http://dx.doi.org/10.1590/1415-52732014000500005

ORIGINAL | *ORIGINAL*

Long-term effects of neonatal malnutrition on microbicide response, production of cytokines, and survival of macrophages infected by *Staphylococcus aureus* sensitive/resistant to methicillin

Efeitos tardios da desnutrição neonatal na resposta microbicida, produção de citocinas e viabilidade de macrófagos na infecção por Staphylococcus aureus *sensível/resistente a meticilina*

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ABSTRACT

Objective

To assess microbicide function and macrophage viability after *in vitro* cellular infection by methicillin-sensitive/resistant *Staphylococcus aureus* in nourished rats and rats subjected to neonatal malnutrition.

Methods

Male Wistar rats (n=40) were divided in two groups: Nourished (rats suckled by dams consuming a 17% casein diet) and Malnourished (rats suckled by dams consuming an 8% casein diet). Macrophages were recovered after tracheotomy, by bronchoalveolar lavage. After mononuclear cell isolation, four systems were established: negative control composed exclusively of phagocytes; positive control composed of macrophages plus

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 http://dx.doi.org/10.1590/1415-52732014000500005

lipopolysaccharide; and two testing systems, macrophages plus methicillin-sensitive *Staphylococcus aureus* and macrophages plus methicillin-resistant *Staphylococcus aureus*. The plates were incubated in a humid atmosphere at 37 degrees Celsius containing 5% CO_2 for 24 hours. After this period tests the microbicidal response, cytokine production, and cell viability were analyzed. The statistical analysis consisted of analysis of variance ($p<0.05$).

Results

Malnutrition reduced weight gain, rate of phagocytosis, production of superoxide anion and nitric oxide, and macrophage viability. Production of nitrite and interleukin 18, and viability of macrophages infected with methicillin-resistant *Staphylococcus aureus* were lower.

Conclusion

The neonatal malnutrition model compromised phagocyte function and reduced microbicidal response and cell viability. Interaction between malnutrition and the methicillin-resistant strain decreased the production of inflammatory mediators by effector cells of the immune response, which may compromise the immune system's defense ability.

Indexing terms: Macrophages. Malnutrition. Methicillin. *Staphylococcus.*

RESUMO

Objetivo

Avaliar a função microbicida e a viabilidade de macrófagos, após infecção celular in vitro*, com* Staphylococcus aureus *sensível/resistente a meticilina, em ratos nutridos ou submetidos a desnutrição neonatal.*

Métodos

Ratos machos Wistar (n=40) foram divididos em dois grupos distintos: Nutrido (ratos amamentados por mães submetidas a dieta com 17% de caseína) e Desnutrido (ratos amamentados por mães submetidas a dieta com 8% de caseína). Os macrófagos foram recuperados após procedimento cirúrgico de traqueostomia, através da coleta do lavado broncoalveolar. Após o isolamento dos mononucleares, foram estabelecidos quatro sistemas: controle negativo, composto apenas pelos fagócitos; controle positivo, macrófagos mais lipopolissacarídeo; e dois sistemas teste, macrófagos mais Staphylococcus aureus *sensível e resistente a meticilina. As placas foram incubadas por 24 horas, à temperatura de 37ºC, com atmosfera úmida e 5% de dióxido de carbono. Transcorrido esse período, foram realizados ensaios para análise da resposta microbicida, produção de citocinas e viabilidade celular. Na análise estatística, utilizou-se analysis of variance, admitindo-se p<0,05.*

Resultados

A desnutrição acarretou redução do crescimento ponderal dos animais, da taxa de fagocitose, da produção de óxido nítrico, do ânion superóxido e da viabilidade de macrófagos. Houve menor produção de nitrito, de interleucina 18 e da viabilidade dos macrófagos infectados com Staphylococcus aureus *meticilina-resistente.*

Conclusão

O modelo de desnutrição neonatal adotado comprometeu a função dos fagócitos, com redução da resposta microbicida e da viabilidade celular. A interação de desnutrição com cepa resistente induziu baixa produção de mediadores inflamatórios por células efetoras da resposta imunológica, o que poderá resultar em comprometimento da defesa.

