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Effect of postnatal malnutrition on hyperoxia-induced newborn lung development

M.M.G.B. Mataloun[1], C.R. Leone[1], R.S. Mascaretti[2], M. Dohlnikoff[3] and C.M. Rebello[2]

[1]Pediatria Neonatal, [2]Laboratório de Pesquisa Experimental, Departamento de Pediatria, Instituto da Criança "Prof. Pedro de Alcântara", Universidade de São Paulo, São Paulo, SP, Brasil
[3]Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil

Correspondence to: M.M.G.B. Mataloun, Rua Albuquerque Lins, 902-93, 01230-000 São Paulo, SP, Brasil
Fax: +55-11-3821-5865. E-mail: mataloun@uol.com.br

Several factors are associated with bronchopulmonary dysplasia. Among them, hyperoxia and lung immaturity are considered to be fundamental; however, the effect of malnutrition is unknown. Our objective was to evaluate the effects of 7 days of postnatal malnutrition and hyperoxia on lung weight, volume, water content, and pulmonary morphometry of premature rabbits. After c-section, 28-day-old New Zealand white rabbits were randomized into four groups: control diet and room air (CA, N = 17), control diet and ≥95% O_2 (CH, N = 17), malnutrition and room air (MA, N = 18), and malnutrition and ≥95% O_2 (MH, N = 18). Malnutrition was defined as a 30% reduction of all the nutrients provided in the control diet. Treatments were maintained for 7 days, after which histological and morphometric analyses were conducted. Lung slices were stained with hematoxylin-eosin, modified orcein-resorcin or picrosirius. The results of morphometric analysis indicated that postnatal malnutrition decreased lung weight (CA: 0.83 ± 0.19; CH: 0.96 ± 0.28; MA: 0.65 ± 0.17; MH: 0.79 ± 0.22 g) and water content, as well as the number of alveoli (CA: 12.43 ± 3.07; CH: 8.85 ± 1.46; MA: 7.33 ± 0.88; MH: 6.36 ± 1.53 x 10^{-3}/mm) and elastic and collagen fibers. Hyperoxia reduced the number of alveoli and increased septal thickening and the mean linear intercept. The reduction of alveolar number, collagen and elastic fibers was intensified when malnutrition and hyperoxia were associated. These data suggest that dietary restriction enhances the magnitude of hyperoxia-induced alveolar growth arrest and lung parenchymal remodeling. It is interesting to consider the important influence of postnatal nutrition upon lung development and bronchopulmonary dysplasia.

Key words: Postnatal malnutrition; Hyperoxia; Lung development; Rabbit

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Introduction

Bronchopulmonary dysplasia involves histopathological pulmonary changes resulting from an inflammatory process induced by hyperoxia (1,2), infection (3), and mechanical ventilation (4), conditions associated with the peculiarities of the developing immature lung.

Several experimental studies have described the acute lung injury caused by hyperoxia in adult animals (5,6) as well as in premature animals (7-14) during the period in which the lung is still in the process of alveolarization. This injury is characterized by arrested alveolar and pulmonary microvascular development (5,6,15,16).

Regarding the influence of nutrition on lung development, it has been shown that tissue elasticity was decreased in the lungs of starved adult rats (17,18) and that this effect was not reversed after resumption of feeding, probably due to irreversible damage to airspaces caused by the loss of connective tissue (17). Several investigators have described a reduction of somatic growth, lung volume and weight (19-21) in term newborn rats subjected to nutritional restriction during the neonatal period. It was also demonstrated that the total number of alveoli and the internal surface area of the lung were significantly reduced in starved newborn rats (19). However, other investigators did not observe any influence of malnutrition on the pulmo-

nary architecture (20,21). It is noteworthy that, although the effects of hyperoxia and malnutrition on lung development have been individually demonstrated, the consequences of the combined treatment on lung growth and development have not been described.

Therefore, we hypothesized that malnutrition will decrease alveolarization as well as interrupt and disorganize the deposition of elastic fibers and collagen in preterm rabbits exposed to hyperoxia. The objective of this study was to evaluate the effects of postnatal malnutrition alone or in combination with 7 days of hyperoxia on lung morphometry in premature rabbits.

