Experimental models of malnutrition and its effect on skin trophism *

Modelos experimentais de desnutrição e sua influência no trofismo cutâneo

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Abstract: BACKGROUND: The skin requires adequate levels of nutrients to function properly. Objective: To analyze skin trophism in well-nourished and undernourished rats using two models of malnutrition.
METHODS: In the marasmus model, 60 Wistar rats were kept on a controlled diet, 30 being randomly selected to receive half the established diet for 60 days. In the gelatin model, 60 rats were used, 30 of which received a diet consisting of poor quality protein (gelatin) for 30 days. The nutritional status of the animals was evaluated according to body mass index, clinical signs and serum albumin measurement. After the period of malnutrition, histology was performed on the animals' skin to analyze the thickness of the dermis and epidermis using the Leica Application Suite software. Collagen was analyzed on slides stained with Gömöri trichrome using the ImageJ software program.
RESULTS: The body mass index of the malnourished animals in the marasmus and gelatin groups was significantly lower than that of the well-nourished animals in the two groups ($p<0.0001$ in both models). With respect to serum albumin, there was no difference between the groups in either of the two models. In relation to the histological analysis of skin thickness, the dermis of the malnourished animals was significantly thinner compared to that of the well-nourished animals ($p<0.0001$ in both models). The percentage of collagen was lower in the malnourished animals compared to the well-nourished animals ($p<0.0005$ and $p<0.003$ in the marasmus and gelatin model, respectively).
CONCLUSIONS: Skin thickness measurements were lower in the malnourished animals in both models, and this finding was histologically confirmed by the lower percentage of collagen, showing the negative effect of malnutrition on skin trophism.
Keywords: Collagen; gelatin; image processing, computer-assisted; nutritional marasmus; public health; skin.

Resumo: FUNDAMENTOS: A pele, para exercer suas funções, necessita de níveis adequados de nutrientes.
OBJETIVO: Analisar o trofismo cutâneo de ratos nutridos e desnutridos por meio de dois modelos de desnutrição.
MÉTODOS: No Modelo Marasmo, utilizaram-se 60 ratos Wistar em controle dietético, dos quais 30 foram selecionados aleatoriamente para receber metade da dieta diária durante 60 dias. No Modelo Gelatina, empregaram-se 60 ratos, dos quais 30 receberam dieta associada a proteína de baixa qualidade (gelatina) durante 30 dias. Avaliou-se o estado nutricional dos animais por meio da massa corporal, dos sinais clínicos e da dosagem de albumina sérica. Após o período de desnutrição, fez-se a histologia da pele dos animais para análise da espessura da derme e epiderme com o *software Leica Application Suite*; nas lâminas coradas com tricrômio de Gomori, analisou-se a colagênese com o *software* ImageJ.
RESULTADOS: A massa corporal dos animais desnutridos pelo marasmo e gelatina foi significativamente menor ($p<0,0001$ e $p<0,0001$) do que a dos grupos nutridos. Quanto à albumina sérica, não houve diferença entre os grupos nos dois modelos. Em relação à análise histológica da espessura da pele, os desnutridos apresentaram a derme significativamente menos espessa em comparação aos nutridos ($p<0,0001$ e $p<0,0001$). No que respeita à colagênese, os grupos desnutridos apresentaram menores percentuais de colágeno em relação aos nutridos ($p<0,0005$ e $p<0,003$).
CONCLUSÕES: Os animais desnutridos pelos dois modelos apresentaram diminuição na espessura dérmica, confirmada histologicamente pelo menor percentual de colágeno, mostrando a influência negativa da desnutrição no trofismo cutâneo.
Palavras-chave: Cicatrização; Colágeno; Gelatina; Marasmo nutriciona; Modelos animais; Processamento de imagem assistida por computador

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INTRODUCTION

The skin is the largest organ in the human body and performs various important functions such as protecting against toxins and microorganisms present in the environment, preventing dehydration and participating in the immune system, in addition to its sensorial and healing properties. [1-3] However, to maintain its structure and function, adequate levels of nutrients such as proteins, carbohydrates, vitamins and minerals are required. [4-6]