Termos de Indexação: *Macrófagos. Desnutrição. Meticilina. Staphylococcus.*

INTRODUCTION

Environmental insults during vulnerable periods of an organism's development can permanently affect the structure and function of organs and tissues. This vulnerability is associated with the intense differentiation and maturation that organ systems undergo during these periods[1]. Studies have shown that neonatal malnutrition can compromise the formation of homeostatic systems, such as the nervous, endocrine, and immune systems[2,3].

According to Chandra[2], neonatal malnutrition affects the macrophages' functional mechanism, causing enduring changes in the adult organism, even long after nutritional recovery. Malnourished individuals may have deficient phagocytic microbicidal function, such as low production of proinflammatory cytokines, free radicals (nitric oxide and superoxide anion), and macrophage viability, making the body more vulnerable to infections[4,5].

Pathogenic invasion may deregulate the microbicidal responsiveness of immune[6] cells. This mechanism is triggered by various microorganisms to facilitate their growth and increase their survival time in the host[7]. In order to establish an infection, *Staphylococcus aureus* has developed several mechanisms designed to withstand the immune response.

Since the 1960's, infection by Methicillin-Resistant *Staphylococcus Aureus* (MRSA) has been considered a public health problem worldwide, mainly because it is more deadly than Methicillin-Sensitive *Staphylococcus Aureus* (MSSA)[8]. MRSA strains seem to have different virulence mechanisms, more intense than MSSA strains[9]. Numerous clinical studies on morbidity and mortality rates have indicated that MRSA is more virulent than MSSA. However, laboratory studies that assessed the presence and magnitude of pathogenic mechanisms and virulence factors in MSSA and MRSA strains obtained conflicting results[5,10].

Despite the various studies using malnutrition models, there is still a lot to be researched in terms of neonatal malnutrition and its late effects on the immune response. Also, there are hardly any studies evaluating the interaction between infection and malnutrition. The present study aimed to evaluate the impact of neonatal malnutrition on microbicidal function, cytokine production, and viability of alveolar macrophages infected *in vitro* by methicillin-sensitive and MRSA. In this context animal experiments may help to clarify morphological changes in early life stages, their intimate relation with microbicidal response, and infectious disease emergence and evolution.

METHODS

Animals and diet

Forty male Wistar rats (90-120 days) from the *Universidade Federal do Pernambuco* (UFPE) Department of Nutrition animal facility were used for the experiments. The experiments were conducted ethically as recommended by the *Colégio Brasileiro de Experimentação Animal* (COBEA, Brazilian College of Animal Experimentation) and the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the Center for Biological Sciences, UFPE under Protocol nº 23076.026684/2009-38. The animals were kept under controlled temperature (22°C, Standard Deviation-(SD)=1°C) in 12:12 hours light-dark cycles and had free access to water and chow.

One day after birth, litter size was standardized to six male pups per mother. On the same day, their first day of life, the litters were divided into two groups: a Nourished (N) group consisting of pups nursed by dams consuming a 17% casein diet (n=20); and a Malnourished (M) group consisting of pups nursed by dams consuming an 8% casein diet (n=20) for the first 21 days after birth. The 8% casein diet is widely used as an experimental model for the study of malnutrition because it induces protein malnutrition[11] in animals.

During the neonatal period the animals were weighed daily by a digital electronic scale (Marte, model S-4000, with an accuracy of 0.1 g) to monitor their body weight during nutritional manipulation. From the 22nd day of life until the end of the experiment, the animals were weighed three times a week to monitor their nutritional recovery. During this period the animals were

http://dx.doi.org/10.1590/1415-52732014000500005

separated from the dams, kept in cages in groups of three, and fed a standard chow (Vivarium, Labina Purina-Brazil) until adulthood (approximately 90 days).

