The Effect of Probiotics Supplementation on Fecal Calprotectin as an Early Marker of Neonatal Enteropathy

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Abstract. ***Objectives.*** Evaluationof the effect of probiotic supplementation on fecal calprotectin levels which was tested as an early marker of necrotizing enterocolitis (NEC) and sepsis in neonates. ***Patients and Methods.*** A prospective, double blind, randomized, controlled clinical trial was conducted in 30 neonates who were examined for fecal calprotectin (FCP) levels. neonates are divided into two groups, the group I consisted of 15 neonates who were given probiotics (Probiotics group) and group II consisted of 15 neonates who were not given probiotics(non-probiotic group), both groups were followed up and observed until reach full feeding for the occurrence of necrotizing enterocolitis (NEC), feeding intolerance and sepsis. ***Results.*** FCP levels were higher in neonates of group II (non-probiotics group) than neonates of group I (Probiotics group) after 2 weeks of probiotic supplementation in neonates of group I (190.5± 86.9 versus 38.4 ± 32.2) and significant Correlation between the level of FCP and Enteropathy in group I showing + ve correlation and p-value<0.05 with p-value is (0.029,0.024,0.019) at the onset of research and after 1 wk and after 2 wks respectively and Cutoff point of the FCP on which enteropathy occurred was 482 µg/g. ***Conclusions.*** The use of probiotics in neonates could decrease the incidence of NEC and sepsis and FCP could be used as an early predictor of NEC in neonates for early prevention and treatment with better prognosis and outcome

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Keyword: probiotics, calprotectin, neonates, enteropathy

1. Introduction

Neonatal period is the first 28days (4weeks) of life. [1]. Necrotizing enterocolitis (NEC) is the most common severe gastrointestinal emergency that affects the neonates. [2].This disease often has a rapid onset with few antecedent signs that used to predict its occurrence. It can lead to death or severe morbidity, so identifying early markers specific for high NEC risk would offer opportunities for early prevention and intervention. [3,4].

Calprotectin is a 36Kilodalton(kDa) calcium and zinc binding protein that constitutes about 60% of soluble cytosol protein in human neutrophil granulocytes and is found in monocytes, macrophages and epithelial cells[5].Calprotectin is resistant to proteolysis and stability even after a week of storage at room temperature facilitate its determination in feces. [7].

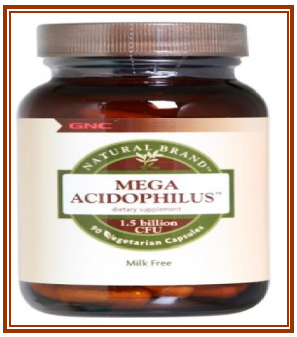
High fecal calprotectin(FCP) levels were shown to correlate with an increased turnover of leukocytes in the intestinal barrier and granulocyte migration towards intestinal lumen. [5].Use of FCP as an early, non-invasive, easy to obtain, and inexpensive marker of gastrointestinal disease, particularly NEC, has been explored in some studies. [5].

Probiotics are microorganisms that have beneficial properties for the host. The list of such micro-organisms includes strains of lactic acid bacilli (eg, Lactobacillus and Bifidobacterium). [6]. Probiotics have been successfully applied to treat several gastrointestinal disorders with beneficial effects which observed in NEC, diarrhea especially antibiotic-associated diarrhea. [8].

2. Materials and Methods

This prospective randomized double blind placebo controlled study was conducted at Tanta University Hospital, Neonatal Intensive Care Unit (NICU) on 30 neonates during the period from 5/2012 to 11/2013 as there were many exclusions criteria.

The group I consisted of 15 neonates who were given probiotics (mega acidophilus) in a dose of 1.5 billion CFU once daily for 2 weeks. (Probiotics group).



The group II consisted of 15 neonates who were not given probiotics (non-probiotic group).

The inclusion criterion was: preterm and full term neonates (gestational age<38wks).

The exclusion criteria were: GIT anomalies, congenital anomalies, sepsis, hypoxia, chest diseases and cardiac diseases .Written informed consent was obtained from the parents of all subjects of the study. The study was approved by The Ethics Committee of Faculty of Medicine, Tanta University.

Methods:

Preparation and administration of probiotics:

The probiotics group received standard formula supplemented by Lactobacillus acidophillus + Bifidobacterium bifidum with an added dose of 1.5x109 colony forming units once daily for 2 weeks. Lactobacillus acidophillus + Bifidobacterium bifidum were prepared using a sterile technique utilizing a suspension of dried powder in sterile distilled water obtain 1.5x109 CFU and stored in a refrigerator at a temperature between 2˚C to 8˚C. This suspension was added to infant formula once daily. Feeding was started when the infant had stable vital signs, active bowel sounds without abdominal distension, gastric aspirate (feeding tolerance).

