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Effect of Enteral Lactoferrin Administration on Invasive Fungal Infections In Preterm Neonates

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ABSTRACT

Background: Lactoferrin is an iron binding protein and one of the main component of breast milk that showed in the last decade very promising results in the prophylaxis against neonatal sepsis, NEC, fungal infections, anaemia of prematurity and infancy.

Objective: Our primary aim was to prove that LF has a beneficial effect in the prevention of IFIs in the preterm neonates in NICUs.

Methods: Our study was conducted on 40 preterm neonates in the NICUs of maternity and pediatrics hospitals, faculty of medicine, Ain Shams University in the period from March 2017 to May 2017. The 40 neonates were subdivided into 2 groups: Group A: 20 neonates who received LF in a dose of 100 mg/day for 4- 6 weeks starting from day of enrolment. Group B: 20 neonates who received placebo in form of distilled water for 4-6 weeks starting from day of enrolment. The neonates included in this study were subjected to full history taking, clinical examination laying stress on clinical signs of infection and laboratory investigations including CBC with differential cell count, CRP, blood culture and fungal cultures that were withdrawn on day of enrolment, day 7, 14, 21 and 28.

Results: Our 1ry outcome was to prove the antifungal effect of LF and it really helped to decrease the incidence as well as the severity of IFIs in the preterm neonates who received it versus those who didn't. As a 2ry outcome, our study showed that LF played a beneficial role in the enhancement of hemoglobin and hematocrit status and as a prophylaxis against anemia of prematurity. In addition to all this, LF

promoted the general condition of the studied neonates and significantly decreased the signs of sepsis, the feeding intolerance, the length of hospital stay and the mortality rate as well as helped those who received it to progress rapidly in feeding, need less invasive procedures and less antibiotics.

Conclusion: Lactoferrin is a safe drug that generally promotes the wellbeing of neonates and promotes their health and need to be taken into consideration by all the neonatal care health units in the developed and developing countries as it might help to decrease the incidence of complications of prematurity and its burdens on the individual and the society.

INTRODUCTION

Preterm birth is defined as the birth of a living neonate before the 37th gestational week ***(Blencowe et al., 2012).***

Those preterm babies especially very low birth weight (VLBW) (< 1500 g) and extremely low birth weight (ELBW) (<1000 g) are more susceptible to the risk of invasive fungal infections (IFIs), this risk is inversely proportional to the gestational age and birth weight ***(Kaufman and Manzoni, 2010).***

Many risk factors contribute in the increase of IFIs in the preterm neonate such as: Invasive procedures, such as central vascular catheters and endotracheal tubes, exposure to broad-spectrum antibiotics and parenteral nutrition, the occasional use of postnatal steroids and gastric acid inhibitors ***(Chitins et al., 2012 and Kaufman et al., 2012).***

The most common organism causing nosocomial fungal infections is *Candida* which is the 2nd most common cause of infectious disease related death in the neonatal intensive care unit (NICU) ***(Testoni et al., 2012).***

Although, the VLBW infant with candidiasis can present with many of the nonspecific signs and symptoms associated with invasive bacterial infection, symptoms are often more subtle and indolent and the lab results are not readily available ***(Greenberg et al., 2012).***

Fluconazole has been shown to be safe and effective in a number of randomized controlled trials (RCTs) and it is recommended in settings of patients with high incidence of IFIs ***(Kaufman et al., 2010).***

Despite reassuring reports، some concerns still exist related to long-term safety and modification of the fungal ecology induced by this azole with emergence of resistant strains ***(Manzoni et al., 2008 and Kaufman and Manzoni, 2010).***

Hence comes the importance of the use of prophylactics against IFIs to decrease the financial burden on the health system related to long term sequelae of IFIs in addition to long hospital stay.