Strains of *Staphylococcus aureus*

Strains of *Methicillin-Sensitive Staphylococcus Aureus* (The American Type Culture Collection - ATCC 33591) and methicillin-sensitive *Staphylococcus aureus* were used due to their distinctive resistance and importance in terms of public health. The bacteria were maintained in Tryptic Soy Broth (TSB) supplemented with 20% glycerol, at -20°C, until use. Twenty-four hours before each experiment, the strains were plated on blood agar (agar supplemented with 5% sheep blood) and incubated at 37°C. At the beginning of the test, some colonies were transferred to tubes containing Phosphate-Buffered Saline (PBS) to provide a turbidity of approximately 0.15 nm at a wavelength of 570 nm. This absorbance, according to Lu & McEwan[12], corresponds to a concentration of approximately 10^6 bacteria/mL of PBS.

Bronchoalveolar lavage

Bronchoalveolar Lavage (BAL) was done as recommended by Castro *et al.*[13]. The animals were anaesthetized with chloralose-urethane (0.5 and 12.5%, respectively) at 8 mL/kg ip. BAL was collected by injecting 0.9% saline through a plastic cannula into the trachea. Several aliquots of 3 mL were then injected and collected in 50 mL conical polypropylene tubes (Falcon, Sigma).

Culture of alveolar macrophages

Bronchoalveolar Lavage samples were centrifuged at 1500 rpm for 15 minutes. The precipitate that corresponds to the cells was resuspended in RPMI 1640 (Roswell Park Memorial Institute medium - Gibco, Invitrogen Corporation) containing 3% fetal bovine serum (Gibco-Invitrogen Corporation) and antibiotics (100 U penicillin/mL and 100 μg streptomycin/mL).

The cells were transferred to 35 mm diameter (6-well Falcon) cell culture dishes, in which a 2 mL suspension was dispensed in a proportion of 10^6 cells/mL in RPMI 1640. After 1 hour in an incubator at 37°C and 5% CO_2, the supernatant was discarded with non-adherent cells and 2 mL of half RPMI were added, leaving the plates for another 1 hour in the incubator in order to stabilize the cells.

Systems

Three systems were established in order to evaluate the rate of adherence, phagocytosis, and superoxide production: Control (C), with only alveolar macrophages; MSSA, Alveolar Macrophages (AM) plus 100 μL of bacterial inoculum on the methicillin-sensitive strain (ATCC 29213); and MRSA, AM plus 100 μL of bacterial inoculum of methicillin-resistant strain (ATCC 33591). To evaluate the kinetics of nitric oxide and alveolar macrophage viability, a positive control was added - PC containing AM plus 10 μL of Lipopolysaccharide (LPS) (*Escherichia coli* serotype; 055: B5, Sigma). Subsequently, the plates were incubated at 37°C in a wet atmosphere containing 5% CO_2.

Assessment of the adhesion rate

After incubation of cell cultures for 1 hour, aliquots were collected from the supernatant containing non-adherent cells and wells of the filtration plate were resuspended with RPMI. These aliquots containing non-adherent cells were added to trypan blue stain (1:10 dilution) and cells were counted using a hemocytometer. The Adhesion Rate (AR) was calculated using the formula described by De la Fuente *et al.*[14]: AR=100 - non adhered cells/mL/the initial number of cells/mL x 100.

Determination of the rate of phagocytosis

For this assay the bacterial inoculum was added to a suspension of 10^6 cells/mL in RPMI 1640 for both strains to a concentration of 10^6 CFU/mL in PBS, with a remainder volume of 1.5 mL in each tube. The contents of the tubes were homogenized and gently/evenly distributed on slides for optical microscopy. Slides were placed in an oven for 1 hour. After this period, they were washed to remove non-adherent cells and then stained and viewed by a trained, "blind" observer (for the system under analysis) using a light microscope. The result was expressed in percentage of phagocytic cells in a total count of 100 cells[15].

Analysis of low superoxide anion (O_2^-) release

O_2^- was induced by adding phorbol myristate acetate/PMA (Sigma) to Hank's solution (HBSS, Gibco-Invitrogen Corporation®) at a concentration of 2 µg/mL. Analysis of discontinuous systems was prepared with hourly assessments for 2 hours. Assay specificity was confirmed by the addition of Superoxide Dismutase (SOD) from bovine erythrocytes, containing 3000 U/mg protein in the final solution of 3 mg/mL in distilled water (Sigma)[13].