Material and Methods

Protocols were approved by the Ethics Committee for the Analysis of Research Projects, University Hospital, School of Medicine, University of São Paulo. The study was carried out in the Experimental Research Laboratory of the Department of Pediatrics.

Animal preparation

Timed-pregnant New Zealand white rabbits (Benjamin Fleder®, Moji das Cruzes, SP, Brazil) at the 28th day of gestation were used. The pregnant rabbits received intramuscular ketamine (10 mg/kg) and acepromazine (0.1 mg/kg), and rachianesthesia with 2% marcaine-xylocaine solution (1:1, v/v, 2 mL). After c-section, the pups were identified by a number marked on the dorsal region and weighed over a thermal mattress and under radiant heat. The pups were exposed to 30% O_2 during the first 30 min of life and the dams were euthanized with an injection of 5-mL pentobarbital.

After the initial period of adaptation, the surviving newborns were randomly divided into one of four groups: CA: control diet and room air (N = 17), CH: control diet and hyperoxia (≥95% O_2) (N = 17), MA: malnutrition and room air (N = 18), or MH: malnutrition and hyperoxia (N = 18). The offspring were sacrificed on day 7.

The animals were kept in incubators (Fanem®, Brazil) at 30-32°C. To prevent infection, the incubators were disinfected, distilled water was used in the nebulizers, the diet was sterilized, and the sawdust was autoclaved. From the 3rd day of life, the animals received crystalline penicillin (20,000 units·kg^{-1}·day^{-1}) and streptomycin (20 mg·kg^{-1}·day^{-1}) by the intramuscular route once a day as prophylaxis against infection, which was the most frequent cause of death in the pilot studies. Pups also received a single intramuscular dose of vitamin K (0.002 mg/kg) on day 3 in order to prevent pulmonary hemorrhage.

A lacteal formula with a composition similar to that of rabbit milk (22,23) and to that described in the literature (8,13,24) was developed because the pups were unable to suck. The control diet consisted of a mixture of 5 g AL110 Formula (Nestlé®, Brazil), 5 g casein (Support®, Brazil), 15 mL triglyceride CMAGE (Support®), 1 drop Vitanove® (USP, Brazil), and distilled water (100 mL qsp), which supplied 5.3 g protein, 16.2 g fat, 2.8 g dextrin-maltase, and 155 kcal per 100 mL. Malnutrition was defined as a 30% reduction of all nutrients of the control diet, based on experimental models of malnutrition and hyperoxia in rats (21,24). The ponderal evolution of pups in this study was similar to a study that used the same formula (8). All animals were fed twice a day through an orogastric feeding tube (4 Fr) adapted to a graduated syringe, because the premature rabbits were unable to suck. The feeding volumes were increased over time from 50 mL/kg on day 0 to 100 mL/kg on day 1, to 150 mL/kg on day 2 and to 200 mL·kg^{-1}·day^{-1} on days 3-10. The same volume was given to all groups.

The animals were weighed daily in the morning on a precise analytical balance (TR 403, Denver Instrument Company®, USA). All measurements were repeated three times and averaged.

Exposure to hyperoxia

Heated and humidified oxygen was administered through a sealed acrylic chamber with neonatal nebulizers (Intermed®, Brazil) with a continuous flow of 3 L/min. This continuous flow prevented CO_2 accumulation and achieved a constant level of 95% O_2. The oxygen concentration was continuously monitored with an environment oximeter (Dixtal®, Brazil).