In the present study we have taken in account the cases of NEC occurring after 7 days of supplementation, such a period was considered the minimal time required by probiotics to colonize the bowel and to exert its action[11].

All the cases were subjected to the following:

1) Complete history taking

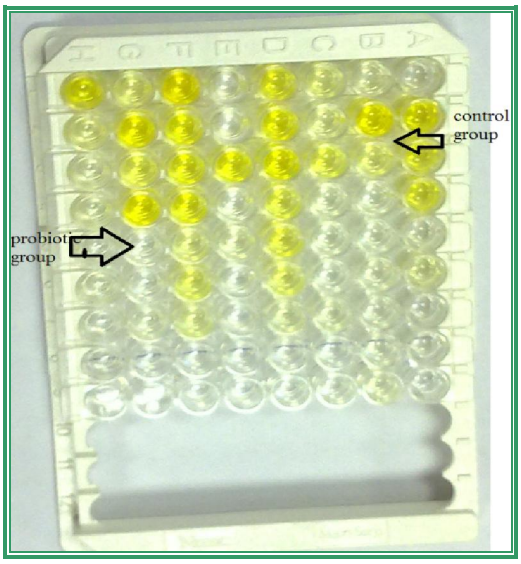
2) Full clinical examination

3) Laboratory investigations: Complete blood picture-reactive protein, Blood gases, Stool PH,Stool calprotectins (ELISA).

Both groups will be observed for two weeks after initiation of entral feeding with special concentration on the following: Occurrence of feeding intolerance, Signs of NEC, Measurement of fecal calprotectin before treatment and one week and 2 weeks from probiotic supplementation.

Principle of the assay:

After a short extraction procedure using one volume of feces and 49 volumes of extraction buffer, the test allows for the selective measurement of calprotectin-antigen by sandwich ELISA technique. Amonoclonal capture antibody (mAb) highly specific to calprotectin heterodimeric and polymeric complexes, respectively, is coated onto the microtiter plate. Calibrators, controls and patients extracts are incubated at room temperature of 30 minutes. After a washing step a detection antibody(Ab) conjugated to horseradish peroxidase (HRP) detects the calprotectin molecules bound to the monoclonal antibody coated onto the plate after incubation and a further washing step, tetrametylbenzidine (TMB) is added (blue color formation) followed by stopping reaction (change to yellow color) the absorption is measured at 450nm.



Yellowish color were more prominent in the non-probiotic group means high level of calprotectin and faint yellowish in probiotic group means low calpotectin level.

Specimen collection and storage:

50 to 100 mg of native stool sample are needed for extraction procedure. Stool samples were collected in plain tubes and stored refrigerated at 2-8ºC for up to 6 days. As freezing may result in slightly increased calprotectin concentrations due to Neutrophiles present in the sample, the extraction were kept at -20ºC for longer storage. The samples were collected without any chemical or biological additions in the collection device.

ELISA procedure:

Assay was performed according to the extended range ELISA procedure. It depends on the expected calprotectin concentration of the sample using a sample dilution of 1:150 (working range 30-1800 microgram).

Statistical analysis:

It was performed by using SPSS for Windows, version 9. Data were expressed as range and mean ± standard deviation (SD)/ or numbers and percentages. Differences between groups in continuous variables were tested for significance with paired t-test while univariate analysis was done with the *Chi-square*test. For all statistical tests done, *P*value< 0.05 was considered significant. Linear Correlation Coefficient was used to assess different correlations.

3. Results

Group I: Consisted of 15 neonates were given probiotics (probiotics group).

Group II: Consisted of 15 neonates who were not given probiotic (non-probiotics group).

Table (1): Comparison between both groups according to sex, gestational age (GA)and weight(Wt) showing no significant difference (P.>.05).

		GI	GII	t. test or X^2	p. value
sex	Male	3(20%)	8(53.3%)	3.589	0.058
	Female	12(80%)	7(46.7%)		
	Total	15(100%)	15(100%)		
GA(wks)	Range	26-37.4	26.4-37.6	0.484	0.632
	Mean ±SD	32.6±3.42	31.97±3.71		
Wt(grams)	Range	800-2500	800-2820	0.635	0.225
	Mean ±SD	1756.6±476	1672.6±630		

GI(GroupI),GII(GroupII),* P.value is significant if < .05

Table (2): Comparison according to incidence of NEC at the onset of the research and after 2 weeks of onset showing significant difference in group I (P.< .05).