Kinetics of nitric oxide production by alveolar macrophages

The production of NO was given by the concentration of nitrite in the culture supernatant[10]. Every two hours, 100 µL of the supernatant were taken from the cultures, in a total incubation period of 24 hours. To quantify nitrates and nitrites, 50 mL of Griess reagent (1.5% sulfanilamide in 5% H_3PO_4, 0.1% in N-(1-naphthyl)ethylenediamine H_20) were added to supernatants. After standing 15 minutes at room temperature, an reader Enzyme Linked Assay (ELISA, ImmunonoSorbent - BIO-RAD, Model 680), with 550 nm filter, was used for the procedure. The nitrite concentration was calculated using average values of a $NaNO_2$ standard curve, data expressed in µM.

Quantification of cytokines IL-1β and IL-18 (interleukin-1β and interleukin-18)

After 24 hours of cell culture incubation, 100 µL of supernatant were collected. From this, IL-1β and IL-18 cytokines were counted by ELISA immunoenzyme test, using the *Quantikineâ m* (R&D Systems) kit.

Viability of alveolar macrophages

Cell viability was assessed by mitochondrial reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) on formazan[16]. After 24 hours of incubation, cell cultures were washed with PBS (1X) at room temperature They were incubated with 550 µL of PBS and 55 µL of MTT solution for two hours protected from light. After this period, 200 µL of PBS and 200 µL of DMSO were added and the cell monolayer was scraped. Quantification of solubilized formazam was performed in an ELISA reader (Bio-Rad, model 680) with 570 nm filter. Results were expressed in absorbance of formazan ($1x10^6$ cells).

Statistical analysis

The Student's t-test was used for the body weight and the rate of phagocytosis analysis. The other parametric data were analyzed by Analysis of Variance (Anova). When Anova revealed a significant difference, the Tukey test was used for identifying which groups differed from each other. The significance level was set at $p<0.05$. The statistical program used for the analyses was Sigma Stat 3.5 version.

http://dx.doi.org/10.1590/1415-52732014000500005

RESULTS

Body weight on malnutrition and nutritional recovery

The body weights (g) of the nourished and malnourished groups were similar until the 3rd day of life. From the 4th to the 21st postnatal day, the malnourished animals were lighter than the nourished animals ($p<0.001$). Between the 22nd and 90th days of life the two groups were fed the same chow, but the malnourished group remained lighter than the nourished group ($p<0.001$) (Figure 1).

Grip index

There were no differences between the nourished and malnourished groups (C-N=87.5±3,0; C-M=86±2,0; MSSA-N=90.3±2.6; MSSA-M=91±2.0; MRSA-N=93.3±3.1; MRSA=93±3.0), $p>0.05$. Also, no differences were observed between the systems under analysis, $p>0.05$.

Rate of phagocytosis

The rate of phagocytosis was lower in the malnourished group (MSSA-N=12.1±2.0; MSSA-M=4.1±3.2; MRSA-N=10.4±3.1; MRSA-M=4.3±3.0), $p<0.001$. However, when analyzing the MSSA and MRSA systems, there was no difference in the rates of phagocytosis of alveolar macrophages ($p>0.05$).

Production of superoxide

The malnourished group produced less superoxide than the nourished group for all systems ($p\leq0.001$) for both incubation times ($p<0.05$). However, the MSSA and MRSA systems did not differ ($p>0.05$) (Table 1).