Histology

The animals were euthanized with an intraperitoneal injection of pentobarbital sodium (25 mg/kg) and by sectioning of the abdominal aorta. The trachea was cannulated with a Silastic cannula measuring 1 mm in diameter, after which bilateral pneumothoraces were produced by puncture of the diaphragm from the abdominal surface. The tracheal cannula was connected to a water column, and the lungs were inflated with air at a pressure of 30 cmH_2O, followed by tracheal ligature. The lungs were removed from the thorax and submerged into a 10% buffered formol solution for 24 h. The lungs were weighed three times, and the arithmetic mean was calculated. Lung volume was measured three times by the water displacement method (25) and the mean value was obtained. These measurements were repeated if two values differed by more than 0.05 mL. A correction factor was not used for humid and dehydrated (wet and dry) values because all lungs were processed identically. The lung weight/body weight ratio was determined at the time of

euthanasia, and the specific lung volume was determined by dividing the lung volume by 100 g body weight (mL/100 g body weight) (26).

The lung water content was quantified as the difference between the wet and dried lung weights. The left lung was used for this measurement and was weighed both in the humid state and after exposure to 80°C for 72 h or until a constant lung weight was obtained (27).

Sagittal 1-mm tissue slices were removed from the distal portion of the inferior right lobe and preserved in 70% ethyl alcohol until paraffin embedding and serial sectioning to a thickness of 5 µm. The sections were stained with hematoxylin-eosin (HE), with modified resorcin-orcein for elastic fiber visualization, and with picrosirius for collagen visualization.

Morphometric analysis

Morphometric analysis was performed by the same investigator in a blind manner, using a light microscope (Nikon E-600, Japan) with a grid (100 points/50 lines) in the eyepiece and an image analysis software (Image-Pro®, Media Cybernetics Inc.™, USA).

Each HE-stained slide was captured at 100X magnification with the image analysis software. Serial images from slides, but not overlying parenchymal regions, with three straight lines traced at distinct levels from pleura to pleura were captured, and the intercepted alveolar walls were counted. The sum of the intercepted alveolar walls was divided by two to obtain the number of alveoli intercepted. The length of each straight line was measured with the image analysis software. The mean number of alveoli per slide was calculated by dividing the number of alveoli obtained by the sum of the length of the three lines. This gives the number of alveoli per unit length (alveolar number x 10^{-3}/µm) (28).

The mean linear intercept (Lm) was calculated in ten 100X fields per slide (29). Fifty alveolar septa were measured with the image analyzer (400X) and graded according to the distance between epithelial basement membranes (30). The proportions of collagen and elastic fibers in the pulmonary parenchyma were determined as the relation between the number of points falling on stained and unstained tissue. Measurements were performed in twenty 400X fields per animal using a 100-point/50-line grid (31). The parenchyma points were estimated by counting points through the grid, considering all points that spanned the tissues as parenchyma points, except blood vessels and bronchi measuring >2 mm in diameter.

Statistical analysis

Analysis of variance for repeated measures was used for group comparisons of the changes in body weight during the study period (32). The assumption of a variable normal distribution was confirmed by the construction of descriptive graphics, and multiple comparisons were made based on Wald Statistics (33). One-way analysis of variance (ANOVA) was used to compare lung weights, numbers of lung weight/specific lung volume alveoli, alveolar septum thickness, Lm values, and collagen and elastic fiber proportions. The sample size calculation was based on the assumption that there would be a difference of 10% between the groups in mean number of alveoli, an α value of 5% and a power of 80%. On the basis of our pilot study, the *n* calculated for each group was 17. A P value <0.05 was considered to be statistically significant.

Results

There were 17 rabbits in the CA group, 17 in the CH group, 18 in the MA group, and 18 in the MH group. Analysis of variance for repeated measures did not reveal any differences in weight between groups from birth to sacrifice. However, the average birth weight of the CA group was lower than that of the MA group. The differences between mean birth weight and weight at the time of sacrifice were calculated, showing a higher weight gain for the CH group than for the MH group (P = 0.0001, with no differences between the CA and MA groups (Figure 1).