Malnutrition may be described as a deficit of energy, protein and/or any other specific nutrient that leads to a measurable alteration in body function and that is associated with increasing the severity of a disease. It may be reversed with adequate nutritional support. [7] It is highly prevalent in developing countries and is generally associated with socioeconomic and educational problems as well as with issues related to health and basic sanitation. [8] Extreme cases of nutritional imbalance such as hunger and malnutrition induce a series of biochemical and organic changes in the individual such as protein, carbohydrate and lipid metabolism disorders, leading to a state of malnutrition either as the result of poor diet or induced by situations of stress that alter the protein requirements of individuals and what they need to compose amino acids. [9]

Proteins are macromolecules that are important to the body, since, in addition to their structural functions, they act as biological catalyzers and hormones, and also participate in the immune system, regulate cell development and participate in the transport of various substances. When proteins fail to play their structural and enzymatic role, a state of metabolic imbalance sets in and anemia, hypovitaminosis and protein-energy malnutrition may develop in the individual, this latter condition being one of the foremost social problems prevalent in Brazil. [10]

Protein-energy malnutrition (PEM) results from inadequate diet and is characterized by energy deficiency due to a reduction in the intake of all macronutrients and often many micronutrients. Clinically, PEM is classified into three major syndromes: marasmus, kwashiorkor or marasmic kwashiorkor, a combination of the two. Marasmus is the predominant form of PEM in most developing countries and may affect all age-groups. It occurs when energy intake is insufficient to satisfy the body's demands, causing the body to begin using its own stores of energy in the form of glycogen, skeletal muscles and, finally, the triglycerides located in the adipose tissue. [11] Consequently, the individual suffers a chronic reduction in weight due to loss of muscle mass and body fat, stunted growth and muscle atrophy, mental alertness being typically retained. Serum albumin levels remain practically unchanged, with decreases only being detected at a later stage. [4, 6-8, 12]

In addition to this type of malnutrition, another type may be triggered when the dietary intake of specific nutrients such as protein is insufficient or when intake is based on poor quality protein such as gelatin. Gelatin is a denatured collagen, the amino acid composition of which consists of a high concentration of glycine, proline and hydroxyproline associated with a low or negligible concentration of tyrosine, tryptophane, isoleucine, cystine and histidine. [13] A deficit of these essential amino acids causes protein depletion in animals, leading to growth stunting. [14]

PEM affects the skin, causing significant morphological and functional alterations and predisposing it to damage (ulceration) with a consequent difficulty in healing. Nevertheless, little is known with respect to the skin alterations triggered by the specific depletion of proteins such as that occurring following the ingestion of gelatin. Malnutrition results in changes in inflammatory reaction, immune function and tissue regeneration, leading to an increase in proinflammatory cytokines, a delay in the healing process and a greater risk of infection. [2,6,12,15,16]

Frade et al. analyzed a sample of 124 patients in Juiz de Fora and surrounding region and found that leg ulcers represented a protracted, recurrent condition that is generally associated with other chronic illnesses, principally affecting the low-income elderly population. [17] Within the present scenario of increased incidence and prevalence of generally malnourished patients with ulcers and the various therapeutic options available to accelerate the healing process, it has become important to standardize and obtain more information on the experimental models of malnutrition in order to evaluate the mechanisms involved in tissue repair and, consequently, to assess the safety and efficacy of these products. [18]

Therefore, the objective of this study was to analyze the changes that occur in the trophic skin of rats subjected to different forms of malnutrition such as that achieved with the experimental model of marasmus and with a normal protein diet associated with low quality protein (gelatin).

MATERIAL AND METHODS

This experimental, randomized controlled clinical trial involving two models of malnutrition was conducted in accordance with the ethical principles and guidelines for animal testing defined in the Brazilian College of Animal Experimentation (COBEA). The study was approved under protocol CETEA/FMRP #274/2005).