Kinetic analysis of the nitric oxide production by alveolar macrophages

The quantification of NO was expressed in µM nitrite. The production of NO by macrophages was lower in malnourished animals in all systems ($p<0.05$). Differences were found between NC and PC systems in both groups after

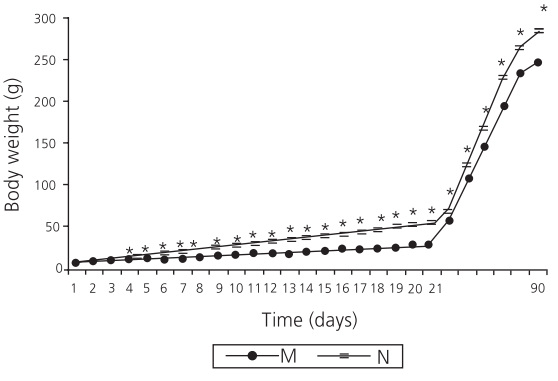

Figure 1. Weight curve during the neonatal malnutrition period (21 days) and nutritional supplementation period (23-90 days) of the groups (N: Nourished- and M: Malnourished). *Recife* (PE), 2009.

Note: *$p<0.05$ in comparison between Nourished and Malnourished groups.

Student's *t* test. Values are expressed as Mean ± Standard Deviation (n=40).

Table 1. Absolute figures for superoxide production in the groups (N:Nourished; M:Malnourished), in the systems (C: Negative Control; MSSA: Methicillin-Sensitive *Staphylococcus Aureus*; MRSA: Methicillin-Resistant *Staphylococcus Aureus*) and incubation periods (1 and 2 hours). *Recife* (PE), 2009.

Time	1 hour			2 hours		
Systems Groups	C	MSSA	MRSA	C	MSSA	MRSA
	M ± SD	M ± SD	M ± SD	M ± SD	M ± SD	M ± SD
N	1.47 ± 0.08	1.69 ± 0.07	1.70 ± 0.07	2.79 ± 0.14#	2.88 ± 0.19#	3.54 ± 0.17#
M	1.07 ± 0.08*	1.10 ± 0.14*	1.11 ± 0.08*	2.12 ± 0.28#*	2.34 ± 0.11#*	2.48 ± 0.14#*

Note: *$p<0.05$ in comparison of Nourished and Malnourished groups; #$p<0.05$ in comparison of 1 hour and 2 hours.

Analysis of Variance and Tukey test. Values are expressed as Mean ± Standard Deviation (n=40).

8 hours of incubation, with high production of nitric oxide in the PC (p<0.001). The peak NO production for the PC system occurred after 22 hours, both for the nourished and the malnourished group (p<0.001). From 4 to 10 hours, the average NO production was lower than in the nourished group in the malnourished group in the MSSA and MRSA systems (p<0.05). The peak NO production occurred after a 4h incubation period in MSSA system, group N, after 6h for group D, and after 8h for MRSA in both groups. Up to 12 hours of incubation, both for the nourished and malnourished groups, there was a reduction of NO production for MSSA and MRSA systems (p<0.001), similar to those of CN (Figure 2).

IL-1β Levels

The levels of IL-1β of nourished versus malnourished controls did not differ (p>0.05). However, when analyzing the testing systems, there was a lower concentration of IL-1β in the supernatant of the MRSA testing systems (MSSA-N=15.94±0.53 pg/mL; MSSA-M=11.81±3.01 pg/mL; MRSA-N=4.24±0.26 pg/mL; MRSA-M=6.41±0.3 pg/mL) p<0.05 (Figure 3).

IL-1β Levels

The levels of IL-18 of nourished versus malnourished controls did not differ (p>0.05). The production of IL-18 was higher in MSSA (MSSA-N=5.87±0.59 pg/mL; MRSA-N=3.11±0.23 pg/mL) p<0.05. However, when analyzing the testing systems, there was a lower concentration of IL-18 in MRSA testing systems of the malnourished group (MRSA-N=3.11±0.23 pg/mL; MRSA-M=0.27±0.01 pg/mL) p<0.05 (Figure 4).

Viability of alveolar macrophages

The malnourished group had lower macrophage viability than the nourished group in all systems under analysis (PC-N=69.2±0.8; PC-M=51.3±0.51; MSSA-N=18.5±0.22; MSSA-M=12.2±0.11; MRSA-N=20.7±0.2; MRSA-M=6.7±0.03), p<0.05. The number of macrophages in the MRSA system of the malnourished group was significantly smaller (p<0.001).