Lactoferrin (LF) has immersed as a new tool for prevention of IFIs, it is an iron binding glycoprotein that is naturally present in mammalian milk, colostrum, tears, saliva, CSF and vaginal secretions, it's considered as a cell-secreted mediator that bridges the innate and adaptive immune responses ***(Valentini and Antonini, 2005 and Siqueiros-Cendon et al., 2014).***

Many studies had shown its important role in the innate immunity by its anti-bacterial, anti-viral, anti-fungal, anti-parasitic, anti-cancer, anti-inflammatory, anti-oxidant, anti-allergic, iron absorption modulatory functions ***(Pierce et al., 2009 and Trend et al., 2015).***

Its antifungal activity in particular is due to its fungistatic effects, and the activity of the N-terminal, 11 aminoacidic peptide of LF called lactoferricin [hLF(1-11)] ***(Lupetti et al., 2007).***

Aim of the Work

To evaluate the efficacy of enteral lactoferrin in the prevention of invasive fungal infections in preterm infants admitted in the neonatal intensive care unit (NICU).

PATIENTS AND METHODS

The study was a prospective single blinded study which included 40 preterm neonates who were admitted at NICUs of maternity and children hospitals, Faculty of Medicine, Ain Shams University, over a duration of 3 months recruitment, from March 2017 to May 2017.

The patients:

The study included 40 preterm neonates (<37 weeks gestation), with a gestational age ranging from 28-36 weeks and a birth weight ranging from 750 gm to 2.600 Kg within the 1st 72 hours of life and were divided into two groups:

Group A (20 preterm neonates) received lactoferrin (100mg/day) by enteral route for 4-6 weeks or until discharge whichever comes first.

Group B (20 preterm neonates) received placebo for 4-6 weeks or until discharge whichever comes first

The gestational age was determined by maternal last menstrual period and confirmed by the neonatologist's physical examination using Ballard score ***(Ballard et al., 1991).***

The Inclusion criteria were Age: < 37 weeks gestational age, postnatal within 72 hours of life and the baby has already started enteral feeding.

While the Exclusion criteria were failure to enroll in the 1st 72 hours of age, conditions necessitating nothing per os such as intestinal obstruction, NEC, congenital anomalies, and suspected inborn error of metabolism.

- **Systematic Randomization was done.**
- **Blindness was done using aliquots covered with opaque plaster.**
- ***Sample size:***

Sample size; determination assuming a rate of IFIs ranging between 0.7% and 2.0% in placebo group and lactoferrin group respectively, sample size of 20 patients in each group is enough to detect such difference if true, at 0.05 alpha error and 0.08 power of the test.

The methods:

All studied neonates were subjected to the following:

Detailed history taking:

- Prenatal history: for pre-existing maternal or fetal problems; maternal hypertension either chronic or pre-eclampsia, maternal vascular disease, maternal diabetes, antenatal steroids, maternal drug use, maternal infection, placental infarction, placental abruption, cord accidents, abnormalities of umbilical vessels, fetal Anemia, fetal infection and intrauterine growth retardation.
- Natal history: Abnormal uterine contractions, cord accidents, prolonged labor, mode of delivery, amniotic fluid (normal, offensive or meconium stained), and the need of resuscitation by oxygen, ambu bag, endotracheal intubation, chest compressions, medications and assisted APGAR score at 1, 5 and 10 minutes ***(Apgar, 1953).***
- Postnatal history: for pulmonary, cardiovascular or neurological abnormalities.
- Age of start of enteral feeding, type of milk and time to reach full enteral intake.
- Determination of the birth weight, length and occipto-frontal circumference
- Full clinical examination including; chest, heart, abdominal and neurological examination.
- **Clinical signs of infection:** temperature instability, irritability, apathy, feeding intolerance, prolonged capillary refill, apnea, tachycardia and tachypnea, and determination of onset of sepsis.

- **Assessment of the hemodynamic state of the neonates:** mean arterial blood pressure (MABP), urinary output, temperature, capillary refill time.

Laboratory investigations

- **Complete blood picture (CBC)** with differential leucocytic count. It was done using coulter GEN-S (Coulter corporation, USA). Manual differential count was obtained by clinical pathologists.
- **C-reactive protein (CRP)** quantitative assay using Latex agglutination slide test (Omega Diagnostics LTD, UK).
- **Blood cultures**:

Bacterial blood cultures were routinely withdrawn in the NICU on the baby's admission using conventional blood culture technique and we withdrew **fungal cultures** on the day of enrolment, day 7, 14, 21 and 28 as explained below:

1- Blood samples collection:

Under strict aseptic technique we collect blood samples into fungal blood culture vial in a ratio 1:10, special supplement was used (anti-saprophytic and bacterial antibiotic).