Experimental induction of malnutrition

a) **Marasmus model**

Sixty adult male Wistar rats (*Rattus norvegicus*) of 180.0 to 200.0 grams in weight, obtained from the animal laboratory of the Ribeirão Preto School of Medicine, University of São Paulo (FMRP-USP), were fed rat chow *ad libitum* for three days in order to calculate mean daily calorie intake. The animals were then randomly assigned to receive rat chow *ad libitum* (well-nourished group) or half the daily ration (malnourished group) and were followed up for two months. The animals received water *ad libitum* and were kept in individual cages at a constant temperature of 22ºC, with the relative humidity of the air around 60%, automatic air exhaustion and artificial light on a 12-hour dark/light schedule (lights on from 6 am to 6 pm).

b) Malnutrition model consisting of a normal protein diet associated with low quality protein (gelatin).

Sixty adult male Wistar rats were given a special diet supplied by the Nutrition Department of FMRP-USP. Thirty of these animals received a normal protein diet and the remaining 30 received a normal protein diet associated with gelatin (Table 1). All were followed up for one month. The animals were kept in individual cages under the same conditions as those used in the marasmus model.

Confirmation of malnutrition

In the marasmus model, three days after the animals had arrived at the laboratory, their nutritional status was evaluated according to their body mass measured using a calibrated digital scale and according to serum albumin levels measured at the Nutrition Laboratory of FMRP-USP. These measurements were repeated on the 60th day of the experiment.

In the gelatin model, nutritional status was evaluated in the same way as in the marasmus model but measurements were taken on the 30th day of follow up in view of the rapidly deteriorating condition of the animals.

Table 1: Nutritional composition of the experimental diets

	Experimental Diets (g/1000g of chow)	
Nutrients	**Well-nourished**	**Malnourished**
Protein (casein)	200	50
Gelatin	-	150
Lipid (soya oil)	70	70
Carbohydrate (corn starch)	532.5	532.5
Sucrose	100	100
Fiber (cellulose)	50	50
Mix minerals	35	35
Mix vitamins	10	10
Choline	2.5	2.5

Sampling and histopathological analysis

In the marasmus and gelatin models, 10 animals were sacrificed in each group on the 60th and 30th days, respectively. Samples from the dorsal skin of the rats were taken by 8 mm punch biopsy and fixed in 4% buffered formalin. The histological slides were stained with hematoxylin-eosin to measure skin thickness and Gomori trichrome to analyze collagenesis.

Measuring epidermal and dermal thickness

A Leica®DM 4000B optic microscope was used to capture the histological images at a magnification of 400x. Five images of the epidermis were taken in sequence as far as the subcutaneous cellular tissue. These images were automatically grouped using the Photomerge function of the Adobe Photoshop CS4 software. Using the Leica Application Suite (LAS) software, the mounted image was calibrated from 242 pixels to 50 ∝m. A line was then drawn from the granular layer of the epidermis to the transition with the dermis (epidermal thickness) and from this point to the transition of the dermis with the subcutaneous cellular tissue (dermal thickness). When this was complete, the software supplied the distance in Ìm.

Analysis of collagenesis

The images of the histological slides stained with Gomori trichrome were captured using the same microscope at a magnification of 100x. The standard 500 x 100 pixel region of interest (ROI) was defined and 10 photographs were taken of each sample from each group using the LAS software. Later, the images were analyzed using the color deconvolution plugin function of the ImageJ software (US National Institutes of Health, Bethesda, MD, USA), which supplies the percentage of blue staining (collagen) in each ROI. [18-22]

Statistical analysis

The measurements of body mass, serum albumin levels, skin thickness and collagenesis were analyzed using Student's t-test and the Mann-Whitney test for the comparison of two non-parametric samples. P-values <0.05 were considered statistically significant.

RESULTS

The initial objective was to evaluate the sample power of 30 rats per group using body mass as the principal comparable variable in the study. Differences ≥ 44 grams were considered sufficient for a variance (^{2}) of 3650 grams, with an alpha of 5% and a ß error of 20%.

