

Comparative Assessment of Markers of Oxidative Stress in Human-milk and Plasma of Lactating Mothers

Moses O. Akiibinu^{1#}, Adekunle A. Adesiyani² and Susanah O. Akiibinu³

¹Department of Biochemistry and Chemistry, Caleb University Lagos, Nigeria.

²Department of Biomedical Sciences, Ladoke Akintola University, Ogbomoso, Osun state, Nigeria.

³Department of Nursing Services, University College Hospital, Ibadan, Nigeria.

corresponding author.

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ABSTRACT

Background: Human-milk is a complex extracellular fluid with different biochemical composition compared with blood. The present study assessed the levels of markers of oxidative stress in human-milk and plasma of healthy lactating mothers.

Methods: Forty lactating mothers (22-36 years) volunteered to participate in this study. They were non-smokers and apparently normal nursing mothers who had normal delivery without postnatal complications. Blood and breast-milk samples were collected from the lactating mothers between 6th and 18th weeks after delivery. Total antioxidant potential (TAP), total plasma peroxides (TPP) and malondialdehyde (MDA) were determined in both human-milk and blood of the mothers using spectrophotometric methods. Oxidative stress index (OSI) was determined as the percent ratio of the TPP and TAP.

Results: The results showed that TAP, TPP and OSI increased significantly ($p < 0.001$) in human-milk compared with the plasma. The plasma / human-milk ratios of TAP, TPP and OSI were 1:3, 1:5 and 1:2 respectively. The level of MDA was significantly ($p = 0.01$) lower in the breast-milk, compared with the plasma. In the plasma, levels of OSI correlated significantly with TAP ($r = -0.46$; $p = 0.015$) and TPP ($r = 0.90$; $p < 0.001$) while in the breast-milk, OSI correlated significantly with only the TAP ($r = -0.76$; $p < 0.001$).

Conclusion: It could be concluded that the higher TPP in the human milk would be beneficial against pathogens, while the bio-accumulated antioxidant molecules regulate free radical load to avert the consequences of oxidative stress in the breast tissue.

Keywords: Oxidative Stress, Human-milk, Lactating Mothers

Introduction:

Human-milk is a complex extracellular fluid containing basic nutritional needs of neonates / infants. It is characterized by heterogeneous populations of cells (i.e. lymphocytes, neutrophils, macrophages and epithelial cells), nutrients (including antioxidants) and non-nutritional bioactive components like antimicrobial factors, digestive enzymes, hormones, trophic factors, growth modulators^{[1][2]} and casomorphins[3][4]. Human-milk may also contain harmful components, such as pollutants, drugs, allergens, and viruses [1][5][6][7][8].

Neonatal protection by the human-milk is associated with passive immunity (IgG) from the mother but may also involve several other constituents in the human-milk. Available evidences suggest that the protective role of human-milk in the immunologically naive newborns is enhanced by continuous migration of leukocytes into mammary tissue from the blood. This makes the human-milk aseptic and provides immunological defense against bacterial invasion [9][10][11]. Such cellular distribution is in favor of immunologic processes (i.e. production of cytokines and phagocytic activities) for the protection of the mammary gland and human-milk against infectious agents [9]. The activated macrophages in the mammary tissues release chemical messengers, or chemo-attractants, which trigger the migration of polymorphonuclear cells towards the mammary tissue [12]. Since one of the important roles of the human-milk is to protect against common childhood infections through oxidative killing mechanism established in the phagocytes, this study assessed the status of cellular activation and antioxidant system in human-milk and plasma by determining the levels of markers of oxidative stress in the human-milk and corresponding plasma of the lactating mother.

Materials and Methods:

Materials:

Human-milk and blood were taken from forty lactating women attending postnatal clinic at Jericho Nursing Home, Ibadan, Nigeria. They were non-smokers (age range 22-36 years) who had normal delivery without postnatal complications. Mature breast-milk samples were collected between 6th and 18th weeks post-delivery.