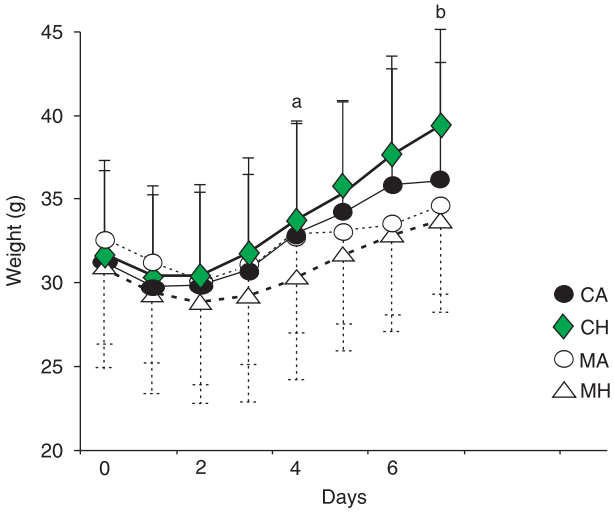

Figure 1. Daily weight curves for the study groups. Data are reported as means ± SD. CA = control diet and room air; CH = control diet and hyperoxia; MA = malnutrition and room air; MH = malnutrition and hyperoxia. [a]CH *vs* MH; [b]CA *vs* MA. P = 0.05 (two-way ANOVA).

Pulmonary effects

Malnutrition induced lower of lung weight, lung weight corrected for birth weight, specific lung volume and water content ($P < 0.05$) in premature rabbits. This treatment also led to a reduction in alveoli number ($P < 0.05$) and elastic fibers ($P < 0.05$) and in collagen deposition ($P < 0.05$) at 7 days. Hyperoxia resulted in a reduced number of alveoli ($P < 0.05$), greater septal thickness ($P < 0.05$) and an increased Lm ($P < 0.05$) at 7 days. When the animals were exposed to both malnutrition and hyperoxia, more pronounced reductions in alveolar number ($P < 0.05$) and collagen deposition ($P < 0.05$) were detected.

A thickening of the elastic fibers with a rough aspect was observed in malnutrition and/or hyperoxia in contrast with thin and delicate fibers in control diet and room air.

These results are summarized in Table 1 and Figures 2, 3, and 4.

Table 1. Effect of postnatal malnutrition and hypoxia on rabbits at 7 days of life.

	CA (N = 17)	CH (N = 17)	MA (N = 18)	MH (N = 18)
Lung weight (g)	0.83 ± 0.19	0.96 ± 0.28	0.65 ± 0.17[a]	0.79 ± 0.22[bd]
Lung weight/body weight	0.027 ± 0.006	0.027 ± 0.008	0.019 ± 0.004[a]	0.023 ± 0.005[d]
Specific lung volume (mL/100 g)	7.77 ± 3.57	7.30 ± 5.80	4.78 ± 1.61[a]	3.67 ± 1.43[bd]
Lung water content	91.77 ± 1.94	90.30 ± 2.48	80.37 ± 4.13[a]	77.54 ± 4.60[b]
Mean linear intercept (µg)	59.10 ± 9.12	73.70 ± 13.40[c]	60.33 ± 10.46	75.83 ± 14.50
Alveolar septum (µm)	9.62 ± 2.48	12.59 ± 4.68[c]	5.23 ± 3.41[a]	12.48 ± 7.01[d]

CA = control diet and room air; CH = control diet and hyperoxia; MA = malnutrition and room air; MH = malnutrition and hyperoxia. $P < 0.05$: [a]CA *vs* MA; [b]CH *vs* MH; [c]CA *vs* CH; [d]MA *vs* MH (one-way ANOVA).