In the marasmus model, body mass was lower in the animals in the malnourished group compared to the animals in the well-nourished group after 60 days of follow-up, with a difference of 294 grams between the measurements ($p<0.0001$) (Figure 1A). Serum albumin levels remained similar in the two groups during the entire evaluation period (Figure 1B). In addition, it was found that the malnourished animals developed typical clinical signs of malnutrition such as decreased weight and growth, muscle atrophy, broken nails, hair loss, a state of mental alertness, intense agitation and hunger.

There was no statistically significant difference in mean epidermal thickness between the animals in the well-nourished group and those in the malnourished group. However, mean dermal thickness values were lower in the malnourished group compared to the well-nourished group and this difference was statistically significant ($p<0.0001$) (Figures 2A and 2B). Figures 2C and 2D show the histological evidence of this difference in dermal thickness between the well-nourished and malnourished groups.

The collagen percentage, evaluated by analyzing the images of slides stained with Gomori trichrome stain at 60 days of follow-up, was found to be lower in the malnourished group compared to the well-nourished group ($p<0.0005$) (Figure 3A). In the qualitative histological analysis, collagen was denser and patterns were more organized in the animals of the well-nourished group compared to those of the malnourished group in which collagen was significantly looser (Figures 3B and 3C).

In the gelatin model of malnutrition, the sample power of 30 rats per group was established by a difference in mean body mass ≥ 25 grams for a variance (^{2}) of 657 grams, an alpha of 5% and a ß error of 20%, which was confirmed by the difference of 116.8 grams found between the two groups. The malnourished animals experienced a rapid loss of body mass as shown by the significant difference found between the two groups on the 30th day of follow-up ($p<0.0001$) (Figure 4A). Serum albumin levels were similar in the two groups at the end of the follow-up period (Figure 4B). The malnourished animals in the gelatin group experienced weight loss and stunted growth; their skin was thinner and more fragile; they were lethargic and experienced hair loss.

After a 30-day period, mean epidermal thickness in the well-nourished group was statistically similar to that of the animals in the malnourished group; however, dermal thickness was significantly lower in the malnourished group compared to the well-nourished group ($p<0.0001$) (Figures 5A and 5B). Figures 5C and 5D show this difference in dermal thickness between the two groups from a histological point of view.

The percentage of collagen was higher in the group of well-nourished animals and this difference was statistically significant ($p<0.003$) (Figure 6A). In the qualitative histological analysis, the area of collagen was better organized in the well-nourished group compared to the malnourished group (Figures 6B and 6C).

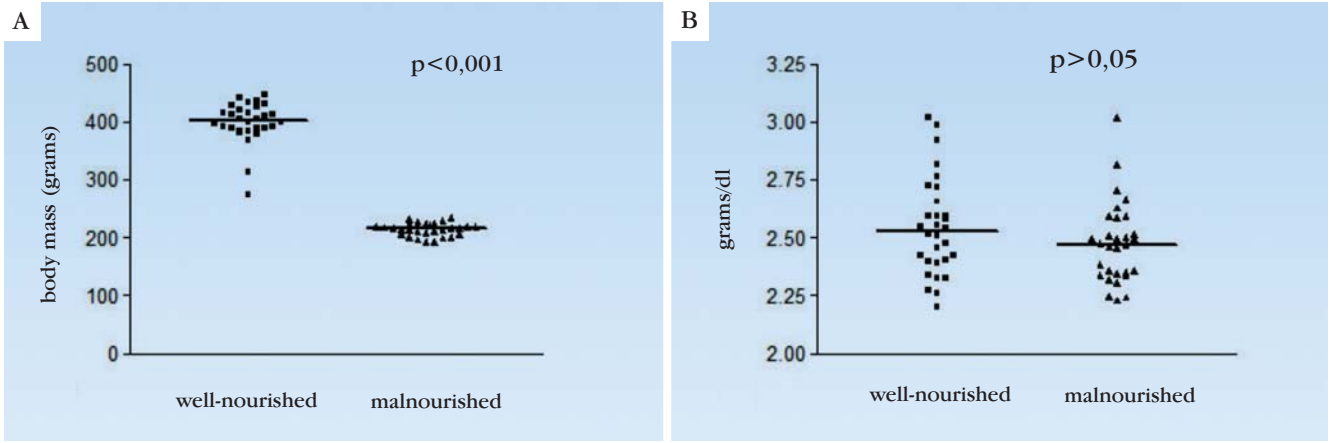

Figure 1: Distribution of: **A.** body mass (g) and **B.** serum albumin levels (g/dl) in the rats in the well-nourished and malnourished groups after 60 days of follow-up