Twenty milliliters (20 ml) of breast-milk and 5 ml of blood were collected simultaneously from each lactating mother for the determination of markers of oxidative stress. The institutional committee for ethics and research approved the study protocol. A written informed consent was taken from the participants of the study after responding to the contents of the questionnaire. The samples were kept at -20°C immediately after collection to ensure inactivation of the cellular constituents and thus stopping free radical generation and lipid peroxidation (as earlier reported by Lawrence [13]). Human-milk samples were separated into fat, supernatant and cellular fractions by centrifugation at 6000 g for 20 minutes. The fatty layer was removed and the supernatant (milk plasma) was collected and stored until ready for analysis.

Methods:

Determination of MDA:

Level of malondialdehyde was determined in both human-milk and the corresponding blood plasma using the method of Varshney and Kale [14]. The principle is based on the fact that malondialdehyde (MDA), a product of lipid peroxidation reacts with the chromogenic reagent; 2-thiobarbituric acid (TBA) under acidic conditions to produce a pink-colored complex measured colorimetrically at 532nm.

Determination of TAP:

TAP, and index of total antioxidants was determined in both human-milk and blood plasma using the ferric reducing / antioxidant power (FRAP) assay [15][16]. 50 μl of test sample and standards mixed with 1.5 ml of FRAP reagent pre-warmed at 37°C (300mM acetate buffer - pH -3.6, 10mM 2,4,6- tripyridyl-s-triazine in 40mM HCl and 20mM FeCl_3 at ratio 10:1:1) in corresponding test tubes. Absorbance was read at 593 nm against a reagent blank. The result was reported as $\mu\text{mol Trolox equiv. / L}$.

Determination of total plasma peroxide (TPP):

Determination of TPP was based on the principle that ferrous-butylated hydroxytoluene-xylene orange complex reacts with plasma hydrogen peroxide to form a color complex that was measured spectrophotometrically at 560nm [15].

Determination of oxidative stress index (OSI):

OSI, an indicator of the degree of oxidative stress is the percent ratio of the TPP to the TAP [15].

Statistical analysis:

Statistical analyses were done using SPSS version 21. The data were expressed as Mean \pm SD. Student (t) test was used for comparison of levels of TAP, TPP and MDA in human-milk and corresponding blood plasma of the lactating mothers. Pearsonian correlation coefficient (r) was calculated. *P* values less than 0.05 were considered significant.

Results:

Comparative assessments of markers of oxidative stress are demonstrated in Table 1. The mean levels of TAP, TPP and OSI increased significantly ($p < 0.001$) in human-milk compared with the plasma. The human-milk: plasma ratios of TAP, TPP and OSI were 3:1, 5:1 and 2:1 respectively, while that of MDA was 1:2 (Table 2). There was a significant ($p = 0.01$) decrease in the level of human milk MDA compared with the blood plasma. When correlations were computed (Tables 3 and 4), levels of OSI in the plasma correlated significantly with TAP ($r = -0.46$; $p = 0.015$) and TPP ($r = 0.90$; $p < 0.001$) while in the human milk, OSI correlated significantly only with the TAP ($r = -0.76$; $p < 0.001$).

Discussion:

Breast tissue has distinct cellular constituents with peculiar membrane receptors, which confer its specialized metabolic activities. Several studies confirm that while cells in the blood confer both specific and non-specific

immune-protective effects (with higher level of IgG), the cellular constituents of the human-milk confer majorly non-specific immunological factors that prevent the survival of pathogen in breast tissue and in the neonate [17][18][19]. The non-specific immunologic function was demonstrated by higher level of TPP (5 fold compared to plasma) observed in the human-milk of our lactating mothers. This might enhance the oxidative killing of pathogens and compensate for low level of humoral immune responses in the breast-milk. Significantly higher level of TPP observed in this study could constitute protection against microbial agents. Our result corroborates that of Brooke et al [20] who observed 1.8-fold higher levels of reactive oxygen species in human-milk than plasma levels taken from the same mother. Sardesai[21] also reported an overwhelming reactive oxygen species in the breast-milk. They associated their findings with the potentials of the human-milk to protect breast tissues against microbial infections. These higher levels of free radicals observed in their study have no significant effect on the micronutrient constituents of the breast-milk [22]. Further studies on the effects of free radicals on nutritionally essential constituents of the breast-milk showed that beta-casomorphin-7, a product of human-milk, contributes to the protection of the tissue structure and micronutrients against free radical-mediated oxidative stress through inhibition of NF-kappa-B / iNOS / NO signaling pathways [3].