2- Blood sample incubation:

a. Each culture bottle was swapped and wiped by fresh ethanol.
b. Each bottle was labelled with the name, number of patient and date, time of collection.
c. Then incubated aerobically at 37°C for 2-3 days for yeast and for 2-3 weeks for fungi (molds).
d. The incubated media is then sub cultured on Sabauroud agar (Himedia company, India) ® for detecting fungal species and Hichrome candida differential agar (Himedia company, India) ® to detect candida species.
e. Sabouroud and Hichrome agars were incubated at 37°C and at room temperature and weren't discarded until 2 weeks.

Oral lactoferrin supplementation:

1. Lactoferrin (Pravotin)® by Hygint was given to all neonates in group (A) in a dose of 100mg/day (1 sachet) once daily starting on day 1 of enrollment, in 2 ml of distilled water with start of feeding and continued for 4-6 weeks or until discharge whichever comes first.
2. Group (B) received placebo in the form of 2 ml of distilled water starting on day 1 and continued for 4-6 weeks or until discharge whichever comes first.
3. Randomization was done using closed envelope technique aliquots were covered with opaque plaster.

RESULTS

The results of our study are summarized in tables from 1-4 as follows:

In our study, lactoferrin group started feeding significantly earlier than the placebo group, also, the rate of increment of feeding was significantly higher in lactoferrin group than placebo as shown in table (1).

Table (1): Comparison between placebo group and lactoferrin group regarding start of feeding and rate of feeding increment

		Placebo group	**Lactoferrin group**	**Test value**	**P-value**	**Sig.**
		No. = 20	**No. = 20**			
Start of feeding (days)	Median(IQR)	2 (1 – 3)	1 (1 – 1)	-2.083‡	**0.037**	**S**
	Range	1 – 3	1 – 3			
Rate of increment (cc/kg/day)	Mean±SD	14.00 ± 4.76	18.75 ± 6.04	-2.762•	**0.009**	**HS**
	Range	10 – 25	10 – 30			

HS: Highly significant; S: Significant
•: Independent t-test; ǂ: Mann Whitney test

There was also a significantly higher incidence of IFIs in placebo group more than lactoferrin group as regards the number of positive and negative fungal cultures results as shown in table (2).

Table (2): Comparison between placebo and lactoferrin group regarding the incidence of IFIs proved by fungal culture:

Total	**Placebo group**		**Lactoferrin group**		**Test value***	**P-value**	**Sig.**
	No.	**%**	**No.**	**%**			
Negative	13	65%	19	95%	5.625	**0.0177**	**S**
Positive	7	35%	1	5%			

In addition, lactoferrin proved to enhance the haemoglobin and haematocrit status in those who received it by having a significantly higher haemoglobin and haematocrit than the placebo group as shown in table (3).

In our study, there was no mortality in the Lf group and 4 neonatal deaths in the placebo group and the LF group has significantly lower length of hospital stay than placebo group as shown in table (4).

Table (3): Comparison between lactoferrin group and placebo group regarding the 5th CBC

CBC5		Placebo group No. = 20	Lactoferrin group No. = 20	t-test	P-value	Sig.
Hemoglobin (gm/dl)	Mean±SD	12.24 ± 3.74	16.31 ± 3.64	**-3.056•**	**0.005**	**HS**
	Range	5.9 – 20.9	8 – 21			
Hematocrit (%)	Mean±SD	35.56 ± 9.06	43.45 ± 8.92	**-2.431•**	**0.021**	**S**
	Range	25 – 60.4	23 – 65			

Table (4): Comparison between placebo and lactoferrin groups as regards the mortality rate and the duration of hospital stay.

		Placebo No. = 20	Lactoferrin No. = 20	Test value	P-value	Sig.
Outcome (n, %)	Discharged	16 (80.0%)	20 (100.0%)	4.444	**0.035**	**S**
	Died	4 20.0%)	0 (0.0%)			
Duration of hospital stay (days)	Mean±SD Range	29.55±9.87 9 - 50	23.0±9.52 11 – 45	2.292	**0.028**	**S**

DISCUSSION

In the present study, both studied groups showed no significant difference as regards demographic characteristics in terms of sex, gestational age, postnatal age, mode of delivery, cause of prematurity and Apgar score at 1 and 5 minutes.