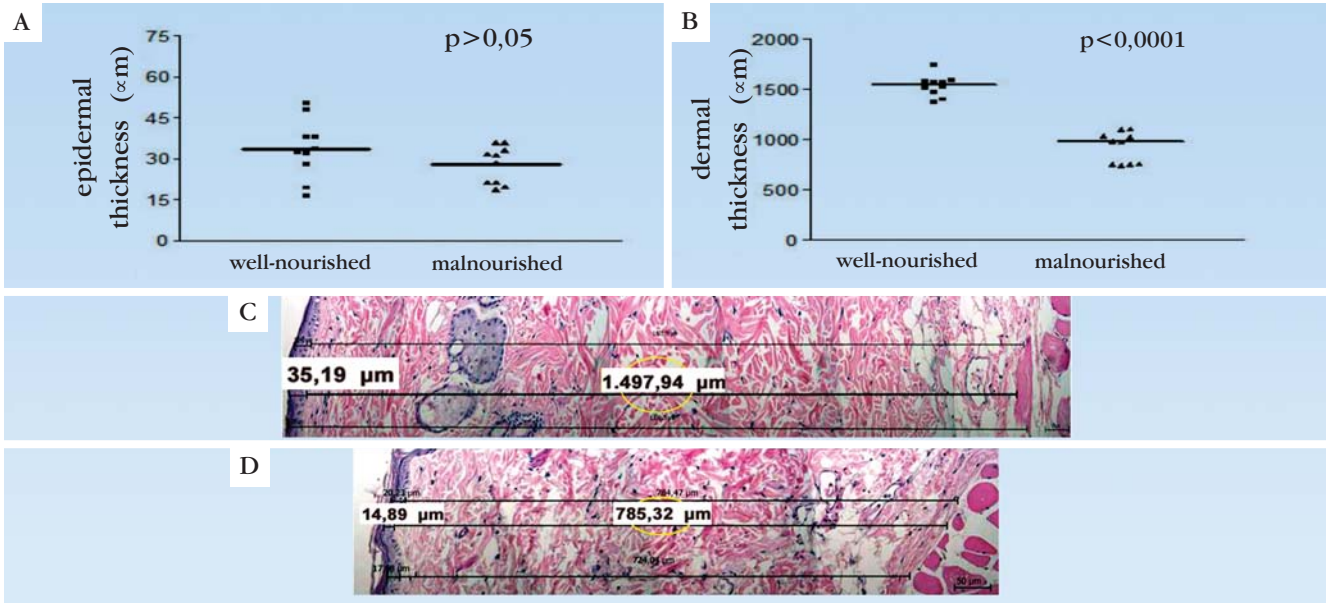

Figure 2: Distribution of: **A.** epidermal thickness (Ìm) and **B.** dermal thickness in the well-nourished and malnourished groups after 60 days of follow-up. Photomicrography (hematoxylin-eosin) of skin samples from **C.** the well-nourished and **D.** malnourished groups

DISCUSSION

In the marasmus model, the protein-energy malnutrition model was used to evaluate the effects of malnutrition on the skin tissue of Wistar rats. In agreement with reports published in the literature, the experimental induction of marasmus in rats was confirmed by their weight and by the clinical signs presented by the animals. [2,6,8,11,12] With respect to serum albumin levels, the results were similar in the well-nourished and malnourished groups after 60 days of follow-up, possibly due to the slow, belated reduction in albumin levels in marasmus. [2,6]

Similar results were found with the gelatin model in which a diet containing low quality protein was used. Gelatin has an amino acid score of zero, meaning that tryptophan is absent in the protein; therefore its conversion to niacin is reduced, leading to growth stunting. [23,24] In addition to a total lack of tryptophan, gelatin contains hydroxyproline and glycine, amino acids that also lead to growth stunting.