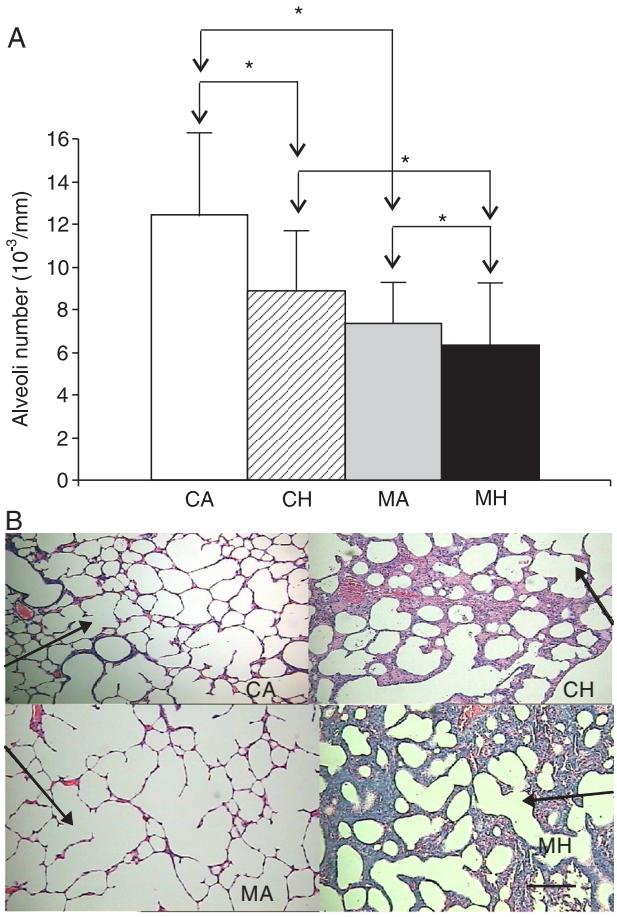

Figure 2. *A*, Alveolar number (x 10^{-3}/mm) in the control and malnutrition groups at 7 days of life. Data are reported as means ± SD. CA = control diet and room air; CH = control diet and hyperoxia; MA = malnutrition and room air; MH = malnutrition and hyperoxia. Alveolar number decreased in the groups submitted to malnutrition or hyperoxia, and a more intense reduction was observed when both challenges were combined. *$P < 0.05$ (one-way ANOVA). *B*, Lung sections showing the alveoli. Hematoxylin-eosin staining. Magnification: 100X. Hyperoxia and malnutrition reduced alveolar number. When the animals were exposed to both malnutrition and hyperoxia, more pronounced reductions in alveolar number ($P < 0.05$) were observed. Alveoli are shown by arrows. Inflammatory process is observed in the hyperoxia groups. One-way ANOVA. Magnification bar = 100 µm for all panels.