However, LF group had a higher birth weight than placebo group, LF group received O2 and suction more than placebo group while there was more unattended deliveries in placebo group.

As regards the nutritional characteristics of the studied groups, in our study we found that in LF group, neonates started feeding significantly earlier and had significantly more increment of feeding rate than placebo group and though not significant, the LF group reached full enteral feeding earlier than placebo group. This can be attributed to the prebiotic function of lactoferrin which enhances the growth of the normal bifidogenic gut microflora with predominant healthy commensals such as bifidobacteria and lactobacilli ***(Rahman et al., 2009 and Awad et al., 2017).***

Similarly, ***Akin et al. (2014)*** study showed no statistical significance in age to reach full enteral intake among studied groups, although age to reach full enteral feeding was earlier in bovine lactoferrin group, perhaps due to the small sample size.

In addition to ***Manzoni et al. in (2009) and (2011) and Ochoa et al. in (2015)*** who proved that there were no significant differences between both groups regarding feeding.

Regarding the invasive procedures, although there were significantly more cannula insertion in the LF group than placebo group, there was no change in frequency of fungal infection and there was significantly more central line and ETT insertion in the placebo group than LF group.

This can be due to the better medical course in the LF group with less need for CVC insertion, less need for parenteral nutrition and rapid increment of feeding.

In the present study we found that the hemoglobin and hematocrit were higher in LF group than in placebo group and in the 4th CBC there was significantly lower WBC in LF group than placebo and this was in accordance with ***Chierici et al. (1992)***, ***King (2007)*** *and* ***El Farsy et al. (2017).***

The role of LF in the enhancement of iron concentration in the neonates was explained by ***Ke et al. in (2015)*** who mentioned that saturated iron concentration of LF in human milk is about 10-30 % in addition to its role in the transport of two ferric iron in synergy with two bicarbonate ions, LF has a 300 times higher iron binding capacity than transferrin ***(Du et al., 2017).***

LF has its own receptors in the enterocytes, but with the lack of these receptors in some animals other studies showed that iron absorption is not affected which leaves the mechanism of LF in iron absorption uncertain and this explains why neonates up to 6 months of age have no iron deficiencies when breastfed ***(González-Chávez et al., 2009).***

In the present study, there was no significant difference between both studied groups as regards fungal cultures 1, 2, 3 and 4, however as regards 5th fungal culture, 75% of LF group were negative, 25% were discharged and no reported mortality, while in the placebo group 85% showed negative results and 15% mortality.

But there was a significantly higher incidence of IFIs in the placebo group more than lactoferrin group which proved that LF had a preventive effect against IFIs. This is in accordance with ***Manzoni and his***

colleagues in (2011), where they proved that LF alone or in combination with lactobacillus is effective in reducing the incidence of IFI in preterm VLBW neonates but has no effect on fungal colonization.

In our study, we found that there was a no significant difference between both studied groups as regards antibiotics used except for vancomycin which was significantly used in the placebo than LF group, this can be due to the longer length of stay of the placebo group and more use of CVC and ETT, delayed start of feeding and less increment of feeding than LF group.

In their study, ***Ochoa et al. in (2015)*** commented on the prolonged use of 3rd and 4th generation cephalosporin in placebo group.

In the present study, we found that in LF group the patients required less MV and less duration on MV than placebo group although it didn't reach statistical significance. This was comparable to ***Manzoni et al. (2011)***.

As for inotropes used, dopamine and dobutamine were significantly more used in placebo group with 65% of patients versus 0% in LF group.

In the present study, we found that in LF group, there were significantly less signs of sepsis which is in accordance with ***Manzoni et al. (2009)***, who compared the effect of lactoferrin alone at 100 mg/day or in combination with lactobacillus Rhamnosus versus placebo and proved that late-onset sepsis occurred less frequently in the BLF and BLF plus Lactobacillus Rhamnosus GG (LGG) groups.

In addition to his latest study in ***(2018)*** where ***Manzoni et al.,*** proved that LF counteracted the effect of gastric acid inhibitors which increases the risk of infection in preterm neonates.