Despite higher level of TPP in the human-milk, we still observed a higher level of TAP (3 fold higher than in plasma) in the breast-milk of the lactating mothers who participated in this study. Tsopmo et al.[23] and Aycicek *et al* [24] earlier reported that human-milk-fed infants demonstrated higher level of plasma antioxidant and lower level of plasma TPP than in formula-fed infants. Goldman et al.[1], Friel et al.[25] and Lugonja et al.[26] have also reported increased levels of antioxidants (i.e. vitamins C, A, and E and lactoferrin) in the human-milk of lactating mothers compared with the blood plasma levels. These confer anti-oxidative protection against overwhelming reactive oxygen species present in the human-milk [27]. Our findings also show that OSI correlated significantly with TAP in the breast-milk, but correlated with both TAP and TPP in the plasma of lactating mothers. This may suggest that antioxidant molecules play significant roles in the regulation of the level of oxidative stress index in the breast-milk and blood plasma of lactating mothers.

Lipid peroxidation occurs when hydrogen atom is abstracted from the methylene groups (CH₂ group) of long-chain polyunsaturated fatty acids (LC-PUFA) due to increased free radical load beyond the neutralization capability of the antioxidant system [28][29]. Surprisingly, in this study, the level of lipid peroxidation decreased significantly in the breast-milk despite the higher level of the TPP. This reduced lipid peroxidation could be associated with increased activity of antioxidant system and other possible anti-peroxidative constituents that may be present in the human-milk. Therefore, the authors of this study may assume that there is a physiologic anti-peroxidative machinery protecting the essential n-6 and n-3 PUFA and controlling the level of lipid peroxidation in the human-milk, since the human-milk lipids play critical roles in the growth and development of infants[30], structural components of cellular membranes of the neurones and retina during perinatal development [31][32].

In conclusion, levels of free radical generation and antioxidant system in blood are different from that of human-milk. Higher free radical generation in the breast tissue could be beneficial against pathogens, while the bio-accumulation of antioxidants molecules regulates free radical load and avert the consequences of oxidative stress in the breast tissue. The resultant lower plasma level of TAP in the corresponding blood plasma samples of lactating mothers may call for adjuvant micronutrient therapy to avert the risk of oxidative stress during lactation.

Competing interest: Authors declared that there was no competing interest.

Authors' contributions: Moses O. Akiibinu and Susanah O. Akiibinu designed the study; Moses O. Akiibinu and Adekunle A. Adesiyun did the analysis, all authors read and approved the final manuscript.

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Tables (Refer Results & Discussion)

Table 1: Markers of Oxidative Stress in Human-milk and Blood Plasma of Lactating Mothers

Samples	N	TAP ($\mu\text{Mol Trolox}$ Equiv./L)	TPP ($\mu\text{Mol H}_2\text{O}_2/\text{L}$)	OSI (%)	MDA (nMol/ml)
Human-milk	40	2371.3 \pm 275.9	45.02 \pm 13.6	2.28 \pm 1.0	3.07 \pm 1.3
Blood Plasma	40	784.7 \pm 252.3	9.1 \pm 4.9	1.27 \pm 0.8	5.93 \pm 2.1
p-values		<0.001*	<0.001*	<0.001*	<0.01*

*= significantly different from controls.

Table 2: Human-milk/Plasma Ratios of Markers of Oxidative Stress in Lactating Mothers

	TAP ($\mu\text{Mol Trolox}$ Equiv./L)	TPP ($\mu\text{Mol H}_2\text{O}_2/\text{L}$)	OSI (%)	MDA (nMol/ml)
Human-milk : Plasma ratio	3:1	5:1	2:1	1:2

Table 3: Correlation of Markers of Oxidative Stress in Blood Plasma (N=40) of Lactating Mothers.

Group	r-values	p-values
OSI/TAP	-0.46	0.015*
OSI/TPP	0.90	<0.001*
OSI/MDA	0.24	0.21

*= significantly different from controls.

Table 4: Correlation of Markers of Oxidative Stress in Human-milk (N=40) of Lactating Mothers

Group	r-values	p-values
OSI/TAP	-0.76	<0.001*
OSI/TPP	0.31	0.091
OSI/MDA	-0.21	0.26

*= significantly different from controls.