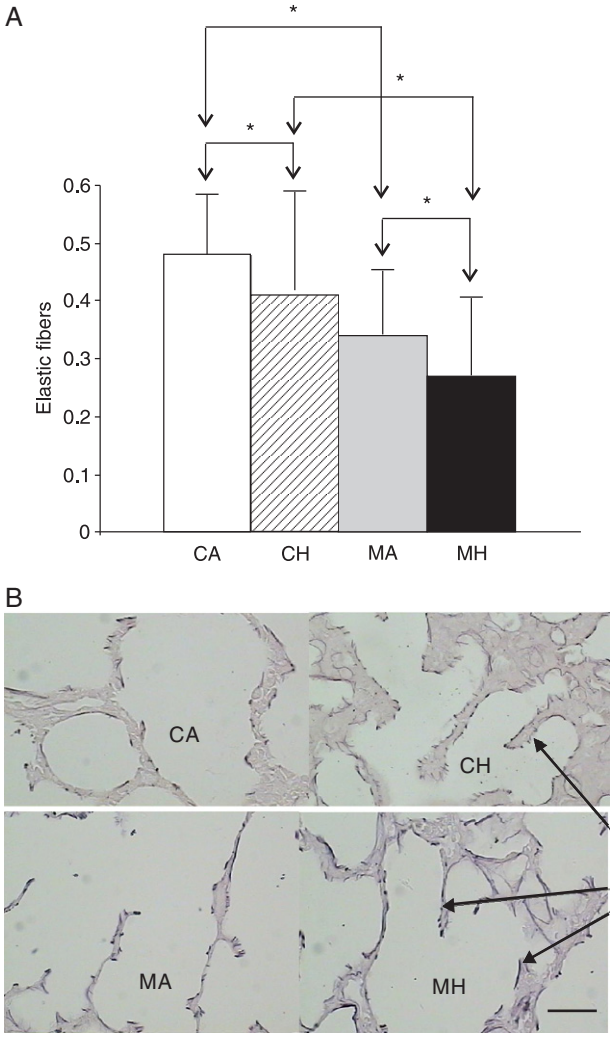

Figure 3. *A*, Elastic fibers in the control and malnutrition groups at 7 days of normoxia or hypoxia exposure. CA = control diet and room air; CH = control diet and hyperoxia; MA = malnutrition and room air; MH = malnutrition and hyperoxia. *B*, Lung slides of premature rabbits at 7 days of life - modified resorcin-orcein staining (magnification: 400X) - a thickening of the elastic fibers with a rough aspect was observed in the malnutrition and/or hyperoxia groups in contrast with thin and delicate fibers in the control diet and room air groups. Elastic fibers - stained in black (arrows). *P < 0.05 (one-way ANOVA). Magnification bar = 100 µm for both panels.

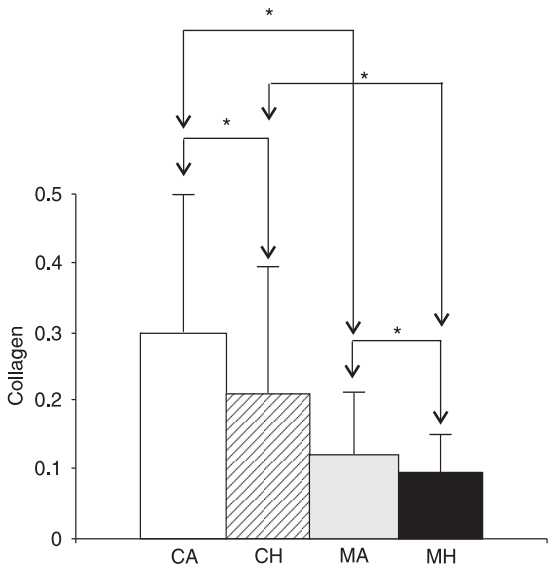

Figure 4. Collagen proportion in the control and malnutrition groups at 7 days of normoxia or hypoxia exposure. Data are reported as means ± SD. The collagen proportion decreased in the groups submitted to malnutrition or hyperoxia, and a more intense reduction occurred when both challenges were combined. CA = control diet and room air; CH = control diet and hyperoxia; MA = malnutrition and room air; MH = malnutrition and hyperoxia. The proportions of collagen and elastic fibers in the pulmonary parenchyma were determined as the relation between the number of points falling on stained and unstained tissue. Measurements were performed in twenty 400X fields per animal using a 100-point/50-line grid. *P < 0.05 (one-way ANOVA).

Discussion

The present study showed that malnutrition reduced lung weight, lung weight/birth weight ratio, specific lung volume and water content, as well as the number of alveoli and elastic fibers and collagen deposition. Hyperoxia reduced alveolar number and led to increased values of septal thickness and mean linear intercept. Combined malnutrition and hyperoxia caused a more drastic reduction in alveolar number and collagen deposition.

This premature rabbit model is particularly suitable for analysis of factors that influence alveolarization. In rabbits, a gestational age of 28 days is the lower limit of viability (8), as well as the point at which lung development is at the end of the saccular phase and at the beginning of the alveolar phase (10,11). Preterm rabbits at 27 days of gestation are a validated animal model for surfactant deficiency and are widely used for surfactant replacement studies (34). The preterm rabbit at 28 days of gestation has an increased surfactant pool and is not appropriate for studies of surfactant deficiency, but it permits the evaluation of the effects of hyperoxia and malnutrition on immature lungs in the early stages of alveolarization. In addition, this rabbit model is less costly and more easily reproduced, thus permitting easier execution of procedures as well as greater precision with respect to gestational age, as ovulation may be induced.