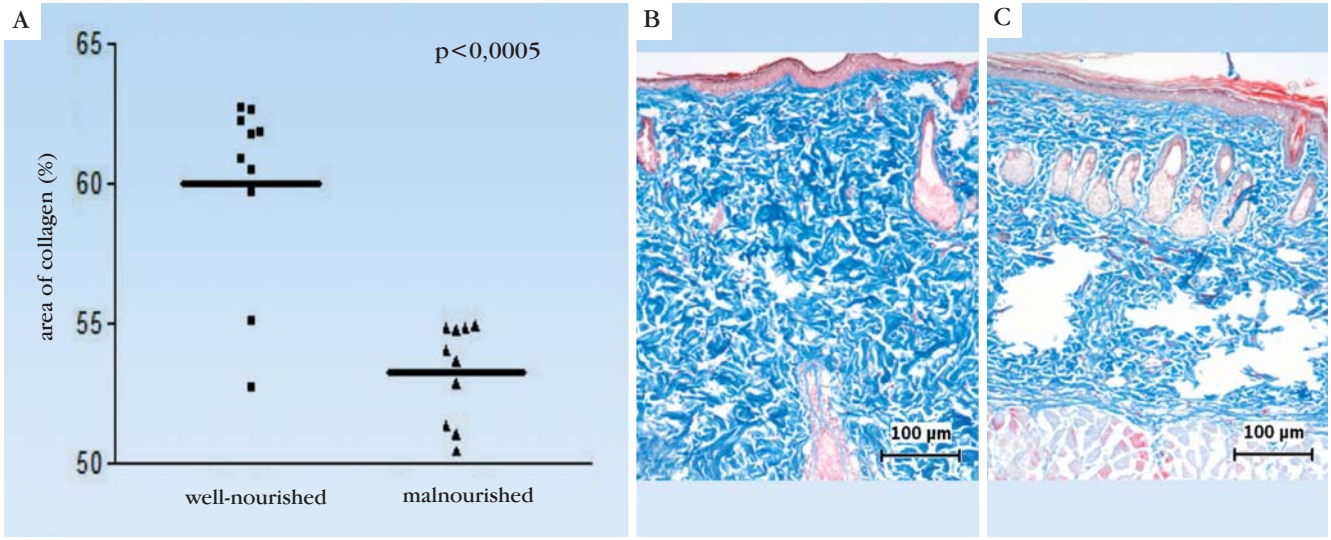

Figura 3: **A.** Distribution of the percentage of areas of collagen in the well-nourished and malnourished groups after 60 days of follow-up. Photomicrography of the slides stained by Gomori trichrome, magnification 100x, showing **B.** dense collagen in the well-nourished group; **C.** loose collagen with the presence of artifactual spaces in the samples from the malnourished group