		At onset of research		After 2 week		X^2	p. value
		N	%	N	%		
Incidence of NEC	GI	3	75	1	25	4.520	0.038*
	GII	4	57.1	3	42.9	1.639	0.112

*** P.value is significant if < .05**

Table (3): Correlation between the level of FCP and Enteropathy in group I showing +ve correlation.

GI		Fecal calprotectin (µg/g)		
		At onset	First week	After 2 weeks
Enteropathy	r.	0.412	0.362	0.98
	p	0.029*	0.024*	0.019*

*** P.value is significant if < .05**

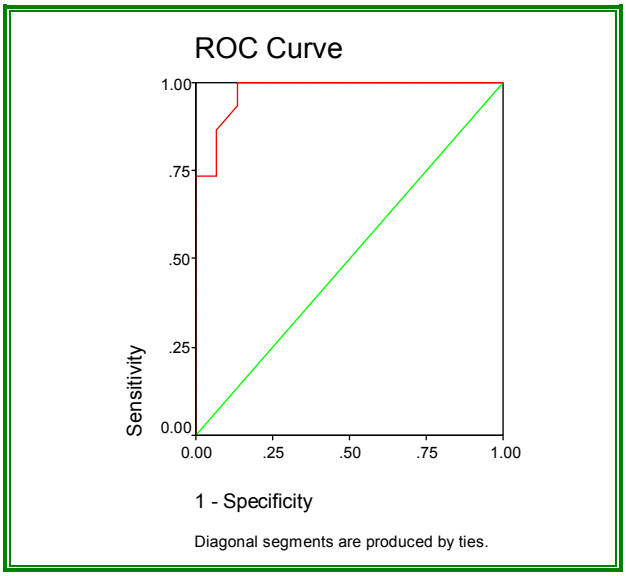


Fig. (1): Cutoff point of the calprotectin on which enteropathy was occured.

Table (4): Cutoff point of the FCP on which enteropathy was occurred.

(Fecal calprotectin) (μg/g)					
Cutoff	**Sens.(%)**	**Spec.(%)**	**PPV**	**NPV**	**Accuracy**
482	91.7	83.9	78.4	82.5	80.8

4. Discussion

In the present study, there was significant difference between the probiotics group(G **I**) and non-probiotics group(G **II**) in the incidence of sepsis and NEC with decreased incidence in the probiotics group, this was in agreement with ***Awad et al., (2010)***[12], ***Indrio et al., (2008)***[13] who stated that there was decrease in NECand sepsis rate in neonates of probiotics group, and the study also in agreement with ***Samanta et al., (2009)***[6] who stated that prophylactic probiotics supplementation reduced the incidence of NEC in neonates,but this is different from the results of ***Fernández-Carrocera et al., (2013)***[14] who reported that no difference in NEC and sepsis rate between neonates of probiotic group and those of control group

The present study demonstrated significant reduction in neonatal sepsis and NEC in probiotic than non-probiotics group. In agreement with our results ***Ren and Wang, (2010***) [15] who confirmed that appropriate and early gut colonization with beneficial bacteria lower the incidence of sepsis and NEC in neonates.

The present study showed that there was correlation between FCP and severity of NEC and this is in agreement with ***Aydemir et al., (2012)***[16] who stated that the increased levels of FCP could predict the occurrence of NEC and on the contrary, ***Selimoglu et al., (2011)***[17] said that FCP did not play a role in diagnosis of NEC, particularly in the early stages of disease.

The present study showed that fecal calprotectin could be an easy,non-invasive marker for early prediction of NEC in neonates with early prevention and treatment with better prognosis and outcome so decreasing the morbidity and mortality of this serious disease and this is in agreement with ***Campeotto, et al (2009)***[10]

From our study we can conclude that probiotic decrease the level of FCP which is a marker of NEC and this is in agreement with **Mohan *et al., (2008)***[9] who found that bifidobacterial supplementation (probiotics) was associated with a significant decrease in calprotectin level and NEC occurrence in neonates.

Conclusion

From this study, we can conclude that, the use of probiotics (mega acidophilus) in neonates could decrease the incidence of NEC and FCP could be used as an early predictor of NEC in neonate for early prevention and treatment with better prognosis and outcome so, decrease morbidity and mortality of this serious disease.

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