A weight reduction of more than 20% in pups exposed to malnutrition was observed in the pilot study, leading us to choose this level of malnutrition. The most common experimental model of nutritional restriction in the literature is the one that manipulates the number of pups fed by the same mother, i.e., increasing or reducing the number of pups per female (19,20). The other models use a reduction of several degrees of nutrients administered to animals. As the pups of the present study were unable to suck, a 30% reduction of the nutrients of the control diet was chosen, as also done in other studies (19,20,35,36). This diet is similar to that used in other premature rabbit models (8,13). Based on previous studies with preterm rabbits (8,13), we decided to use a diet volume corresponding to 20% of body weight between the 3rd and 7th days of life based on earlier studies with preterm rabbits (8,13).

The use of 95% oxygen concentration in order to obtain an intense inflammatory response over a short period of time was chosen based on data from previous studies (5,37).

Malnutrition during the immediate postnatal period yielded lower lung weights as also observed in previous studies (19,20), in addition to reduced lung water content and specific lung volume after 7 days of treatment. Although some studies using models of malnutrition found that this treatment induced higher specific lung volumes, other studies obtained contradictory results (14,15). The studies that detected higher specific lung volumes also found greater alveolar size, while a subset also observed emphysematous segments (36). These inconsistent results might reflect differences in the degree of malnutrition or the type of nutrients restricted, as well as the time of onset of treatment.

The reduction in alveolar number attributed to malnutrition was significant, similar to other studies that examined this period of rapid alveolar production in several species, including rabbits (12). This effect occurred without changing the dimensions of the alveoli, as observed in a previous study (14), suggesting the existence of a mechanism other than septation by which malnutrition could alter alveolar formation. It is known that elastic fiber deposition in the alveolar wall is a major mechanism involved in alveolarization that results in further crist formation (38). Our results suggest that the reduction of elastic fibers and collagen deposition due to malnutrition impairs the alveolarization process. This effect of malnutrition has been interpreted to be a consequence of substratum depletion (19,39). Therefore, a reduction in these deposits could interfere with alveolar septation, causing arrested alveolar development.

Significant histopathological pulmonary changes were observed due to malnutrition, despite the fact that there were no differences in body weight gain between the control diet and malnutrition groups. Probably, the duration of malnutrition in this study was not long enough to produce changes in weight gain. However, in an earlier study using this experimental model for a period of 11 days, the authors described a reduction of body weight growth in the malnutrition group (39). These results suggest that body weight may be a poor nutritional marker of short duration malnutrition. The mechanism by which hyperoxia induces alveolar arrest has been shown to be mediated by elastic and collagen fiber disorganization, and secondarily by oxidative stress and the inflammatory process (15). In the present study, hyperoxia decreased alveolar number and increased alveolar septal thickening and Lm, suggesting arrested alveolarization. These results are consistent with earlier studies of lung injury due to hyperoxia (2,3,5,7). Septal thickening could indicate the presence of a fibro-proliferative process (5,7), which may be responsible for the increase of the mean Lm intercept in these samples. However, this Lm increase may also have been due to the inhibition of septation and to alteration in the development of the alveolar capillaries (2,5,6,15,16). Hyperoxia did not increase lung water content in the pres-

ent study, as was expected (27). This may have been due to the shorter time of exposure to hyperoxia in our study. Incidentally, collagen and elastic fiber deposition did not differ between the hyperoxia and room air groups.

Combined malnutrition and hyperoxia treatment induced an enhanced reduction of alveolar number as well as elastic fiber and collagen deposition.

Malnutrition affects lysyl-oxidase production due to the global deficiency of nutrients (40). This enzyme is involved in the initial step of crista formation and consequently elastin deposition. In this manner, nutrition can play an important role in elastin production and deposition and thus enhance the effects of hyperoxia.

The relevance of these results is that they suggest that malnutrition might intensify hyperoxia-induced acute lung injury, mainly with respect to decreased alveolarization, in premature rabbits. The clinical significance of this structural change, secondary to the distortion or inhibition of several processes of lung development and growth, corresponds to the "new" bronchopulmonary dysplasia. In this context, nutrition may either facilitate or enhance this process. New recommendations for the nutrition of preterm infants during the neonatal period should be considered in order to contribute to a better short- and long-term clinical outcome.

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