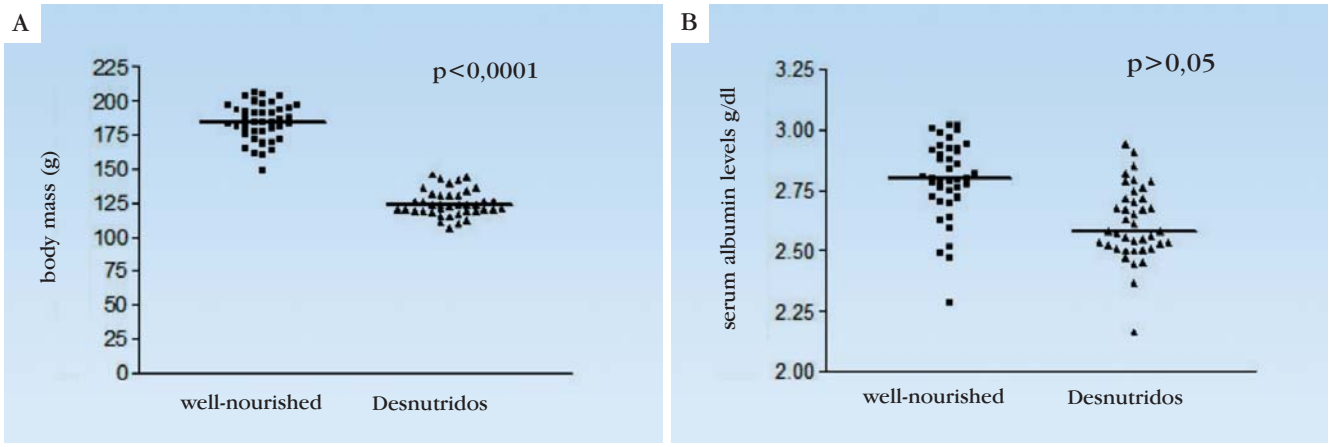

Figure 4: Distribution of: **A.** body mass (grams) and **B.** serum albumin levels (g/dl) of the rats in the well-nourished and malnourished groups after 30 days of follow-up

[25] The malnutrition induced in the rats was confirmed by their excessive weight loss and consequent growth deficit and by the clinical signs shown by the animals. Serum albumin levels were similar in the two groups during follow-up, probably due to the lack of tryptophan in gelatin, leading to a suppression of niacin, with the tryptophan that is present in the body then being used to synthesize more important endogenous proteins, such as serum proteins, in order to maintain normal levels. [26]

With respect to the histological analysis, a reduction in skin trophism (dermal thickness) occurred in the animals in the malnourished groups compared to the well-nourished groups in both models. This fact was confirmed histologically by the lower percentage of collagen per skin area in the malnourished groups. Collagen is the major structural protein present in human beings, constituting three-quarters of the proteins present in skin. It is closely associated with the tensile strength and flexibility of skin and is important in the healing process. Nevertheless, during the malnutrition process, collagen deposition falls, compromising the function of collagen in healing the skin. [15,26,27]

The results shown in the two models of malnutrition were similar irrespective of the model used, showing the negative effect of malnutrition on skin trophism in these animals, an effect that was confir-