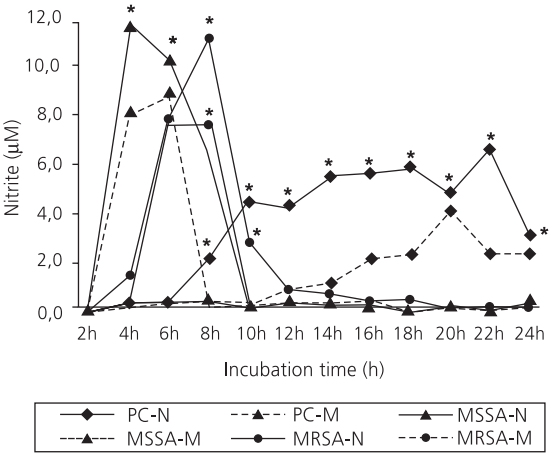

Figure 2. Nitric oxide production in the supernatant of alveolar macrophage cultures in groups (N: Nourished; M: Malnourished) and systems (PC: Positive Control, MSSA: Methicillin Sensible *Staphylococcus Aureus*; MRSA: Methicillin Resistant *Staphylococcus Aureus*). *Recife* (PE), 2009.

Note: *p<0.05 on the comparison of the Nourished and Malnourished groups.

Analysis of Variance and Tukey test. Values are expressed as Mean ± Standard Deviation (n=40).

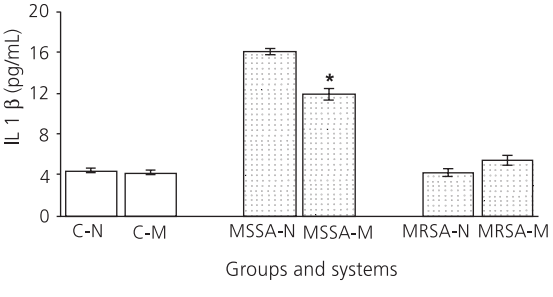

Figure 3. Levels of IL-1â in the supernatant of alveolar macrophage cultures in groups (N: Nourished; M: Malnourished) and systems (C: Negative Control, MSSA: Methicillin Sensible *Staphylococcus Aureus*; MRSA: Methicillin Resistant *Staphylococcus Aureus*). *Recife* (PE), 2011.

Note: *p<0.05 in comparison of Nourished and Malnourished groups.

Analysis of Variance and Tukey test. Values are expressed as Mean ± Standard Deviation (n=40).

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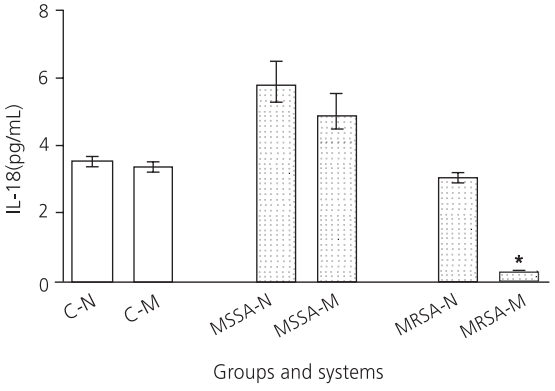

Figure 4. IL-18 levels in the supernatant of alveolar macrophage cultures in groups (N: Nourished; M: Malnourished) and systems (C: Negative Control, MSSA: Methicillin Sensible *Staphylococcus Aureus*; MRSA: Methicillin Resistant *Staphylococcus Aureus*). *Recife* (PE), 2011.

Note: *$p<0.05$ in comparison of Nourished and Malnourished groups.

Analysis of Variance and Tukey test. Values are expressed as Mean ± Standard Error Deviation (n=40).

DISCUSSION

Studies show that neonatal malnutrition models correlate with the deficiency of certain nutrients and gene expression, changing the genotype and phenotype[17,18]. The intensity and duration of malnutrition will determine the extent of systemic consequences[1].

