remove the fixative from plates by sucking 100ul introduced completely when setting the multichannel at 125ul to remove completely.

4. The wells as then washed 2times with PBS using the multichannel set at 150-170ul
5. Putting 100ul of staining solution at room temperature for long checking every six hours or at 37?C for 1 hour and continuously there after for 6 hours
6. The plates are then examine under the microscope to see the blue stained cells

RESULTS

Photograph 2: Normal Cmagi cell (yellow)

Photograph 2 above shows a picture of normal Cmagi cell (yellow)



Photograph 3: Cmagi cell (blue)

Photograph 3 above shows a picture of HIV-1infected Cmagi cell (blue) ß-galactosidase is transcribed when the Cmagi cells are infected with HIV-1 and addition of X-galactosidase react with ß-galactosidase.