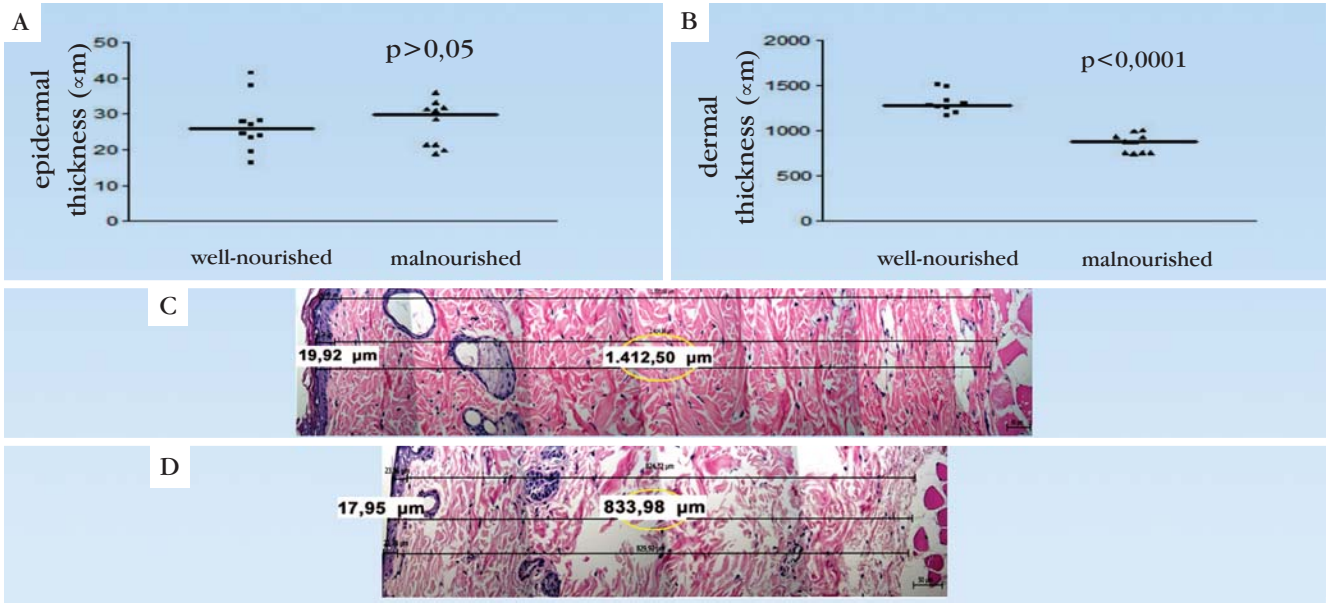

Figure 5: Distribution of: **A.** epidermal and **B.** dermal thickness (∝m) in the well-nourished and malnourished groups after 30 days of follow-up. Photomicrography (hematoxylin-eosin) of skin samples from the **C.** well-nourished and **D.** malnourished groups

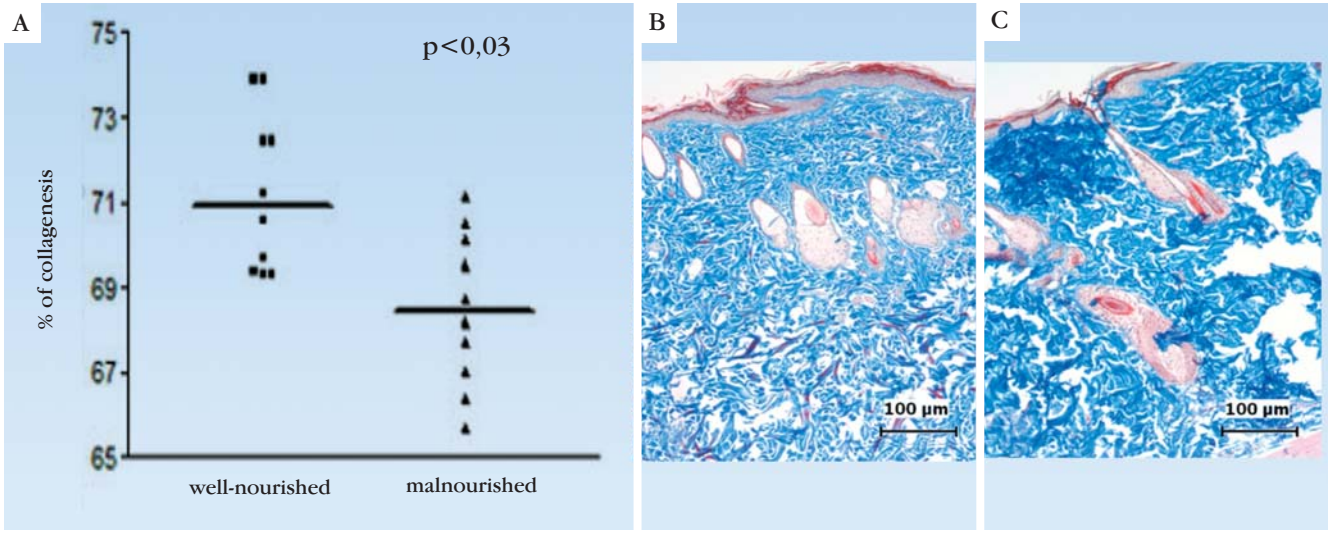

Figure 6: Distribution of: **A.** the areas of collagen in the well-nourished and malnourished groups after 30 days of follow-up. Photomicrography of slides stained by Gomori trichrome (magnification 100x) showing **B.** dense collagen in the animals of the well-nourished group and **C.** loose collagen with the presence of artifactual spaces in the slides of the animals in the malnourished group

med histologically by the reduction in dermal thickness and consequent decrease in the percentage of collagen, which may result in delays in the skin healing process, as has been already described by various authors. [6,11,14,15,26,27]

CONCLUSIONS

Histological alterations in dermal thickness and in collagenesis were found in the malnourished animals in both experimental models, confirming the negative effect of malnutrition on skin trophism in rats and characterizing them as important models for further studies into the healing process. ❑

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