In this study the experimental model of malnutrition consisted of an 8% casein diet, which is considered low protein. The low protein level on the diet offered to dams is characterized by the restricted amount of nutrients available to puppies. Thus, infants develop protein malnutrition, while puppies develop protein-calorie malnutrition. This fact is crucial for the genesis of the deleterious effects observed in the offspring[19].

The animals nursed by these dams were stunted, evidenced by low weight at weaning that persisted to 90 days of age. From the fourth postnatal day, malnourished animals gained less weight than the nourished samples. This result is similar to that found by Melo *et al.*[4], but they used a regional basic diet low in all constituents. Costa *et al.*[5] used an 8% casein diet to induce malnutrition and also found that weight gain decreased after the fourth day of life.

Nutritional insults in the neonatal period seem to interfere with the programming of macrophage functional mechanisms, causing lasting changes detectable in adulthood, even after a long nutritional recovery[3]. Prestes-Carneiro *et al.*[20] found that malnutrition from the first to twelfth day of lactation compromised the microbicidal response, represented by lower rate of phagocytosis. Other researchers have reported a deficit in the production of nitric oxide[4,21]. Dong *et al.*[22] reported lower production of free radicals by alveolar macrophages after *in vitro* stimulation with LPS.

In the present study, the low-protein diet did not change alveolar macrophage adherence regardless of pathogenic stimulus, suggesting that this initial step of the macrophages' immune response may not be impaired by neonatal malnutrition caused by an 8% casein diet. Chandra[2] noticed that malnutrition changes different stages of activated neutrophil and macrophage phagocytosis. Thus, malnutrition may change mechanisms that rely on macrophage activation not necessarily before activation.

According to this premise, the study demonstrated that malnutrition during lactation reduced macrophage phagocytosis in both systems. According to Prestes-Carneiro *et al.*[20] in the case of inflammatory stimuli, macrophages from malnourished animals do not respond with the same intensity as macrophages from nourished animals, which allows the development of inflammation and/or infection. Other studies have also reported low phagocytic capacity in animals subjected to malnutrition, whether neonatal or not[4,22] .

Regarding oxidant activity, alveolar macrophages from neonatally malnourished rats produced less superoxide, both under normal conditions and under bacterial stimuli. Corroborating this finding, Kawakami *et al.*[23] found that phagocytes' antimicrobial systems are potentially affected by malnutrition. Prestes-

Carneiro *et al.*[20] also found that superoxide production decreases during severe protein malnutrition.

In the present study, nitric oxide production was analyzed every two hours for a total of 24 hours of incubation. Both groups produced more than the PC, peaking at 22 hours, but malnutrition decreased production. Corroborating this result, Melo *et al.*[4] found that alveolar and peritoneal macrophages produced less nitric oxide after 24 hours of incubation with LPS in rats submitted to early malnutrition. Ferreira-Silva *et al.*[24] also found that nitrite concentration decreased in cell culture supernatant during nitric oxide production in the undernourished group after LPS stimulation. These data indicate that neonatal malnutrition induces changes in the macrophages, with significant repercussions during adulthood[9].

Pumerantz *et al.*[25] stated that the nitric oxide produced by alveolar macrophages plays an important microbicide role against *Staphylococcus aureus*. By comparing the nitric oxide release in the MSSA and MRSA systems of nourished and malnourished groups, the malnourished groups presented the lowest production. Low synthesis of this free radical may allow resistant bacteria to proliferate inside phagocytes because this important defense mechanism is compromised[10].

According to Richardson *et al.*[26], *S. aureus* can evade multiple components of the innate immune response, including the microbicidal action of nitric oxide. These authors found that *S. aureus* can adapt metabolically to nitrosative stress because it has an inducible NO-L-lactate dehydrogenase enzyme. The production of NO-L-lactate dehydrogenase enables *S. aureus* to keep homeostasis during nitrosative stress, and antibiotic resistance does not seem to interfere on this mechanism.