Ochoa et al. (2015) enrolled 190 neonates weighing less than 2500 g at birth. BLF in a dose of 200 mg/day in 3 divided doses over first four weeks of life versus maltodextrin as placebo were used.

There was decreasing trend in incidence of sepsis in the BLF group compared to the placebo group.

It also goes in agreement with ***Kaur and Gathwala (2015)***, where they enrolled 121 low birth weight (less than 2000 grams) neonates. BLF was supplemented daily over first 28 days of life and the control group received placebo for the same duration. The authors reported that incidence of first episode of culture proven LOS was significantly lower in the BLF group than in the placebo group.

It also goes in concordance with ***Actor et al. (2009), Ando et al. (2010)*** where BLF is proved to have immune-modulatory role, ascribed to its immunotropic and anti-inflammatory effects.

While on the other hand it goes in contrast to ***Akin et al. (2014)*** who published their trial of oral BLF to prevent nosocomial sepsis and NEC in premature neonates and studied effect on T-regulatory cells. Where a prospective, placebo-controlled, double-blind, randomized trial, infants either VLBW or born before 32 weeks were assigned to receive either placebo, or 200 mg BLF daily throughout hospitalization. Episodes of culture proven nosocomial sepsis and NEC were recorded. They reported fewer sepsis episodes in intervention group with none developing NEC, though without statistical significance. This may be attributed to the small sample size.

And with ***Awad et al. (2017)*** who proved that the incidence of late onset sepsis was significantly lower lactoferrin group compared to control group.

Though not statistically significant, LF group showed no signs of NEC or feeding intolerance in comparison to placebo group that had 3 patients who developed NEC which is comparable to ***Ochoa et al. (2015) and Akin et al. (2014)*** who explained this by the small sample size as well as to ***Barrington et al. in (2016)*** who didn't reach statistical significance regarding NEC in both groups by adding lactoferrin to the babies feeds.

Also, ***Pammi and Abrams (2011)*** and ***Venkatesh and Abrams (2010)*** reported that there is no evidence of efficacy of oral lactoferrin in the prevention of NEC in preterm neonates.

This in contrast to ***Manzoni et al. (2014)*** who evaluated the effect of administration of BLF alone or in combination with Lactobacillus Rhamnosus GG (LGG) on incidence of NEC ≥2 Bell's stage, the main finding of the study was that oral BLF administration, alone or with LGG, significantly decreased NEC incidence in VLBW neonates, as well as the death-and/or-NEC outcome. NEC incidence was significantly lower in BLF and BLF + LGG (2.0% and 0%, respectively) than in controls (5.4%).

BLF plays an important role in protecting against the virulence and the toxicity of the microbes in the small intestine which may be effective in the prevention of NEC ***(Sherman, 2016 and Awad et al., 2017)***. It also has a trophic and pro-proliferative activity on the enterocytes, regulating gut permeability ***(Buccigrossi et al., 2007 and Awad et al., 2017)*** and downregulating pro-inflammatory cytokines unexpressed in intestinal

epithelial cells so has an anti-inflammatory role as well ***(Berlutti et al., 2006 and Awad et al., 2017)***. In addition, BLF has an antioxidant effect by suppressing free radical activity and decreasing levels of oxidative products ***(Raghuveer et al., 2002 and Awad et al., 2017).***

There are no known risks related to LF intake and it was used in many previous studies with no LF-related side effects. However, because there was the remote possibility of an allergic reaction to cow milk proteins, we closely monitored for possible signs (allergic rhinitis, diarrhoea, vomiting and eczema). There were no signs of allergy or treatment intolerance in 97.5% of observed neonates; there were only 2 episodes of vomiting during the intervention period in one patient which is in accordance with ***Ochoa et al. (2015) and Barrington et al. (2016).***

Conclusion

Lactoferrin, being an iron binding protein and broad spectrum non pathogen-specific antimicrobial protective protein offers perspectives of beneficial effect in neonates especially the preterm ones and proved to have an effective role in the prevention of IFIs, improvement of clinical signs of sepsis, prevention of NEC, improvement of hemoglobin status, shortening of the duration needed to reach full enteral intake as well as of hospitalization and decreasing the mortality rate.

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