Based on analysis of IL-1β production, the nourished and malnourished groups differed only on the testing systems. High IL-1β production was detected in the MSSA system of the nourished group, but in the MRSA system, it was higher in the malnourished groups. IL-1β is a potent endogenous pyrogen (a fever inducer), and a potent stimulator of leukocyte migration into tissues and cytokine and chemokine expression[27]. IL-1β is an important mediator for defense against *Staphylococcus aureus*. In *S. aureus* infection, the production of IL-1β acts in the recruitment of neutrophils and the subsequent degradation of the bacterial cell wall by lysozyme enzyme. However, *S. aureus* has an O-acetyltransferase enzyme that transforms the cell wall resistant to the action of lysozyme and thus escapes the microbicidal response[28]. These findings justify the high MRSA-related mortality rates.

IL-18 production was higher in the positive than negative control. When analyzing the testing systems of the nourished and malnourished groups, the production in the malnourished groups was small and even smaller in the MRSA system. IL-18 induces the production of IFN-y (interferon-gamma) by cells of the immune system. This cytokine is important for the activation of macrophages, T lymphocytes, and other cells[28]. In MRSA infections of malnourished animals, the pro-inflammatory profile (Th1) may be compromised, favoring the persistence of the bacteria in the host organism.

When comparing macrophage viability in the PC, MSSA, and MRSA systems, viability decreased intensely after infection with *S. aureus.* This finding was more evident in the malnourished group infected by MRSA. This indicates that macrophage vulnerability is greater during MRSA infection, especially in immunocompromised individuals.

Protein-calorie deficiencies may induce irreversible cell damage that triggers the mechanism of programmed cell death[20]. Ferreira-Silva *et al.*[24] found a reduction in the viability of alveolar macrophages after perinatal malnutrition. Corroborating these authors, Rivadeneira *et al.*[29] found that malnutrition is associated with increased apoptosis. Apoptosis can be triggered by external stimuli or internal stimuli that result in mitochondrial dysfunction, DNA damage, and

http://dx.doi.org/10.1590/1415-52732014000500005

alteration of nutritional and growth factors. These different routes induce activation of caspases, which generate the cleavage of structural proteins, impairing cytoskeleton integrity, resulting in cell death[29].

Staphylococcus aureus is able to produce a variety of potent cytotoxins, allowing the bacteria to resist microbicidal response. Leucocidin is a toxin associated with new methicillin-resistant *Staphylococcus aureus* strains that destroys leukocytes by forming pores in the cell membrane[30]. Thus, we suggest that *S. aureus* infection induced phagocyte death by triggering cell lysis, and that neonatal undernutrition further promoted this effect. The study results may explain the high morbidity and mortality rates associated with MRSA infection in immunocompromised individuals.

CONCLUSION

The study neonatal malnutrition model compromised some functional parameters of innate immunity, such as rate of phagocytosis and production of nitric oxide, superoxide anion, and IL-18. Phagocytosis and the production of these inflammatory mediators are critical for the effective destruction of invading microorganisms. Adherence rate and production of IL-1β were not affected, but neonatal nutrition does impact the programming of macrophage microbicidal mechanisms. Methicillin-sensitivity in *Staphylococcus aureus* strains seems to influence their ability to evade the microbicidal response, decreasing immune defense. Interaction between neonatal malnutrition and MRSA infection increased phagocyte susceptibility, which may allow severe and fatal infections. However, many gaps remain to be filled regarding the structure and performance of immune defense components during infections, such as those caused by *Staphylococcus aureus* in adults who have endured environmental insults. Thus, it is important to conduct studies using more sensitive and specific methods, such as biological molecular analyses. These may provide better data on this topic and contribute to the clarification of the morphological changes that occur in early life and the impact of such changes on the microbicidal response of phagocytes and on the emergence and evolution of infectious diseases.

COLLABORATORS

NG MORAIS helped to design the study and experimental strategy; tabulate the data; discuss the results; and write the article. TB COSTA helped to prepare the experimental groups, maintain the animals, and collect the data. MS SEVERO helped to design the study and the experimental strategy. CMMB CASTRO helped to design the study, tabulate the data, discuss the results, and write the article.

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Received on: 1/29/2014
Final version on: 7/15/2014
Approved on: 8/5/2014